

Differential proteomic analysis of serum exosomes reveals alterations in progression of Parkinson disease

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Abstract

Exosomes are nanometer-sized vesicles with intercellular communication functions, and their encapsulated proteins may participate in the pathological process of neurodegenerative disorders. The aim of this study was to identify the protein changes of serum exosomes in Parkinson disease (PD) patients with different disease progress types, and to identify potential biomarkers. The exosomes of PD patients with different severity and healthy control group were isolated from serum. The exosome proteins were analyzed by mass spectrometry with label-free quantitative proteomics. A total of 429 proteins were identified, of which 14 were significantly different in mild and severe PD patients. The expression levels of 7 proteins, including pigmented epithelium-derived factor, afamin, apolipoprotein D and J, were significantly increased in PD patients. The expression levels of 7 proteins, including complement C1q and protein Immunoglobulin Lambda Variable 1-33 (IGLV1-33)Cluster -33, were decreased in PD patients. These differentially expressed proteins were analyzed by gene ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis, which confirmed that the interaction between prion diseases and ECM receptors was the most significant pathways of enrichment. The changes of proteins and pathways may be related to the pathophysiological mechanism of PD. Therefore, some of these proteins could be considered as potential biomarkers for early PD diagnosis.

Abbreviations: A β = amyloid beta-peptide, ApoD = apolipoprotein D, CNS = central neural system, CSF = cerebrospinal fluid, DEPs = differentially expressed proteins, GO = gene ontology, M:F = ratio of male and female, PD = Parkinson disease, PEDF = pigment epithelium-derived factor, SN = substantia nigra.

Keywords: exosome, label-free MS/MS, Parkinson disease, proteomics

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RJ and CR contributed equally to this work.

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1. Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease in the United States, with an estimated 640,000 elderly people aged 65 years and over (1.6% of elders) suffering from this disease.^[1] Neuropathologically manifestations of PD patients are motor disorders, including changes in muscle rigidity, tremors, and gait.^[2] In addition to the motor symptoms, nonmotor features of the disease include autonomic failure, urinary incontinence, hallucinations, and dementia.^[3] Studies have shown that in the first clinical manifestations of PD, patients lost about 60% of their striatum dopaminergic nerve terminals, and about 30% of their substantia nigra (SN) neurons.^[4] Since the disease has entered the advanced stage of neuropathology at the time of diagnosis, it is of great significance to find potential biomarkers that can identify the disease early in the experiment of disease modification therapy.

Exosomes are cell-derived vesicles between 30 and 100 nm in diameter, protected by lipid-rich binding membranes. These vesicles were originally considered to be vesicles produced by mature blood reticulocytes and secreted into extracellular milieu with topically expressed transferrin receptors.^[5,6] They are secreted by hematopoietic and nonhematopoietic cells, and are present in serum, urine, cerebrospinal fluid (CSF), bronchi alveolar lavage, synovial fluid, and other body fluids due to their specific cell-derived components on their surfaces. Their components include proteins, lipids, and different amounts of DNA, RNA, and small cytoplasmic RNA. These components are

known to may be involved in many physiological processes and the progression of diseases, such as neurodegenerative diseases. In neurodegenerative diseases, the progressive accumulation of protein aggregates and lack of sensitive biomarkers hamper the development of disease-specific therapies. In addition, although protein aggregates can be detected in CSF and blood, their extremely low levels limit their use as potent biomarkers. In view of these limitations, the focus of research on biomarkers in neurodegenerative diseases has shifted from proteins to the vesicles in biological fluids. The presence of disease-specific molecular signatures in exosomes makes them powerful diagnostic candidates.^[7] As exosomes protect their cargo from degradation, screening exosomes in CSF and serum provides a way to identify the origin of their cells, thus providing insights into the cellular and pathogenic processes of the central nervous system. The exosomes produced by tumor cells in the brain display immunosuppressive and carcinogenic factors, which is a good example of disease-specific molecular characteristics in exosomes.^[8]

In the present study, we quantitatively analyzed the serum exogenous proteomes of PD patients using mass spectrometry-based label-free technique and compared them with healthy controls. This study was designed to identify difference of exosome protein content between PD patients and control. A total of 429 proteins were identified. Some of them, including apolipoprotein J, pigment epithelium-derived factor (PEDF), and gelsolin, may be potential target for early PD pathogenesis.

