Isolation and identification of serum exosomes and correlation of its contents with acute ischemic stroke

Pei Yu (pdxyupei@gmail.com)
Youjiang Medical University for Nationalities https://orcid.org/0000-0002-6239-8654
Wencheng Chen
Youjiang Medical University for Nationalities

Research

Keywords: acute ischemic stroke, exosomes, non-coding RNA, Erythrocyte

Posted Date: November 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-108426/v1

Abstract

Background

Erythrocyte deformability is one of the pathophysiological changes of high-risk factors such as smoking, hypertension and atherosclerosis in stroke. It mainly affects blood viscosity and fluidity in the occurrence, development and outcome of the disease. Exosomes are a new type of biological activity test target, and it mainly contents miRNA, but its effect on stroke is still not clear.

Objective

To detect the serum exosome-derived miR-150-5p expression in patients with acute ischemic stroke and explore its diagnostic potential for acute ischemic stroke.

Methods

A total of 84 samples were collected with matched age and gender, collecting relevant laboratory indicators and general clinical data of the research subjects. The kit was used to extract serum exosomes, real-time fluorescent quantitative polymerase chain reaction was used to determine the expression of serum exosomal miR-150-5p, evaluate its value as a diagnostic marker through the ROC curve. Through Randa, Target Scan and other online databases, bioinformatics methods predict the target genes of miR-150, the results are drawn Venny to take intersection analysis, literature screening and ischemic stroke related target genes.

Results

The exosomes showed elliptical or round membranous vesicles with a diameter between 30–200 nm and fusion phenomenon and detected the exosomal marker proteins CD63 and HSP70; Compared with the control group, the relative expression of exosomal miR-150-5p in patients with acute ischemic stroke was increased ($T = 8$, $P < 0.001$). The expression of serum exosomal miR-150-5p in the disease group at different time points after stroke, the difference was not statistically significant(Chi-square = 2.925, $P = 0.232$); the area under the ROC curve was 0.883. bioinformatics prediction analysis of miR-150 target genes, which may be involved in the development of stroke disease through EGR2 and PLP2, as well as erythrocyte membrane protein-related genes SLC4A1 and SPTB also belong to the miR-150 prediction targets.

Conclusion

The expression of exosome-derived miR-150-5p is relatively high in patients with acute stroke, and it has a certain potential for early disease diagnosis.

1. Introduction

Ischemic strokes usually happen when blood vessels are blocked by vascular deposits or thrombus in blood vessels supplying the brain. Without timely elimination of this blockage, brain tissue ischemia may lead to permanent neurological deficit or brain death. Approximately 84% of deaths are caused by ischemic strokes [1]. China is witnessing an accelerated urbanization and aging of the society. As unhealthy lifestyles prevail, a growing population is exposed to cardiovascular risk factors, leading to a surging prevalence rate. The prevalence rate shows rapid growth in low-income groups, indicates significant gender and geographic disparities, and experiences a younger trend. Stroke diagnosis currently relies on imaging techniques. However, it might be difficult for physicians to make objective judgment when symptoms are mild or difficult to distinguish from other neurological and non-neurological disorders. In recent years, epigenetics and gene sequence analysis have been increasingly used as adjuncts in the diagnosis of cardiovascular disease and cancer. MiRNA, in particular, has been indicated to be associated with several risk factors for stroke, including hypertension, diabetes, atherosclerosis, etc[2]. Exosomes are small membrane-bound vesicles secreted by most types of cells. They contain a variety of functional substances that can be used for diagnostic and therapeutic purposes, such as mRNAs and miRNAs [3].

