Am J Digest Dis 2020;7(1):1-10
www.ajdd.us /ISSN:2329-6992/AJDD0119676

Original Article

Label-free proteomic analysis of exosomes extracted from the serum of stroke patients

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Received August 6, 2020; Accepted October 13, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Stroke is one of the leading causes of mortality and disability in developed countries. The rapid and reliable diagnosis of stroke is important for patient treatment. Currently, neurological and neuroradiological diagnosis of stroke, especially in its acute phase, is frequently inconclusive and uncertain. Exosomes are 30-150 nm in diameter membrane vesicles, which can cross blood-brain barrier and act on the central nervous system by fusing with cell membrane. In this study, unbiased label-free LC-MS/MS-based quantification analyses of exosomal proteins was used to explore the aberrantly expressed exosomes between the serum of patients with stroke and healthy control. In total, 525 protein groups and 3637 peptides were identified in serum of stroke patients and control. 15 proteins were differentially regulated in stroke patients, including 6 overexpressed proteins and 9 downregulated proteins. Among the upregulated serum exosomes-encapsulated proteins, F13B, PPBP and HABP2 have been reported to be involved in the development of stroke. We speculated that exosomes-encapsulated F13B, PPBP and HABP2 may be the new biomarkers of the fast and variable diagnosis of stroke.

Keywords: Stroke, label-free proteomic analysis, exosomes, diagnosis

Introduction

Stroke is the second highest cause of disability and death globally, the incidence of which is increasing rapidly [1]. Stroke affects about 13.7 million people worldwide and caused 5.5 million deaths per year [2]. One in four adults in their lifetime will suffer from a stroke and there are more than 80 million stroke survivors worldwide [3]. The cause of ischaemic stroke is important as it can guide therapeutic strategies for the prevention of recurrent stroke.

Exosomes are nano-sized (between 20 and 100 nm in diameter) membrane-enclosed vesicles carrying various components such as proteins, nucleic acids, lipids, mRNA, miRNA and other non-coding RNAs [4]. Exosomes come into being in endocytic compartments and then the extracellular membrane of these vesicles fuse with the cell membrane and secrets them to the extracellular milieu [5]. Exosomes play a vital role in signal transmission or materials transportation between different cells and tissues [6]. In recent years, more and more studies focused on the function of exosomes in the development of various diseases. The occurrence of various diseases is closely related to exosomes for they release the encoded information into blood circulation or cellular fluid, leading changes in the recipient cells [7-9]. For example, exosomes carrying miR-155 and miR-204 can facilitate the occurrence of diabetes via reducing the body sensitivity to insulin, inducing insulin resistance and activating mitochondrial apoptosis in β cells.

An increasing number of findings have demonstrated that exosomes can cross the blood-brain barrier and act on the central nervous system [10]. Adult exosomes from mesenchy-

mal stem cells can be absorbed by neurons and microglia in the motor cortex by the nasal spray administration route, causing alleviating neuronal inflammation [11]. Low-concentration exosomes inhibit neuronal injury by play anti-apoptotic and anti-oxidation roles, while high-dose exosomes play the opposite role in neurons [12]. Exosomes promote angiogenesis, neuronal axon remodeling and neurogenesis and in stroke models [13]. In addition, culturing neurons and glial cells together with exosomes containing miR-133b derived from mesenchymal stem cells promoted neuronal cell growth [14]. These studies reveal that exosomes released from different sources protect the brain by preconditioning cerebral ischemic, thereby ameliorating nervous system diseases in the clinic. While there few reports to reveal exosomes that can be used in the diagnosis of stroke, in view the fact that exosomes have been shown to be able to be used in the diagnosis and treatment of other diseases [15-17].

In this study, unbiased label-free LC-MS/MS-based quantification analyses of exosomal proteins was used to explore the aberrantly expressed exosomes between the serum of patients with stroke and healthy control. We attempted to identify some exosomes that can be used to the rapid diagnosis of stroke.

Methods and materials

Human subjects

This study protocol was approved by the Institutional Review Boards (IRBs) of the participating institutions Affiliated Hospital of Youjiang Medical University for Nationalities. Between January 2018 to February 2019, a total of the serum of 3 consecutive subjects undergoing evaluation for stroke were included in the study. All patients were in accordance with the diagnostic criteria for stroke. Three healthy volunteers were recruited through a posted flyer.