2. Materials and methods

2.1. Participants and sample collection

All subjects received written informed consent for participation from their guardians directly or indirectly, and were approved by the institutional Ethics Committee of the Second People's Hospital of Lishui, Zhejiang province. All participants were assessed including medical history, physical and neurological examinations, and neuropsychological assessments. PD patients were diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINDS-ADRDA) criteria, and their diagnoses were confirmed by the senior neurologist according to the United Kingdom Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria.^[9] The sporadic PD group included 20 subjects (average age 78 years; 11 males; 9 females). According to the Hoehn and Yahr classification,^[10] these PD patients were divided into 2 clinical subgroups: mild (score less than 3; $n=10$), or severe (score greater than 3; $n=10$). Healthy control subjects (average age 79 years; 6 males, 4 females) were community volunteers with good health. In the Cambridge Cognitive Examination (CAMCOG) scale, they have no signs or symptoms indicating cognitive impairment.^[11] Whole blood samples were centrifuged to separate out serum. All serum samples were stored at -80°C until analysis.

2.2. Exosome isolation from serum

The initial volume of serum for the experiments was 5 mL per group. The samples were diluted with an equal volume of Phosphate Buffered Saline (PBS) to decrease the viscosity. The diluted serum samples were centrifuged at 2000 g for 10 minutes, 10,000 g for 30 minutes at 4°C to remove dead cells and cell

debris. The supernatant was transferred into Ultra-Clear tubes (Beckman Coulter, Indianapolis, IN), and centrifuged at 100,000 g using a Beckman Optima XL-70 ultracentrifuge for 70 minutes at 4°C . The supernatant was removed by pipette, and the supernatant was remained 2 mm above the pellet. Five cycles of ultracentrifugation were needed to purify exosomes and remove serum proteins.^[12] After centrifugation, the exosomes appeared as beige in the bottom of the vessel. The exosomes dissolved in 200 μL SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0), and boiled for 5 minutes. Undissolved debris was removed by centrifugation at 14,000 rpm for 15 minutes. The supernatant were collected and quantified with a BCA Protein Assay Kit (Bio-Rad, CA, USA).

2.3. Exosomal protein digestions

Digested protein (50 μg for each sample) was performed according to the filter-aided sample preparation (FASP) procedure described previously.^[13] Briefly, the detergent, DTT and other low molecular weight components were separated by centrifugation using 200 μL UA buffer (8 M urea, 150 mM Tris-HCl pH 8.0) by repeated ultrafiltration (pall, 10 kD). Then 100 μL of 0.05 M iodoacetamide in the UA buffer was added to the sample block to reduce cysteine residues and the samples were incubated for 20 minutes in darkness. The filter was washed with 100 μL UA buffer 3 times and then 100 μL 25 mM NH_4HCO_3 twice. Finally, the suspended protein was digested with 3 μg trypsin (Promega, Shanghai, China) in 40 μL 25 mM NH_4HCO_3 overnight at 37°C , and the resulting peptides were collected as a filtrate. Based on the frequency of tryptophan and tyrosine in vertebrate proteins, the content of the peptide was estimated by ultraviolet spectral density at 280 nm using a solution with an extinction coefficient of 0.1% (g/L).

2.4. Label-free quantitative proteomics

The peptide of each sample was desalted on C18 Cartridges (Empore SPE Cartridges C18 [standard density], bed I.D. 7 mm, volume 3 mL, Sigma-Aldrich, CA), and then concentrated by vacuum centrifugation and reconstituted in 40 μL of 0.1% (v/v) trifluoroacetic acid. MS experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific, San Jose, CA). 5 μg peptide was loaded onto a C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (2% acetonitrile and 0.1% formic acid), and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL/minutes controlled by IntelliFlow technology over 120 minutes. MS data was acquired using a data-dependent top 10 method to dynamically select the most abundant precursor ions from the survey scan (300–1800 m/z) for High Energy Collisional Dissociation (HCD) fragmentation. Determination of the target value is based on predictive automatic gain control. Dynamic exclusion duration was 25 seconds. The resolution of survey scan was 70,000 at m/z 200, and the resolution for HCD spectrum was set to 17,500 at m/z 200. Normalized collision energy was 30 eV and the underfill ratio was defined as 0.1%, which specifies the minimum percentage of the target value that may be reached within maximum fill time.^[14] The peptide recognition mode was enabled when the instrument was running. MS experiments were performed triply for each sample.

2.5. Sequence database searching and data analysis

The MS data were analyzed using MaxQuant (version 1.3.0.5) software. MS data were searched in the UniProt human database (148,327 total entries, downloaded 20,160,207). An initial search setting was 6 ppm in the precursor mass window. The search followed the enzymatic cleavage rule of trypsin/P, allowing for maximum deletion of 2 missed cleavage sites, and a mass tolerance of fragment ions was 20 ppm. Cysteine aminomethylation was defined as a fixed modification, and protein N-terminal acetylation and methionine oxidation were defined as variable modifications for database searches. The cutoff value of the global false discovery rate for peptide and protein identification was set to 0.01. Label-free quantification was carried out in MaxQuant as previously described.^[15] Protein abundance was calculated on the basis of the normalized spectral protein intensity (LFQ intensity).