As thrombus persists during ischemic stroke, changes in the erythrocyte membrane and the resulting material may trigger other possible structural and biochemical changes as red blood cells in the circulatory system [4]. It has been shown that hemorheological parameters deteriorate in acute ischemic stroke. For patients with acute cerebral circulation disorders, their blood viscosity increases and erythrocytes aggregate, erythrocyte deformability reduces and plasma fibrinogen levels elevate [5]. As for patients with thromboembolic ischemic stroke, scanning electron microscope observation indicates 92% of abnormal erythrocytes in the smear[6]. The change of erythrocyte deformation capacity affects both the oxygen transport capacity of erythrocytes and the survival rate of circulating erythrocytes, since deformability of erythrocytes plays a crucial role in the circulation of erythrocytes through the microvasculature system and splenic sinusoids. In addition, reduced cellular deformability is closely related with stroke[7]. MiR-150/c-myb interaction during in vitro experiments is also critical for megakaryocyte-erythroid progenitor cell to form in vitro megakaryocyte and for the determination of erythrocytes spectrum[8]. Researchers found that sustained inhibition of miR-150 is essential for normal final erythroid development. It is also validated that 4.1R gene is a new target of miR-150 during terminal erythropoiesis. Its enrichment ensures mechanical stability and deformability of the membrane. It is the first study to investigate the relationship between miRNAs and membrane proteins[9]. There are also studies revealing the essential correlation between miR-150G > A polymorphism and ischemic stroke for the first time, suggesting a higher frequency of miR-150GA genotype in stroke patients, and that miR-150 may play a role in the prevalence of ischemic stroke[10]. It has been verified that that miRNAs in the blood

are primarily derived from exosomes, which are both miRNA concentrates and transport carriers. The miRNA expression levels of exosome reflect the physiological, pathological and functional status of secretory cells, and are considered to be valuable for the diagnosis of relevant diseases[11,12]. However, reports on the expression level of serum exosomal miR-150 in acute stroke patients remain absent. Therefore, in this study, we extracted and identified peripheral serum exosomes from acute stroke patients to detect exosomal miR-150-5p expression, to investigate whether exosomal miR-150-5p is altered in patients with acute stroke and its diagnostic and therapeutic value as a new biomarker.

2. Materials And Methods

2.1Clinical sample collection

Fourty two patients diagnosed with acute ischemic stroke during hospitalization in the Neurology Department of the Affiliated Hospital of Youjiang Medical University for Nationalities in Baise, Guangxi Province between May and November, 2019 were included as AIS group. Another 42 healthy people who visited the hospital during the same period of time for routine physical examination and were denied a history of strokes were included as the control group. Inclusion criteria: 1.Age ≥ 45 years old with no previous history of stroke; 2. First episode with a duration of no more than 72 h; 3. Diagnosis through examination by clinical neurologist and confirmed by MR and CT scans. Exclusion criteria: recurrent stroke or duration of stroke attack over 72 hours, renal or hepatic failure, acute infectious disease, tumor, hematological diseases and patients who are unable to cooperate with physical examinations.

The National Institutes of Health Stroke Scale (NIHSS) [13] was adopted to evaluate the degree of patients with ischemic stroke. Patients' gender, age, and risk factors associated with cerebral infarction including hypertension, hyperlipidemia, diabetes, smoking and drinking history were recorded. Patients in the disease group were divided into subtypes of cardioembolism group (CE), Large artery atherosclerosis group (LA) and small-artery occlusion group (SA) through TOAST typing[14]. This study was approved by the Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities (Grant No. YYFY-LL-2019-001). By strictly following the principles of the Declaration of Helsinki, the patient should sign an informed consent form. Table 1 indicates statistical and clinical data of AIS and control group.

2.2 Serum collection and Exosomes isolation

Collect 5 mL of whole blood in a clean centrifuge tube and leave it for clot to separate out. Centrifuge at 4000 × g,4℃ for 10 min and remove the supernatant. Centrifuge again at 1500 × g,4℃ for 10 min and separate into 1.5 ml enzyme-free centrifuge tubes. Exosomes were extracted from serum using the Exosome Precipitation Reagent Kit (EZB-exo101,EZBioscience,USA) following the manufacturer's protocol. One portion of the extract was used for total RNA extraction and another portion was used for exosome identification.