Chemicals and antibodies

Antibodies against CD63 (ab216130 and ab68418) was obtained from Abcom (Cambridge, Britain); anti-TSG101 rabbit polyAb (102286-T38) was purchased from sinobiological (Beijing, China); Rabbit IgG was obtained from Santa Cruz (Dallas, USA). BCA quantification kit (P0012) was purchased from Beyotime (Shanghai, China). Ammonium bicarbonate (NH_4HCO_3) (A6141-25G) and trifluoroacetic acid (TFA) (T6508) were obtained from Sigma (St. Louis, Missouri, USA).

Production and isolation of exosomes

The serum was donated by three stroke patients and three normal persons as biological replicates. Collected serum was processed in parallel in subsequent steps. Serum was collected and centrifugated at 2000 g for 30 min at 4°C. Following being filtered through 0.22 μm syringe filter (Millipore, Darmstadt, Germany), the supernatants were further centrifuged at 120,000 g overnight at 4°C using eppendorf 5430R high-speed freezing centrifuge. Exosomes were precipitated from the supernatants. The sediments of exosomes were resuspended by cold PBS, and ultracentrifuged again at 120,000 g for 90 min at 4°C. The final sediments of exosomes were resuspended in cold PBS or SDT lysate buffer, and immediately stored at -80°C.

Electron microscopy

Exosomes purified from serum samples were resuspended by 50 μl PBS. 20 μl exosomes suspensions were added onto copper grid carefully, blotted up, and stained with 2% phosphotungstic acid (PTA). Sample was imaged using a transmission electron microscope.

Western blotting

Exosomes or cell lysate proteins (5 μg) were mixed with 2 × SDS sample buffer, separated on 12% SDS-acrylamide gels, and transferred onto PVDF membrane. The membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with either anti-CD63 (1/200 dilution), anti-TSG101 (1/500 dilution) or anti-GAPDH (1/5000 dilution) antibodies, followed by incubation with horserumdish-peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were detected using ECL Western blot detection reagents (Thermo, USA).

MS sample preparation and FASP digestion

A protein amount of 200 μg per sample, and DTT was added to a final concentration of 100 mM, and the mixture was boiled in water for 5 minutes, and cooled to room temperature. Add 200 μL of UA buffer and mix, transfer to a 30

kD ultrafiltration centrifuge tube, centrifuge 12500 g for 15 min, discard the filtrate (repeat this step once). 100 μL of IAA buffer (100 mM IAA in UA) was added, shaken at 600 rpm for 1 min, and reacted at room temperature for 30 min in the dark, and centrifuged at 12500 g for 15 min. This step was repeated twice by centrifuging 12500 g for 15 min by adding 100 μL of UA buffer. 100 μL of 40 mM NH_4HCO_3 solution was added and centrifuged at 12500 g for 15 min, and this procedure was repeated twice. 40 μL of Trypsin buffer (4 μg Trypsin in 40 μL 40 mM NH_4HCO_3 solution) was added, shaken at 600 rpm for 1 min, and placed at 37°C for 16-18 h. Renew the collection tube, centrifuge 12500 g for 15 min; add 20 μL of 40 mM NH_4HCO_3 solution, centrifuge 12500 g for 15 min, and collect the filtrate. The peptide was desalted by C_{18} Cartridge, and the peptide was lyophilized and then reconstituted by adding 40 μL of 0.1% formic acid solution to quantify the peptide (OD280).

LC-MS/MS

Each sample was separated by Easy nLC system. The buffer A solution was 0.1% formic acid aqueous solution and B solution was 0.1% formic acid acetonitrile aqueous solution (acetonitrile 80%). The chromatographic column was balanced with 100% liquid A, and the sample was separated from the automatic sampler to the analytical column (Thermo Fisher Scientific, Acclaim PepMap RSLC 50 um × 15 cm, nano viper, P/N164943) at a flow rate of 300 nL/min.