2.6. Bioinformatics

Differentially expressed proteins (DEPs) were annotated using the gene ontology (GO) program Blast2GO (<https://www.blast2go.com/>) to create a histogram of GO annotations, including cellular components, biological processes, and molecular functions. For pathway analysis, the differential proteins were mapped to the terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using the KAAS program (http://www.genome.jp/kaas-bin/kaas_main).^[16]

2.7. Statistical analysis

The Perseus software (version 1.3.0.5) was used to analyze the library files obtained by MaxQuant and compare the peak intensities to obtain quantitative data for all the peptides in the sample. The LFQ intensities of peptides from MaxQuant analysis were imported and converted into logarithmic scale with base 2. Student *t* test was used to calculate the quantification of protein and statistical significance. $P < .05$ was considered as a significant difference.

3. Results

3.1. Quantitative protein identification of serum-derived exosomes

Serum-derived exosome proteins were isolated and digested with trypsin. The peptides from the in-gel digestion were analyzed by LC-MS/MS, and the resulting data was submitted to database search using the Mascot (Matrix Science, London, UK) program. In total, 429 proteins were detected in all samples with high confidence. The quantization information on the identification of peptides was listed in the supporting file (Table S1, <http://links.lww.com/MD/D271>). Their molecular weight is mainly distributed in the ranges 10~70 kDa (Fig. S1, <http://links.lww.com/MD/D271>), indicating that a group of middle molecular substances and the basic proteins are enriched. Among these 429 proteins, 355 proteins were found in exosome samples of all groups (Fig. 1A). Nine unique proteins, including protein S100, tyrosine protein kinase receptor, lactoferrin, dermcidin, platelet activating factor acetylhydrolase, and isocitrate dehydrogenase, exist only in serum exosomes of patients with severe PD. On the other hand, some common proteins in serum samples, including keratin 1, ryanodine receptor 2, alternative protein fibroblast growth factor receptor 2 (FGFR2), and Immunoglobulin G (IgG)

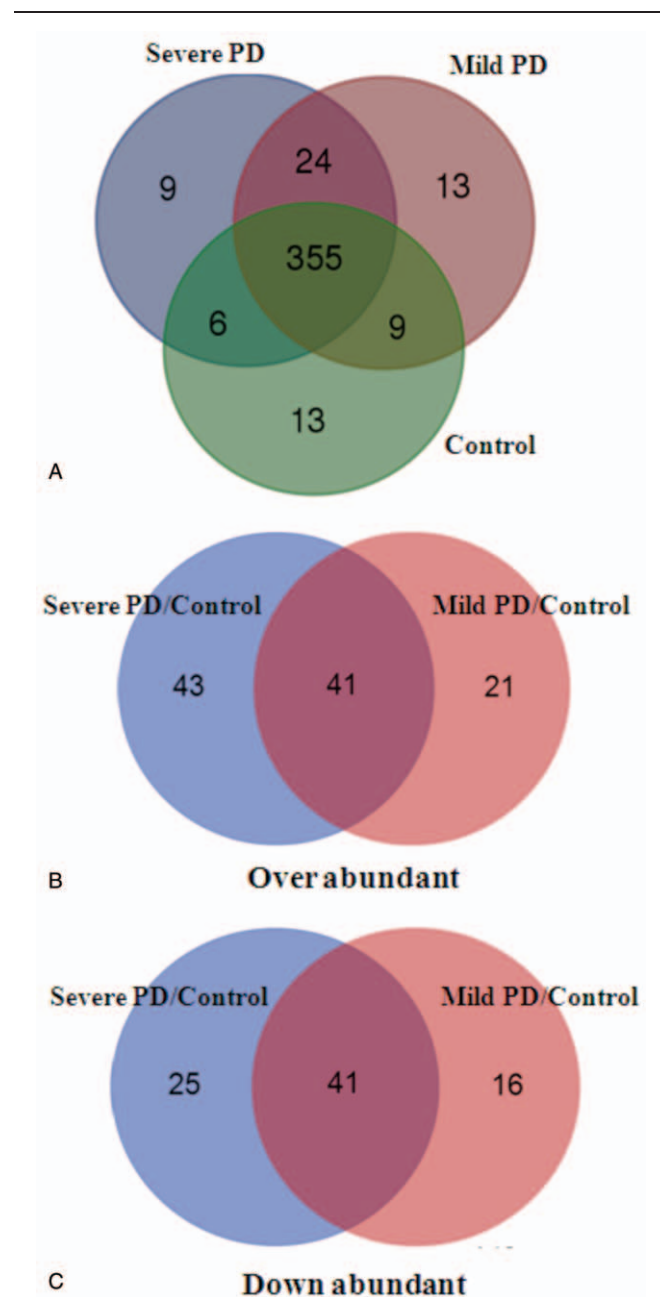


Figure 1. Summary of significant proteins in serum-derived exosomes of PD patients or controls. (A) The number of identified proteins in each group is represented by Venn diagram. The number of upregulated (B) and down-regulated (C) proteins in mild and severe PD compared with healthy control. Up or downregulated of the 2 groups of proteins is at the intersection. PD = Parkinson disease.