2.3Transmission electron microscope

The extracted exosome was resuspended in enzyme-free water and dropped on a copper mesh with carbon membrane support for 3–5 min, then remove the excess liquid with filter paper; 2% phosphotungstic acid was dropped on a copper mesh with carbon membrane support for 2–3 min, and the excess liquid was then removed with filter paper. Dried at room temperature, observed under a transmission electron microscope (TEM, HT7700 Hitachi, Japan) and collected images for analysis.

2.4Western blot

Intact receptors and antigens were preserved on the surface of exosomes. CD63, CD9, CD81. Tsg101, Alix, HSP70, etc [15]. are the common key test indicators. RIPA lysis buffer were used to extract exosomal proteins (Biyuntian, Shanghai, China), and protein concentrations were determined by BCA Protein Assay Kit (Biyuntian, Shanghai, China). Skimmed milk was used to seal the blotting membranes and was incubated overnight with CD63 antibodies (1:1000, Abcam, UK), HSP70 (1: 1000,Abcam,UK). Applied HRP-conjugated Goat Anti-Rabbit Secondary Antibody (1□5000□CST□USA) to CD63, and applied HRP-conjugated Goat Anti-Mouse Secondary Antibody to HSP70 for 1 hour (1: 5000, Sanying, Wuhan, China), applied imaging scans to PVDF membranes through gel imaging system.

2.5 Total RNA extraction of serum exosomes

using Exosome RNA Purification kit (EZBioscience, EZB-exo-RN1, USA) according to instructions. Reverse transcription of RNA using miDETECT A Track miRNA qRT-PCR Starter Kit (Ribo, Guangzhou, China) under the following conditions: mixing with a reverse transcription reaction system. The reaction was performed at 42 °C for 1 hour, followed by incubation at 72 °C for 10 minutes to obtain cDNA, SYBR Green was used as a fluorescent molecule using a three-step process: 95 °C, 10 minutes, 1 cycle; 95 °C, 2 seconds, 60 °C, 20 seconds, 70 °C, 10 seconds, 40 cycles; dissolution curve generated. External reference Cel-miR-39-3p (miDETECTTM miRNA External Control, Guangzhou Yuebo) was used for standardized processing, and Roche Lightcycler480 (Roche Applied Science, Germany) was used for qRT-PCR, all steps were performed according to manufacturer's instructions. The relative expression of miRNA was quantified through $2^{-\triangle\triangle}$Ct method.

2.6 Statistics

All data were processed with SPSS 24. If measures conformed to a normal distribution, T-tests of the two independent samples were used to compare differences between the two groups. One-way ANOVA was used to analyze the difference among the two groups. If measures did not conform to a normal distribution, non-parametric test was performed. Counting data were tested with chi-square test. Receiver operating characteristic curve (ROC) was performed to analyze the specificity and accuracy of sensitivity miR-150-5p. All hypothesis tests were bilateral. Results were considered statistically significant when p-value is less than 0.05.

3. Result

3.1 General clinical information and baseline information sheet

The ratios of men and women in the control group and AIS group were 28:14 and 23:19 respectively($p = 0.264 > 0.05$), indicating no statistical significance in the difference. Average age ± standard deviation were 60.05 ± 8.36 and 57.07 ± 6.56 ($p > 0.05$) indicating no statistical significance in the difference. In terms of high-risk factors for stroke, the differences between the two groups show no statistical significance in history of diabetes, smoking, drinking and hyperlipidemia. However, the difference of hypertension between the two groups shows statistical significance ($P < 0.05$) The AIS group demonstrates a higher incidence rate than the control group. Among the relevant laboratory indicators, TG, HDL-C, LDL-C, WBC, and HCT were higher in AIS group, while RBC and PLT counts are higher in control group($P < 0.05$) indicating that the differences were statistically significant. The differences of other laboratory indicators were not statistically significant. Exosomal miR-150 and NIHSS scores show no correlation.