1-hour gradient: 0 min to 5 min, B liquid 3%, 5 min to 45 min, B liquid linear gradient from 3% to 28%, 45 min to 50 min, B liquid linear gradient from 28% to 38%, 50 min to 55 min, B liquid linear gradient from 38% to 100%, 55 min to 60 min, and B liquid at 100%. 2-hour gradient: 0-5 min, B-liquid 3%, 5-95 min, linear gradient of B-liquid from 3-28%, 95-110 min, linear gradient of B-liquid from 28-38%, 110-115 min, linear gradient of B-liquid from 38-100%, 115-120 min, and B-liquid at 100%. 3-hour gradient: 0 min-7 min, B solution 4%, 7 min-159 min, B-liquid linear gradient from 4% to 28%, 159 min-166 min, B-liquid linear gradient from 28% to 38%, 166 min to 173 min, B-liquid linear gradient from 38% to 100%, 173 min to 180 min, and B liquid at 100%.

The samples were chromatographed and mass spectrometry was performed with a Q-Exactive Plus mass spectrometer. The analysis duration is 60/120 min, the detection mode is positive ion, the scanning range of the mother ion is 350-1800 *m/z*, the resolution of the primary mass spectrum is 70,000, the AGC target is 3e6, and the first-level maximum IT is 50 ms. The mass charge ratio of the polypeptide and the polypeptide fragment was collected as follows: 10 fragment profiles (MS2 scan) were collected after full scan, MS2 Activation Type is HCD, Isolation window is 2 *m/z*, the secondary mass spectrum resolution is 17,500, the Microbe is 1, the second-level Maximum IT is 45 ms, and the normalized Collision Energy is 27 eV.

The original data of mass spectrometry analysis were raw files, and MaxQuant software (version No. 1.5.5.1) [18] was used for library identification and quantitative analysis. Relative ratio quantification was performed using quantities of unique peptides and required a minimum of two unique peptides. Proteins quantified with p-value ≤ 0.05 and a fold change of ≥ 1.50 or ≤ 0.66 were considered to be significantly differential.

Gene ontology analysis and networks, functional and pathway mapping

GO and pathway enrichment analyses were performed to identify potentially biological processes of the differentially expressed miRNA based on the GO (http://geneontology.org/page/go-enrichment-analyses) and pathways (http://www.genome.jp/kegg/pathway.html) database. The *P* value of each GO term was calculated by right-sided hypergeometric tests. Benjamin-Hochberg adjustment was used for multiple test correction [19, 20]. Those terms with a *P* value < 0.05 were considered as significantly enriched terms. The KEGG pathway enrichment analysis was conducted by DAVID. The protein-protein interaction network was generated by STRING 10 (http://string-db.org/) [21].

Statistical analyses

The statistical analysis of label-free discovery study was analyzed by means of unpaired ANOVA implemented in the Progenesis QI software. For functional analyses of differential proteins, the statistical analysis was performed using two-tailed student's T test in Prism 5 (Graphpad) software. All p-values were two-sided, and values less than 0.05 were considered statistically significant.