chains, exist only in serum exosomes of healthy controls. Detailed information on unique proteins in serum exosomes of each group, including protein score, sequence coverage, and molecular weight, is provided in the supporting file (Table S2, <http://links.lww.com/MD/D271>).

3.2. Quantitative comparison of protein expression at different stages

To accurately evaluate the proteomic changes, more than twice the statistical value between 2 groups were defined as significant

expressed proteins. In total, compared with healthy control, 62 and 84 proteins were upregulated in mild and severe PD groups, respectively, while 57 and 66 were downregulated. Among these significantly expressed proteins, 41 proteins were upregulated and 41 were downregulated in PD patients both with mild and severe stages (Fig. 1B and C). Details of these significantly expressed proteins are shown in Table S3, <http://links.lww.com/MD/D271>. More importantly, clusterin, complement C1r subcomponent, afamin, angiotensinogen variant, apolipoprotein D (ApoD), gelsolin, and PEDF were progressively upregulated from mild PD to severe PD (Fig. 2A). In contrast, human neuroblastoma full-length cDNA clone CS0DD006YL02, precursor (AA -19 to 113), complement C1q subcomponent, myosin-reactive immunoglobulin kappa chain, Ig kappa chain V-III region, immunoglobulin mu chain, and immunoglobulin kappa variable 1 to 33 are gradually downregulated in the serum exosomes from mild PD to severe PD (Fig. 2B).

3.3. GO enrichment of DEPs

To obtain a global image of the proteomic changes during PD development, we performed the DEPs were annotated with GO terms based on the biological processes, molecular functions, and cellular component categories. For the classification of biological processes, the top 5 enriched annotation terms were response to stimulus, response to stress, regulation of stimulus response, defense against response, and immune system processes (Fig. 3A). The molecular functions of these proteins included receptor binding, calcium ion binding, cell adhesion molecule binding, growth factor activity, protein binding, and low-density lipoprotein particle binding (Fig. 3B). The cellular component

classes of these changed proteins were the extracellular region, extracellular space, blood microparticle, fibrinogen complex, cell cortex, cytoplasmic region, and sarcoplasm (Fig. 3C).

3.4. Pathway analysis

The label-free MS results were also evaluated using UniPathway. DEPs were divided into up and downregulated pathways. In the upregulated pathways of PD patients, the top 6 enrichment terms of biological process class were prion diseases, Extracellular Matrix (ECM)-receptor interaction, platelet activation, phagosome, proteoglycans in cancer, and regulation of actin cytoskeleton (Table 1). Compared with the healthy control, the expression of complement component C5-9 (P10643, P02748, P01031, P13671, and P07358) in prion diseases pathway, cartilage oligomeric matrix protein (P49747) in ECM-receptor interaction pathway, phosphatidylcholine-sterol acyltransferase (P04180) in glycerophospholipid metabolism, and carboxypeptidase B2 (Q96IY4) in pancreatic secretion were significantly increased in PD patients. Conversely, the 5 pathways were downregulated in PD patients, including prion diseases, fat digestion and absorption, Nucleotide-binding Oligomerization Domain (NOD)-like receptor signaling, and Peroxisome Proliferators-Activated Receptors (PPAR) signaling pathway (Table 1). Compared with the healthy control, complement C1q subcomponent subunit A-C (P02745, P027746, and P02747) in prion diseases pathway, apolipoprotein AI (P02647) in fat digestion and absorption pathway, caspase recruitment domain-containing protein 9 (Q9H257) in NOD-like receptor signaling pathway, and ryanodine receptor 2 (Q92736) in pancreatic secretion were significantly decreased in PD patients.