3.2 Exosome identification

Collect sxosomes from the blood serum of the control group and the AIS. Electron microscopy shows oval or round membranous vesicle, similar in shape and size, with a diameter between 30–200 nm. Some of the vesicles are fused, and vesicles are rare in some exosomes(Shao H et al,. 2018) Fig. 1. Since the exosomes are surrounded by a bi-layer lipid membrane, the cystic structure shows a light central color and a dark peripheral color with a clear border. CD63 and HSP70 protein expression can be tested in the exosomes extracted from both control group and AIS group (Fig. 2).

3.3 Expression trends of exosomal miR-150-5p in patients with acute ischemic stroke

The Comparison of the relative expression levels of exosomal miR-150-5p in the two groups was shown in Fig. 3. With cel- miR-39-3p as a control, exosomal miR-150-5p relative expression in the disease group was 1.79 ± 0.45, higher than that of control group, 1.06 ± 0.38. The difference between the control group and the acute disease group was significant ($t = 8.00$, $P < 0.001$). The exosomal miR-150-5p in normal and control groups were monitored at 24, 48 and 72 hours after the onset of acute stroke (Fig. 4). The expression differences at different time were not statistically significant (Chi-square = 2.925, $P = 0.232$). According to TOAST typing (shown in Fig. 5), expression differences of exosomal miR-150-5p in acute ischemic stroke typing were not statistically significant (Chi-square = 2.925, $P = 0.232$).

3.4 ROC curve analysis of exosomal miR-150-5p

Receiver Operating Characteristic curve (ROC) curves are suitable for the analysis and evaluation of diagnostic tests, especially the evaluation and comparison of clinical diagnostic tests. Performed ROC curve analysis to exosomal miR-150-5p (Fig. 6), the area under the curve (Table 2) was 0.883 (95%CI: 0.810–0.956), indicating certain diagnostic value in exosomal miR-150-5p.

3.5 Target gene prediction

The results of miR-150 target genes are predicted based on four commonly used online tools: DIANA predicts 915 target genes for miR-150;miRanda predicts 525 target genes; Pictar predicts 264 target genes; TargetScan predicted 363 target genes. Finally, eight co-expressed target genes (HILPDA, FOXD3, EGR2, PLP2, ELOVL3, ZNF-335, SHISA4, STX5) were analyzed online using Draw Venn Dia-grams. The literature found that EGR2 is closely related to the occurrence and development of stroke, and the red blood cell membrane protein-related genes SLC4A1 and SPTB are also miR-150 targets.

4. Discussion

MiRNAs constitute a kind of regulatory RNA that regulates gene expression through binding to specific mRNA targets and trigger its degradation or inhibit its translation. MiRNAs consist of approximately 21 to 23 nucleotides, and are widely found in multicellular organisms such as virus, plants and human. Growing evidences demonstrate that aberrant miRNA expression is closely related with tumors, cardiovascular disease, viral infections and many other diseases. The miRNAs concentrated in the brain play an important role in the pathophysiological mechanism of stroke. They are stored in blood and cerebrospinal fluid and show stability in long-term storage[16,17]. Since cerebrospinal fluid is in direct contact with the brain, it reflects changes in the brain's substance content. Yet, since the collection of cerebrospinal fluid through lumbar puncture is invasive, blood miRNA is therefore a more effective and ideal biomarker, especially for the old. Notably, circulating miRNAs are primarily derived from exosomal miRNAs. Although circulating miRNAs and exosomal miRNAs are both potential serum markers of stroke, the very same circulating miRNA or exosomal miRNAs during the same disease may be different. Chen F et al [18]studied the severity idiosyncrasy of the change of serum and exosomal miR-126 levels to cerebral ischemia, and found no correlation between blood serum and exosomal miR-126 level. Circulating miRNAs are usually present in exosomes and sometimes bound by certain carrier protein to maintain stable[19,20]. This stability is considered a result of the fact that exosomes wrapping the miRNA and protecting it from interference of external RNA enzymes [21].