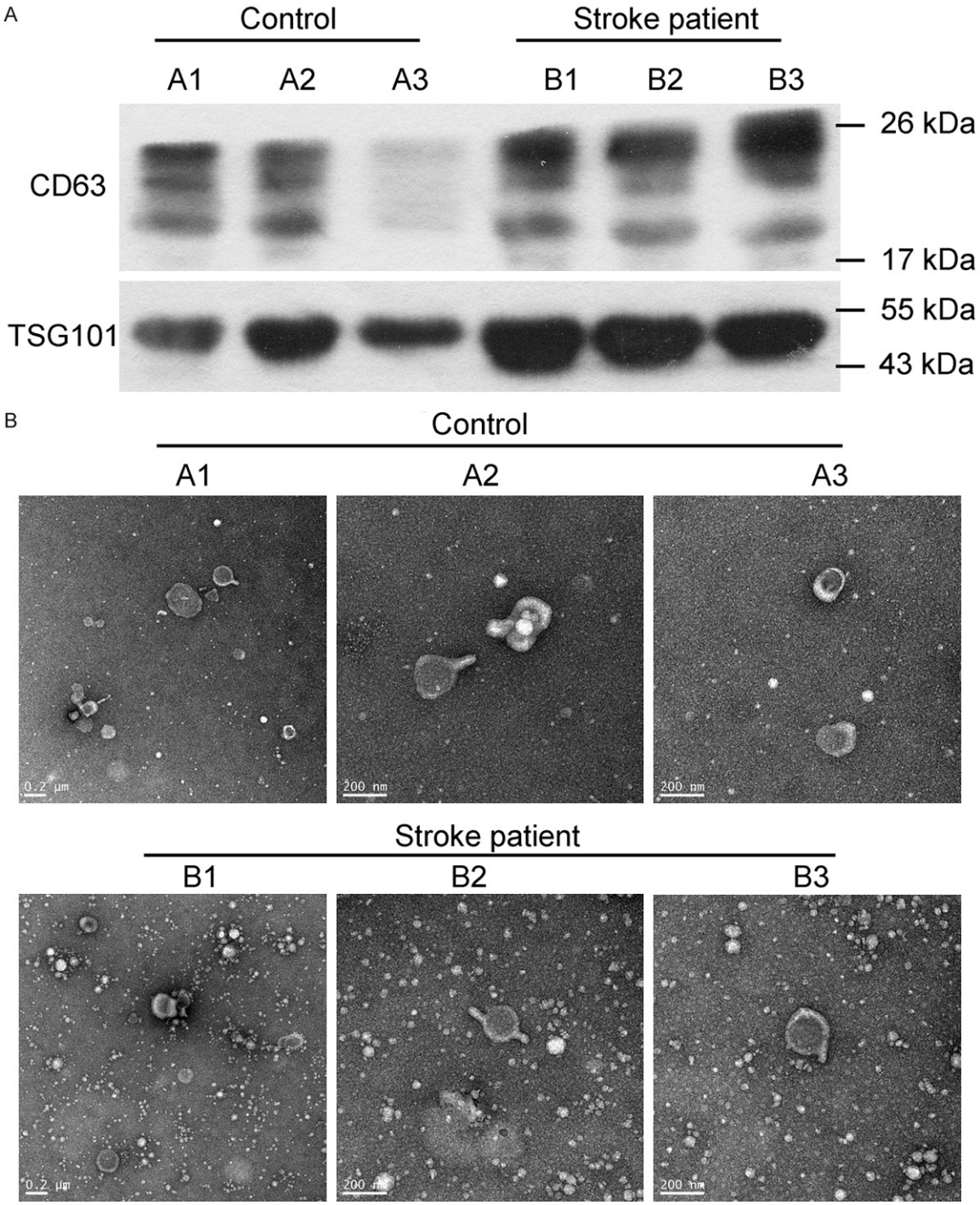

Figure 1. Identification of serum-extracted exosomes. A. Verification of exosomal surface marker protein by Western blotting. B. Transmission electron microscopy showed the morphological characteristics of exosomes. The white arrows represent tipical exosomes in a single field of view under TEM, Scale bar = 200 nm. Use a nanoparticle size analyzer to detect exosomes size.

Results

Exosomes enrichment and characterization

Crude exosomes were prepared from serum of 3 stroke patients and 3 healthy control following a differential centrifugation. To characterize the exosomes in derived from the serum of stroke patients and healthy controls, Western blot was used to detect the expression of the typical exosomal markers CD63 antigen (CD63) and tumor susceptibility gene 101 (TSG101).