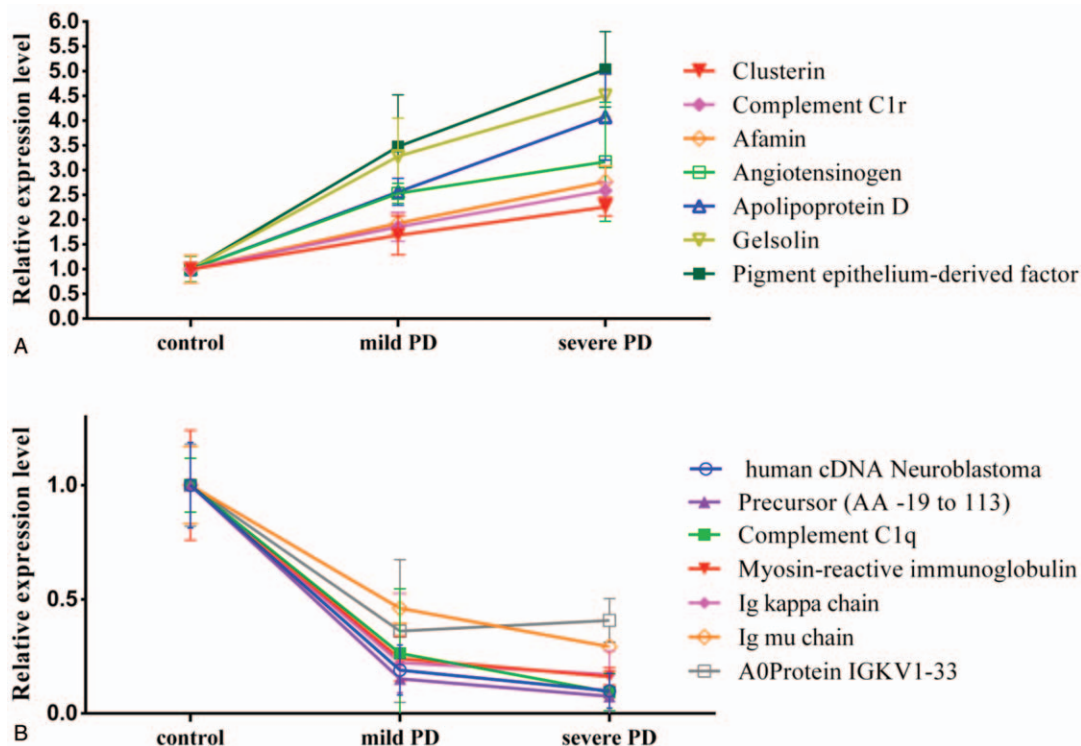


Figure 2. Significant proteins in serum-derived exosomes of PD patients. Upregulation (A) or downregulation (B) of the protein is progressively from mild PD to severe PD. PD = Parkinson disease.