Normal morphological function, morphological plasticity and normal flow of red blood cells in the blood vessels, especially at the site of stenosis is an important factor to ensure the smooth flow of blood vessels and the oxygen supply to the corresponding tissues. If the deformability of red blood cells decreases, peripheral resistance might increase and lead to microcirculation disorders, which in turn affects the exchange of substances in tissues and organs and causes a series of pathological and physiological changes. During ischemic stroke, it will result in oxidation of red blood cells and changes in protein hydrolysis, and therefore lead to changes in cellular rheology and inflammatory processes[4]. Regulated miRNA expression is crucial for erythrocyte chromatin condensation and denucleated terminal red lineage differentiation[22]. Research shows that regulation of miR-451 and miR-150 expression may be an effective alternative for stimulatory cytokines to differentiate CD133 + into erthyrocyte-like spectrum[23]. Recent studies have shown that miR-150 inhibits and promotes terminal erythropoiesis, and that the 4.1 R

gene, whose enrichment ensures stability and deformability of membrane, is the new targets of miR-150 during terminal erythropoiesis[24]. Scherrer et al found that among patients suffering from ischemic stroke, miR-150-5p was highly correlated with mortality rate within 90 days[25]. In the rat model that suffered from cerebral ischemia, upregulation of miR-150 expression reduces vascularity in the infarct border zone of rats after MCAO, and decreases tube formation, proliferation and migration of cerebral microvascular endothelial cells[26]. Studies involving post-stroke blood-brain barrier disruption mechanisms during edema and hemorrhagic transformation indicated that miR-150 can affect the blood-brain barrier permeability after permanent middle cerebral artery occlusion in rats[27]. Our study finds that there is a correlation between the relative expression levels of exosomal miR-150 and erythrocyte counts and the expression of hematocrit in acute stroke patients.

The selection of appropriate internal reference impacts the true variation of microRNA expression levels and the biological interpretation of data. MiRNA extraction, initial template amount, transcriptional efficiency and many other factors affect the real-time fluorescence quantification (RT-qPCR). Currently, the internal references of circulating miRNA can be broadly categorized into non-coding RNAs, artificially adulterated exogenous miRNAs, and circulating miRNAs with stable expression. U6 was usually selected as an internal reference gene in non-coding RNAs. As a small RNA with nucleus inside the cell, it is the major component of RNA spliceosome in the post-transcriptional processing of eukaryotic organism. It is about 100–215 nucleotides in length and involves in the processing of mRNA precursors. It is stable, abundant, and highly conserved, and prevails in all eukaryotic cells. Considering repeated freeze-thawing often occurs during experiments, U6 is not suitable to be the research reference of circulating miRNA, since longer RNAs are more likely to be degraded by RNase. And U6 is significantly longer than miRNA. In addition, U6 contains a large number of nucleotides that may be degraded by RNase in blood after separation from carrier proteins during freezing and thawing[28]. Some researchers prefer to use circulating miRNAs with stable expression as internal reference. In the research on the expression of microRNA in the blood serum of Patients with diffuse large B-cell lymphoma by Charles et al, they first used miR-16 as an internal reference. However, some studies show that miR-16 expression is not stable. Ostenfeld et al[29] found that miR-16 was upregulated in the epithelium-derived extracellular vesicles in the blood serum of Patients with rectal cancer. Qian et al [30]considered that miR-16 is down-regulated in rectal cancer tissues and can be used as a biomarker of prognosis. Given the uncertainty of non-coding RNA expression in blood, exogenous miRNAs of the same amount with internal reference were added into serum or plasma samples. Common exogenous miRNAs include cel-miR-39 of the showy primer nematode and lin-4. These exogenous miRNAs have no human homologous gene sequences. A certain amout of exogenous miRNAs were added during RNA extraction, and experience extraction, reverse transcription and PCR quantification together with the target miRNAs to be tested. This method eliminates the bias in the examination and solidates the results. However, it also cumbersomes the experimental procedures, as well as clinical applications. Yet, manual adulterated miRNAs are free heterologous miRNA molecules that are not affected by changes in blood composition and do not reflect the changes in circulating miRNA expression caused by changes in the internal environment of human body, and therefore cannot improve the accuracy of analysis[31]. In our study, cel-miR-39 was chosen as an internal reference. A

certain amount of standard were added during the lifting process to normalize the relative expression levels of exosomal miR-150-5p.The exosomal miR-150-5p relative expression levels of the control group and the acute disease group were significantly different, with the relative expression of the disease group higher than that of the control group. Monitoring of 24 h, 48 h, and 72 h after onset shows that, the difference in relative expression level of exosomal miR-150-5p was not statistically significant. The difference in the relative expression levels of exosomal miR-150-5p in acute stroke patients with TOSE fraction was neither statistically significant.