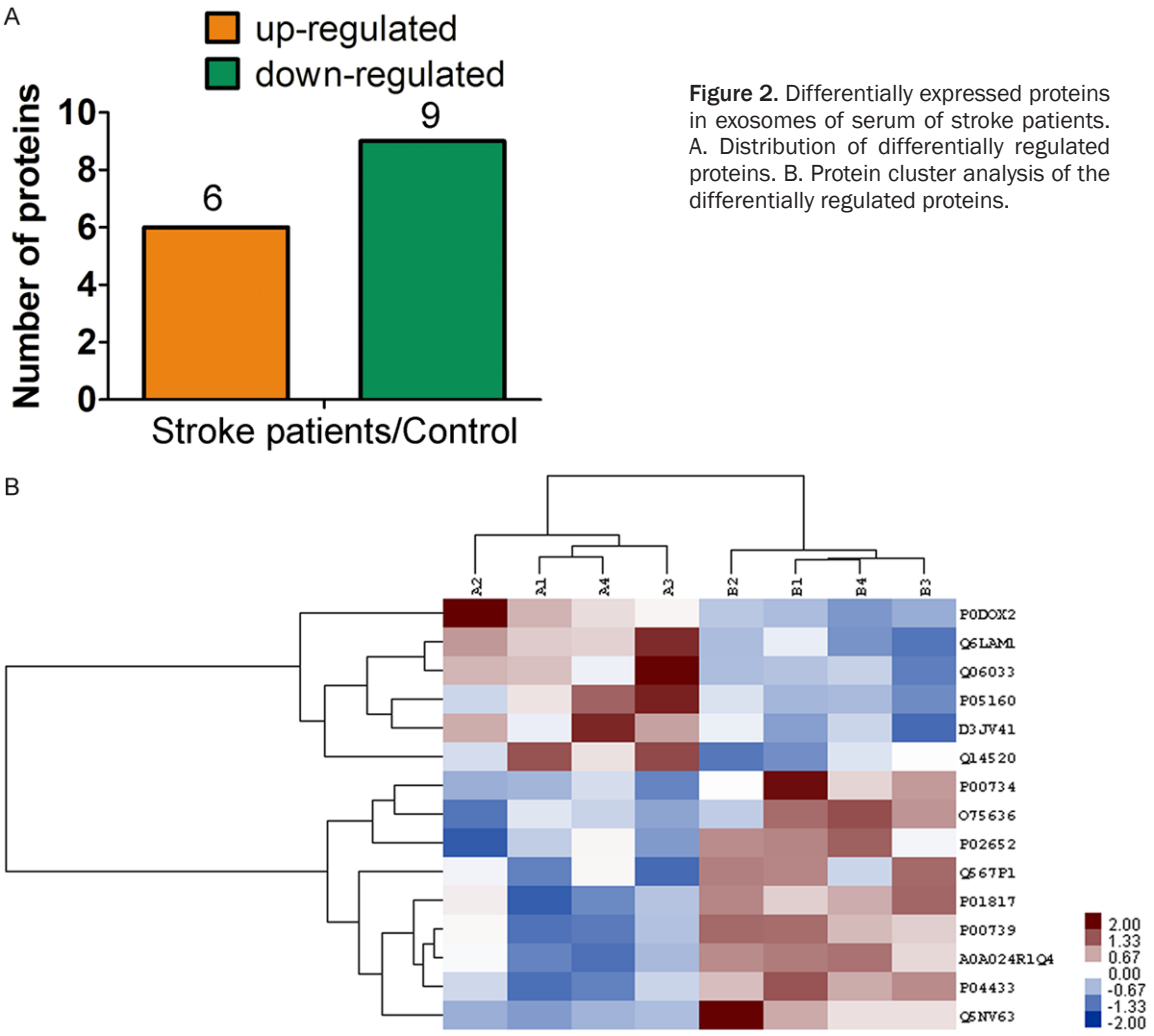

Figure 2. Differentially expressed proteins in exosomes of serum of stroke patients. A. Distribution of differentially regulated proteins. B. Protein cluster analysis of the differentially regulated proteins.

The results showed that CD63 and TSG101 were identified in both stroke patients and healthy controls (**Figure 1A**). The purity of exosomes was analyzed by electron microscopy, which demonstrated a presence of small membrane vesicles of about 30-150 nm in diameter with the cup-shaped structure typical of exosomes (**Figure 1B**).

Global profilling of exosome proteins in stroke patients

As a type of extracellular vesicle that can cross the blood-brain barrier, exosomes have attracted increasing attention as an attractive drug delivery system to delivery drugs to brain against ischemic brain injury [22-25]. While the global profiling of protein content in exosomes in the serum of stroke patients is still not clarified thoroughly. Therefore, we tried to consummate these earlier studies using highly sensitive LC-MS/MS approaches. In total, 525 protein groups and 3637 peptides were identified in serum of stroke patients and control. The identified proteins and peptide information are listed in Tables S1 and S2, respectively.