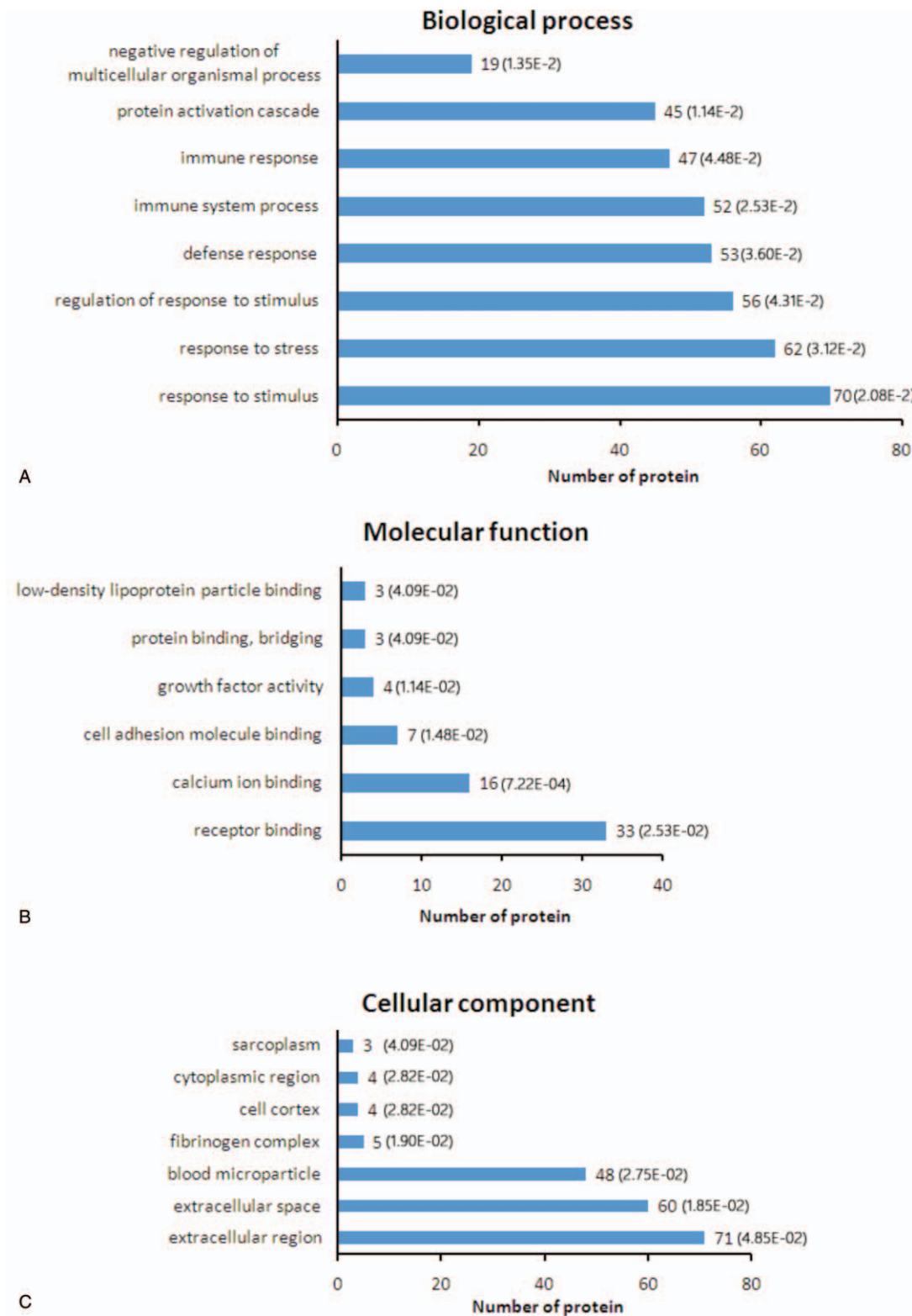


Figure 3. Gene ontology functional classification of serum-derived exosome proteomes. (A) Biological process, (B) molecular function, and cellular component (C). The numbers after comma indicate the number of hits for each subcategory.

4. Discussion

Although exosomes play an important role in intercellular communication and influence both physiological and pathological processes, their function in PD remains still in its infancy. The

working hypothesis was that serum-derived exosomes exhibited distinct biochemical compositions during the development of PD. This hypothesis is important, since the discovery of distinct protein changes in the exosomes might provide novel insights into the

Table 1
Differentially upregulated or downregulated protein pathway analysis in patients with mild and severe Parkinson disease compared with healthy control.

Pathway	Healthy control	Mild PD	Severe PD
Prion diseases	P10643;P02748;P01031; P13671;P07358		P10643;P02748;P01031 P13671;P07358↑
ECM-receptor interaction	P04275;P07996;P04004;P49747	P04275;P07996;P04004;P49747↑	P04275;P07996;P04004 ↑
Platelet activation	P04275;P02675;P0267	P04275;P02675;P02671↑	P04275;P02675;P02671 ↑
Phagosome	P11226;P07996;P49747	P11226;P07996;P49747↑	P11226;P07996↑
Proteoglycans in cancer	P07996;P04004	P07996;P04004↑	P07996;P04004↑
Regulation of actin cytoskeleton	P06396;P00734	P00734;P06396↑	P00734;P06396↑
p53 signaling pathway	P07996	P07996↑	P07996↑
Glycerophospholipid metabolism	P04180		P04180↑
Pancreatic secretion	Q96IY4		Q96IY4↑
Wnt signaling pathway	P36955	P36955↑	P36955↑
Chemokine signaling	P02775	P02775↑	
Prion diseases	P02746;P02747;P02745	P02746;P02747↓	P02746;P02747;P02745↓
Fat digestion and absorption	P02647		P02647↓
NOD-like receptor signaling	Q9H257	Q9H257↓	
PPAR signaling pathway	P02647		P02647↓
Pancreatic secretion	Q92736	Q92736↓	Q92736↓
Calcium signaling pathway	Q92736	Q92736↓	Q92736↓
cAMP signaling pathway	Q92736	Q92736↓	Q92736↓
Metabolic pathways	P27169		P27169↓

cAMP = cyclic adenosine monophosphate, PD = Parkinson disease.

disease-specific systemic responses induced by the pathological processes of PD. In this research, we conducted a case-control study to explore the proteomic profiling of serum-derived exosomes in healthy subjects and patients with PD at different stages. The result revealed the development of distinct protein specific to the PD.