Some limitations still exist in this study: small sample size limits the overall results, therefore, it is necessary to expand the sample amount to better proof that the upregulation of relative expression level of exosomal miR-150-5p in acute stroke patients is not a coincidence; since all subjects come from the Zhuang ethnic group in Guangxi Zhuang Autonomous Region, the geographical restriction blocks the knowledge of difference among regions and ethnic groups; there is no uniformity in the selection of internal reference genes for exosomal miRNA detection; many functions of the organism do not rely on a single regulator mechanism; functional validation of predicted target genes is not performed and the presence of competing RNAs is not fully understood; we did not trace the origin of exosomes, which varies; whether there are differences or correlations between circulating blood miRNA-150-5p and exosomal miRNA-150-5p could be part of the next study.

5. Conclusion

In summary, exosomal miRNA-150-5p has certain risk-predictive value in acute stroke. Since the functions of miRNAs are realized by regulating the expression of downstream target genes, further studies are required to explore the molecular mechanism of exocomal miR-150-5p during acute ischemic stroke.

Declarations

Conflict of interest disclosure

None of the authors has any conflict of interest to disclose.

Funding

This work was supported by the Scientific Research and Technology Open Plan Project of Baise (grant numbers 20202501).

Ethical approval

This study was approved by the Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities (Grant No. YYFY-LL-2019-001). By strictly following the principles of the Declaration of Helsinki. Each subject signed informed consent and agreed to make the data public.

Guarantor

Wencheng Chen

Contributorship

Wencheng Chen guided and revised manuscript. Yu Pei participated in experiment and wrote the manuscript.

Acknowledgments

We are deeply grateful to all participants of this study.

References

1.Goldstein LB, Adams R, Becker K, et al. Primary prevention of ischemic stroke: A statement for healthcare professionals from the Stroke Council of the American Heart Association. Stroke. 2001;32(1):280-99.

2.Schober A, Weber C. Mechanisms of MicroRNAs in Atherosclerosis. Annu Rev Pathol. 2016;11:583-616.

3.Zhang J, Li S, Li L, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics. 2015;13(1):17-24.

4.Pretorius E, Lipinski B. Thromboembolic ischemic stroke changes red blood cell morphology. Cardiovasc Pathol. 2013;22(3):241-2.

5.Ionova VG. State of the Rheological Properties of Blood in the Dynamics of Development of Acute Disturbances of Cerebral Circulation in Atherosclerosis Patients. Journal of Engineering Physics & Thermophysics. 2003;76(3):672-80.

6.Swanepoel AC, Pretorius E. Scanning electron microscopy analysis of erythrocytes in thromboembolic ischemic stroke. Int J Lab Hematol. 2012;34(2):185-91.

7.Tikhomirova IA, Oslyakova AO, Mikhailova SG. Microcirculation and blood rheology in patients with cerebrovascular disorders. Clin Hemorheol Microcirc. 2011;49(1-4):295-305.

8.Grabher C, Payne EM, Johnston AB, et al. Zebrafish microRNA-126 determines hematopoietic cell fate through c-Myb. Leukemia. 2011;25(3):506-14.

9.Sun Z, Wang Y, Han X, et al. miR-150 inhibits terminal erythroid proliferation and differentiation. Oncotarget. 2015;6(40):43033-47.

10.Choi GH, Ko KH, Kim JO, et al. Association of miR-34a, miR-130a, miR-150 and miR-155 polymorphisms with the risk of ischemic stroke. Int J Mol Med. 2016;38(1):345-56.