Cluster analysis is a commonly used exploratory data analysis method, the purpose of which is to group and classify the data on the basis of similarity. In the results of clustering grouping, the similarity of data patterns within groups is higher than that between groups. The results of proteomic analysis revealed 15 proteins (fold change > 1.5 and p-value < 0.05) that were differentially regulated in stroke patients, including 6 overexpressed proteins and 9 downregulated proteins (**Figure 2A**, **2B** and **Table 1**). The expression of P0DOX2, Q6LAMI, Q06033, P05160, D3JV41 and Q14520 is decreased in the stroke patients, while the content of P00734, 075636, P02652, Q567P1, P01817,

Table 1. Proteins differently regulated in exosomes isolated from stroke patients

Protein accession	Gene name	peptides	Unique peptides	Regulated type	A/B	*P* value
P0DOX2		15	3	up	1.89	0.017805
Q6LAM1		11	1	up	1.89	0.019171
P05160	F13B	6	6	up	1.87	0.034486
Q06033	ITIH3	16	16	up	1.73	0.023209
D3JV41	PP8P	3	3	up	1.67	0.048485
Q14520	HABP2	8	8	up	1.58	0.046626
P00734	F2	32	32	down	0.62	0.016028
Q567P1	IGL@	10	0	down	0.58	0.04218
P02652	APOA2	8	8	down	0.57	0.016934
O75636	FCN3	9	9	down	0.53	0.025207
P01817	IGHV2-5	3	1	down	0.49	0.008673
P00739	HPR	27	12	down	0.47	0.006384
U5LKQ0	APOL1	19	19	down	0.46	0.003364
A0A024R962	V1-4	2	2	down	0.43	0.026014
W5U1X0	IGKV3-11	3	2	down	0.38	0.001954

Protein accession is identified from Uniprot database (https://www.uniprot.org/). Fold change >1.5 is considered differently expressed. A/B represents the protein content in exosomes from serum of Control compared with Stroke patients. *p*-value < 0.05 is considered statistically significant.

P00739, A0A024R1Q4, P04433 and Q5NV63 is increased.

Functional enrichment analysis of the differentially regulated proteins

Gene ontology is a standardized classification system of gene function, which provides a set of dynamically updated standardized vocabulary, and describes the attributes of genes and gene products in organisms from three aspects: biological process, molecular function and cell component in participating biological processes. GO enrichment analysis of the differentially expressed proteins derived from stroke patients showed that the principle biological processes included “biological regulation”, “response to stimulus”, “single-organism process” and “metabolic process”. The top 3 cellular components included “extracellular region part”, “membrane” and “organelle” (**Figure 3**). The main molecular function is associated with “transporter activity”, “catalytic activity” and “binding”.

KEGG pathway enrichment analysis demonstrated the proteins that were significantly enriched in 8 pathways (**Figure 4**), included ‘african trypanosomiasis’, ‘complement and coagulation cascades’, ‘cholesterol metabolism’, ‘PPAR signaling pathway’, ‘chemokine signaling pathway’, ‘regulation of actin cytoskeleton’, ‘neuroactive ligand-receptor interaction’ and ‘Cytokine-cytokine receptor interaction’.

Discussion

Stroke is one of the most frequent causes of disability and death in the developed countries, which has no efficient therapeutic strategy in clinic and often leads to irreversible and devastating brain damage currently [26]. Up to now, there is no single biomarker which is available for ischemic stroke to address the above mentioned problems, which is different to the cardiovascular disease. It justifies the need that simple, fast and reliable diagnostic measures for stroke are urgently needed. Exosomes show great potential as biomarkers, drug carriers, in neurovascular remodeling and treatment, owing to their unique advantages of crossing the blood-brain barrier. Exosomal miRNAs have been revealed to paly protective roles in tissue injury of stroke. MiR-17-92 enriched exosomes promote motor electrophysiological recovery and axon-myelin remodeling after stroke [27]. Exosomal microRNA 146b promotes the differentiation of endogenous neural stem cells after ischemic stroke [28]. However, few reports to reveal exosomes that can be used in the diagnosis of stroke have been done. In this study, we evaluated the expression profile of aberrantly expressed exosomes extracted from the serum of stroke patients to screen the fast and effective biomarkers of the early diagnosis of stroke. 525 protein groups and 3637 peptides were identified in this study, including proteins involved in biological regulation, response to stimulus, transporter activity, cholesterol metabolism, PPAR signaling pathway and so on.