4.1. Proteomic characteristics of serum exosomes in patients with PD

The current studies have identified 383 proteins in human serum-derived exosomes from healthy samples. Previous studies evaluated the serum exosomes in healthy control and reported a total of 66 identified proteins.^[17] Differences in the number of proteins detected by healthy subjects in different studies could be explained by the number of samples, where in the previous study only 2 healthy subjects was analyzed.^[18] It has been demonstrated that when samples were aggregated from multiple subjects, the variability of samples reduced.^[19] Perhaps, by increasing the number of samples, the range of detected proteins may increase. On the other hand, other studies have reported that circulating exosomes from healthy or diseased subjects contained more detected proteins.^[20,21] In the study of Tomlinson et al,^[20] 1033 human proteins were identified by nano-Ultra Performance Liquid Chromatography (UPLC)-MS/MS. Another study identified 1369 human proteins in healthy subjects by antibody-coupled monolithic silica.^[21] Differences in the amount of identified proteins can also be explained by the MS technique and the number of peptides used to identify proteins. Studies using only 1 peptide to identify proteins may increase sensibility^[22,23]; however, it may reduce specificity. In this study, we pooled serum-derived exosomes from PD patients at various developmental stages to reduce individual impact and used a peptide to identify proteins. Our results showed that the label-free proteomic approach successfully detected and quantified the overall protein profile alternations in the mild and severe stages of PD development.

4.2. Identifying significant protein alterations in serum exosomes of PD patients

The emergence and development of proteomic technology has brought us new hope for further investigation of its pathogenesis and treatment methods. There are some special advantages due to the unique accessibility of serum samples. This study used a relative quantitative proteomics approach to compare serum samples of healthy controls with those of patients with 2 different progressive types of PD. In total, 14 DEPs are progressively altered in exosomes derived from serum of patients with PD at different stages. According to previous studies, most of these proteins have already been associated with PD or other neurodegenerative disorders, which can be mentioned and analyzed below.

Studies have shown that immunologic systems are found in central neural system (CNS) which involved with PD, in addition, the serum inflammatory factors and blood T cells are involved in the immune dysregulation of PD and inspected as the potential clinic biomarkers for PD prediction.^[24] The PD animal models showed that abundant CD3+, CD4+, CD8+ T lymph cells migrated from blood vessels into SN to attack dopamine neurons.^[25,26] Therefore, it is reasonable to suppose that there may be some potential connections between PD stage and immune dysregulation in periphery blood. The complement system is believed to amplify the effectiveness of both the specific and nonspecific immunological defense system, which is related to the pathology of various neurodegenerative disorders including the pathogenesis of PD.^[27] Complement C1q (C1q) is the first subcomponent of complement activation classical pathway C1 complex, which is essential for the elimination and connection of synapses. In our study, the level of C1q was significantly lower in PD patients. Recent evidence has shown that C1q can mediate the formation of central synapses and contribute to the neuroprotection of the central nervous system, which may be a beneficial therapeutic target in neurodegenerative

disorders.^[28] Interestingly, constitutive expression of C1q was restricted to microglia throughout the brain, and microglial C1q expression was early and transiently upregulated after treatment in the substantia nigra (SN) and striatum, but nigrostriatal dopaminergic injury may be not affected by C1q in PD model.^[29] Therefore, combined with our observation and previous studies, C1q has complicated functions in the pathogenesis of PD.

Some exosomes with significant changes are also metabolic related and primarily expressed in other organs beside of the nervous system. ApoD is a lipocalin transporter of small hydrophobic molecules, which is expressed in and secreted from a variety of peripheral and brain tissues and plays an important role in several neurodegenerative diseases.^[30] In healthy neural system, ApoD is expressed by glia cells and other nonneuronal cells (such as pericytes), which has a profound impact on the function and survival of neurons.^[31] In addition to its role in extracellular lipid transport, ApoD regulates AA metabolism in an antioxidant and anti-inflammatory manner.^[32] Apolipoprotein plays an important role in PD and exhibits vital function in the pathogenesis of PD. apolipoprotein J (or clusterin) is widely expressed in human tissues and has multiple functions in health and disease. Previous proteomic studies showed that the level of clustering protein in PD serum was higher than that in the control group.^[33] Consistent with these results, our proteomic analysis further revealed that the level of cluster proteins in serum-derived exosomes gradually increased from mild to severe patients (Fig. 3). In addition, the dose-dependent manner of inducing oligomerization in patients with α -synuclein PD in CFS liquid has recently been observed.^[34] In view of the close correlation between exosome and synuclein, we speculated that the higher level of exosomal clusterin in serum might modify the formation of synuclein-positive Lewy bodies and thus play a role in PD. In addition to clusterin and ApoD, a small hydrophobic lipocalin transporter was upregulated in PD glial cells, but not in dopaminergic neurons, suggesting its role in neuroprotection and the importance of glia in the amount of this protein in the central nervous system.^[35] Our study further demonstrated the elevation of serum-derived exosome ApoD in PD patients at different stages, indicating its potential as a biomarker of PD.