11.Balusu S, Van Wonterghem E, De Rycke R,et al. Identification of a novel mechanism of blood-brain communication during peripheral inflammation via choroid plexus-derived extracellular vesicles. EMBO Mol Med. 2016;8(10):1162-83.

12.Ajit SK. Circulating microRNAs as biomarkers, therapeutic targets, and signaling molecules. Sensors (Basel). 2012;12(3):3359-69.

13.Brott T, Adams HP, Jr., Olinger CP, et al. Measurements of acute cerebral infarction: a clinical examination scale. Stroke. 1989;20(7):864-70.

14.Tuttolomondo A, Di Raimondo D, Pecoraro R,et al. Inflammation in ischemic stroke subtypes. Current pharmaceutical design. 2012;18(28):4289-310.

15.Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science. 2020;367(6478).

16.Shao H, Im H, Castro CM, et al. New Technologies for Analysis of Extracellular Vesicles. Chem Rev. 2018;118(4):1917-50.

17.Christensen M, Schratt GM. microRNA involvement in developmental and functional aspects of the nervous system and in neurological diseases. Neurosci Lett. 2009;466(2):55-62.

18.Sorensen SS, Nygaard AB, Carlsen AL,et al. Elevation of brain-enriched miRNAs in cerebrospinal fluid of patients with acute ischemic stroke. Biomark Res. 2017;5:24.

19.Chen F, Du Y, Esposito E, et al. Effects of Focal Cerebral Ischemia on Exosomal Versus Serum miR126. Transl Stroke Res. 2015;6(6):478-84.

20.Squadrito ML, Baer C, Burdet F, Maderna C, Gilfillan GD, Lyle R, et al. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. Cell Rep. 2014;8(5):1432-46.

21.Pegtel DM, Peferoen L, Amor S. Extracellular vesicles as modulators of cell-to-cell communication in the healthy and diseased brain. Philos Trans R Soc Lond B Biol Sci. 2014;369(1652).

22.Li DB, Liu JL, Wang W, et al. Plasma Exosomal miR-422a and miR-125b-2-3p Serve as Biomarkers for Ischemic Stroke. Curr Neurovasc Res. 2017;14(4):330-7.

23.Listowski MA, Heger E, Boguslawska DM, et al. microRNAs: fine tuning of erythropoiesis. Cell Mol Biol Lett. 2013;18(1):34-46.

24.Kouhkan F, Hafizi M, Mobarra N, et al. miRNAs: a new method for erythroid differentiation of hematopoietic stem cells without the presence of growth factors. Appl Biochem Biotechnol. 2014;172(4):2055-69.

25.Scherrer N, Fays F, Mueller B, et al. MicroRNA 150-5p Improves Risk Classification for Mortality within 90 Days after Acute Ischemic Stroke. J Stroke. 2017;19(3):323-32.

26.He QW, Li Q, Jin HJ, et al. MiR-150 Regulates Poststroke Cerebral Angiogenesis via Vascular Endothelial Growth Factor in Rats. CNS Neurosci Ther. 2016;22(6):507-17.

27.Fang Z, He QW, Li Q, et al. MicroRNA-150 regulates blood-brain barrier permeability via Tie-2 after permanent middle cerebral artery occlusion in rats. FASEB J. 2016;30(6):2097-107.

28.Xiang M, Zeng Y, Yang R, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun. 2014;454(1):210-4.

29.Ostenfeld MS, Jensen SG, Jeppesen DK, et al. miRNA profiling of circulating EpCAM(+) extracellular vesicles: promising biomarkers of colorectal cancer. J Extracell Vesicles. 2016;5:31488.

30.Qian J, Jiang B, Li M, et al. Prognostic significance of microRNA-16 expression in human colorectal cancer. World J Surg. 2013;37(12):2944-9.

31.Chen X, Liang H, Guan D, et al. A combination of Let-7d, Let-7g and Let-7i serves as a stable reference for normalization of serum microRNAs. PLoS One. 2013;8(11):e79652.