Exosomes are revealed to participate in antigen presentation, immune response, tumor in-

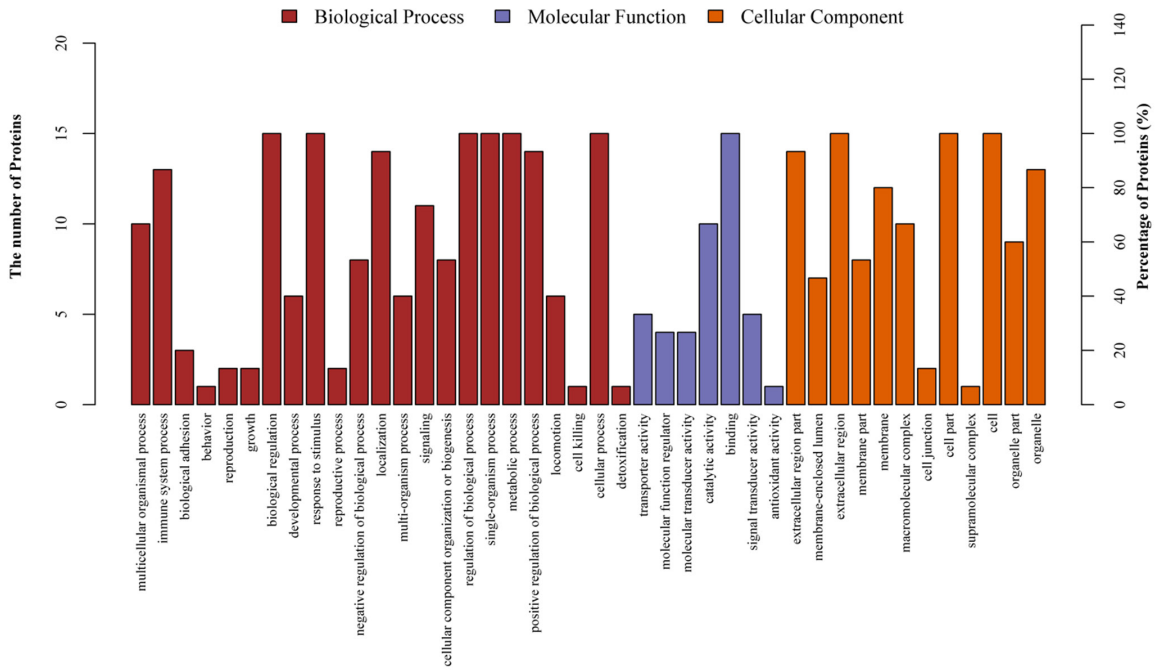

Figure 3. GO enrichment of differentially regulated proteins.