PEDF is a member of the serpin superfamily. It is a nerve growth factor, which has been detected in the CNS and has interesting properties in terms of neuronal survival, as it can protect against oxidative stress and glutamate excitotoxicity. PEDF seems to be a neuroprotective factor for spinal motor neurons, hippocampal neurons, and cerebellar granule cells. Furthermore, PEDF has been shown to improve neuronal survival in a 6-hydroxydopamine PD mouse model. In this study, our results showed that the level of PEDF in serum of PD increased. One explanation for the increase in PEDF might be that PD patients have a compensatory mechanism against neuronal cell death. Similarly, another independent study also has revealed elevated levels of serum PEDF.^[36] Taking together, the data indicated that PEDF seems to be an applicable biomarker.

Gelsolin is an 82 kDa actin-modulating protein regulated by Ca^{2+} and phosphoinositides. Gelsolin exists in 2 isoforms; a secreted protein found in plasma and CSF, which is considered to be part of the actin scavenging system, and the cytoplasmic form that regulated actin cytoskeleton.^[37] Here, our proteomic analysis revealed that the levels of serum-derived gelsolin in PD patients were significantly higher than those of control, suggesting that gelsolin might play a potential role in PD. In line

with our results, previous immunohistochemical studies showed that gelsolin co-occurred with α -synuclein Lewy bodies in PD patients and the presence of α -synuclein calcium chloride, which is closely associated with the aggregation rate.^[38] Similarly, plasma gelsolin also showed binding to amyloid beta-peptide ($A\beta$) and inhibition of fibril formation. In addition, peripherally administrated plasma gelsolin $A\beta$ levels reduced brain transgenic Alzheimer disease mouse model.^[39] These observations, together with our studies, indicate that increased serum gelsolin could be involved in the aggregation process of α -synuclein and thus might affect the formation of Lewy bodies.

Exosomes are secreted membrane nanovesicles that not only transfer membrane components, but also deliver nucleic acids and proteins among different cells. Although this study identified 14 particular proteins in patients with PD, larger prospective cohort studies are required to validate the present results. Moreover, further studies are needed to investigate whether the change occurs originally or may just be the secondary effect of PD or even irrelevant diseases.

4.3. Assessing limitations

There is significant heterogeneity in gender and age risks for PD. Although there is good evidence that men are, in general, about 1.5 times more likely to suffer from PD than women, the results of different studies are not the same.^[40] The ratio of male and female (M:F) in the incidence of PD is more significant in different regional populations, ranging from 1.01 to 2.1.^[41] Similarly, the average age of onset fluctuated from 68 to 79.^[42] According to these observations, the M:F was 1.2 (11/9), and the average age was 79 in our study. These patients were carefully selected within several years in line with strict clinical features and exclusion criteria. The clinical manifestations include motor impairments involving resting tremor, bradykinesia, and rigidity, along with nonmotor symptoms such as autonomic nervous, cognitive, and psychiatric problems. And the exclusion criteria were as follows: (1) secondary Parkinson disease or Parkinsonism syndrome; (2) history of stroke, brain injury, or surgery, Alzheimer disease, motor neuron disease, or other central nervous system diseases; and coexistence of severe systemic disease (such as tumors, hepatitis, nephritis, chronic heart failure, or chronic obstructive pulmonary disease) or infectious diseases at the time of enrollment.^[43] According to Hoehn and Yahr scores, patients were classified as moderate and severe, and those less than 3 were classified as mild, or more than 3 as severe. When the basic symptoms mentioned above and the clinical history of PD or any other disease did not exist, and the results of the detection of neurological diseases were negative, they were considered case control.

There are some limitations of this study that should be discussed. Firstly, small sample size was a limitation for our study, and further research with large sample size are needed to confirm these preliminary results. Secondly, the potential proteins identified in the study need be verified by other molecular methods including Western blot. Thus, further investigations are required to identify whether these proteins are promising biomarkers for PD. Although our sample size is not large, it was enough to show significant differences among the clinical groups through MS analysis. On the other hand, previous proteomic studies were also performed in relatively small sample size. For instance, Bostanci et al^[44] studied 5 healthy subjects and 5 patients with aggressive periodontitis; and Kido et al^[45] studied

8 patients with CP and 1 health periodontal subject. Additionally, some studies analyzed only 10 healthy subjects.^[46]

5. Conclusions

In summary, our proteomics analysis demonstrates that serum-based proteomic can identify disordered pathways and diagnostic biomarkers. The limitation of this study is that the sample size is relatively small, so future work is required to validate potential candidate targets and determine whether the identified serum biomarkers can increase the performance value of PD at different stages and change clinical management. In all, the identification of serum exosome proteins in PD by proteomic should be the basis of future research. According to other proteomic analysis of these proteins reported in the literature, our findings suggest that these proteins can be used as potential biomarkers of PD.

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