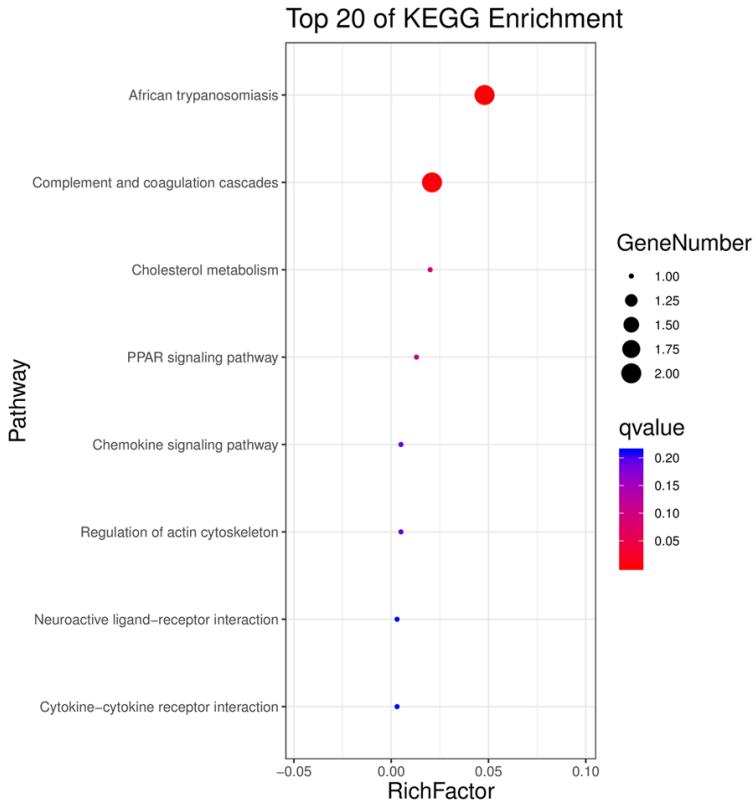

Figure 4. KEGG pathway enrichment analysis of differentially regulated proteins.

vasion, cell migration and so on thus have been one of the research hotspots in the medicine field [29-31]. Serum exosomal long noncoding RNA pcsk2-2:1 is significantly downregulated in the serum of gastric cancer patients and acts as a potential novel diagnostic biomarker for gastric cancer [32]. An increasing number of studies revealed that exosomes play vital roles in the formation of primary liver cancer microenvironment, as well as the development, invasion, progression, metastasis, prognosis, diagnosis and treatment of primary liver cancer, while the specific molecular mechanisms underlying it and the role of exosomes play in these biological processes remain unclear [31]. McKiernan et al (2016) compared the efficiency of combining urine exosomes gene expression assay with standard of care or standard of care alone in prostate cancer. This novel assay together with standard of care improves the accuracy and sensitivity of prostate cancer screening and diagnosis. In this study, among the 525 protein

identified, 15 proteins (fold change > 1.5 and *p*-value < 0.05) that were differentially regulated in stroke patients. The protein expression serum exosomes-encapsulated IGA2, Q6LAM1, F13B, ITIH3, PPBP and HABP2 in stroke patients is increased, while the concentration of exosomes-encapsulated F2, IGL, APOA2, CN3, IGHV2-5, HPR, APOL1, V1-4 and IGKV3-11 is decreased.

Among the upregulated serum exosomes-encapsulated proteins, F13B, PPBP and HABP2 have been reported to be involved in the development of stroke [33-36]. F13B His95Arg variant was more common in stroke patients caused by large vessel disease [33]. HABP2, which encodes an extracellular serine protease, is the risk loci for early-onset stroke [34, 35]. The expression of PPBP is increased revealed by proteomic analysis of plasma from 40 patients with acute ischemic stroke due to large vessel occlusion [36]. It is obvious that exosomes-encapsulated F13B, PPBP and HABP2 play a critical role in the development of stroke. Thus, we speculated that exosomes-encapsulated F13B, PPBP and HABP2 may be the new biomarkers of the fast and variable diagnosis of stroke.

KEGG pathway enrichment analysis revealed 8 pathways that were related with the progression of stroke. Most of these pathways were relevant to neural tissue injury and metabolic disturbance, including cholesterol metabolism, PPAR signaling pathway, neuroactive ligand-receptor interaction, and the role of exosomes is important and complex and demands further studies.

It is undeniable that this study has some shortcomings. First of all, the number of samples collected in this study is not sufficient. Secondly, when it comes to grouping, the stage of stroke patients is not well distinguished. Finally, due to the difficulty of follow-up studies, verification experiment was lacking in this study.

This is the first study reporting the analysis of exosomes extracted from the serum of stroke patients. Our data confirmed that the expression of exosomes-encapsulated F13B, PPBP and HABP2 was significantly upregulated in serum of stroke patients, which indicated that F13B, PPBP and HABP2 had great potential as auxiliary diagnostic markers for stroke.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81760450), Guangxi Natural Science Foundation of China (2017GXNSFAA198322), The First Batch of High level Talent Scientific Research Projects of the Affiliated Hospital of Youjiang Medical University for Nationalities in 2019 (R201963-10), Special Funding for Guangxi Special Experts and Guangxi Medica High-level Leading Talents Training “139” Project.

Disclosure of conflict of interest

None.

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