Expression Profile of Exosome-associated MicroRNA Derived from Human Red Blood Cell Suspensions During Storage: Predicting microRNA-1246 and microRNA-150-3p Exert Essential Roles in Transfusion-related Immunomodulation

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Research

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Abstract

Background: Transfusion-related immunomodulation (TRIM) can be caused by exosomes and microRNA (miRNA) is one of the critical functional components in exosomes. This study intends to investigate the differences in the expression of exosomal miRNAs in red blood cell (RBC) suspensions at different storage times, also the potential functions related to TRIM of the abundant miRNAs.

Methods: Twenty-five bags of RBC suspensions were selected randomly, and exosomes were separated by ultracentrifugation at different storage times. Isolated exosomes were identified by Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and Western Blot (WB). Exosomal miRNA profiles were analyzed using genechip in 5 RBC suspension samples, and the miRNA expressions between different storage times were compared. For the statistically upregulated microRNAs, the bioinformation of their predicted target genes was analyzed. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to identify the miRNAs upregulated more than 10 folds at 5weeks storage time in 20 RBC suspension samples.

Results: The detection of the gene chip showed most exosomal miRNAs were up-regulated as storage time increases. Compared to RBC suspensions stored for 1 week, that kept for 5 weeks had 539 differential miRNA expressions, among which 159 were significantly different (P<0.05) and 148 (93.08%) were up-regulated. For the bioinformatic analysis, significant immunoregulatory annotations related to thyroid hormone, mitogen-activated protein kinase (MAPK), focal adhesion, and ras signaling pathway were found. The top 17 miRNAs were validated by qRT-PCR, and the results showed miRNA-1246 and miRNA-150-3p were the most significantly enriched miRNAs (more than 150 folds during 5weeks storage).

Conclusions: As storage time increased, various exosomal miRNAs in RBC suspensions accumulated and involved multiple immuno-signaling pathways. The predominantly accumulated miRNA-1246 and miRNA-150-3p were confirmed to participate in pro-inflammation responses and immune-regulation, which might exert essential roles in TRIM.

Background

Red blood cell (RBC) suspension is commonly transfused in the clinic for patients suffering anemia. It can be stored for 35 days at 4°C in an additive solution with A-form of acid-citrate-dextrose (ACD-A), and the storage time can extend to 42 days with saline adenine glucose mannitol (SAG-M) [1]. During storage, RBCs undergo a series of morphological and biochemical changes known as RBC storage lesions which affect the quality and functions of RBCs [2]. The effects of RBC storage time on the clinical outcomes of individuals have always been an active and controversial topic in the clinical community [3]. Some studies have reported that aged RBC transfusion can increase the incidence of complications and adverse clinical outcomes. Czubak-Prowizor K, et al. reported that patients with cardiovascular disorders who received “older” RBC transfusion might be associated with thrombotic complications [4]. For trauma

patients, aged RBC transfusion may increase the risk of deep vein thrombosis and bacterial infections [5, 6]. These adverse complications may be related to the changes in RBC and bioactive substances accumulation during the storage period [7].

Subsequently, transfusion-related immunomodulation (TRIM) as a potential explanation for numerous clinical outcomes has emerged. TRIM was first described as immunosuppression function in renal transplantation patient received perioperative blood transfusion [8]. TRIM was also reported to be associated with increases in tumor incidence, pathogen infection, and mortality [9, 10]. The mechanism of TRIM, especially how stored RBC transfusion interacts with the recipients' immune cells and whether the interactions are related to poorer clinical outcomes, needs further investigation [11]. Exosomes, a kind of bioactivity mediators, are produced continuously during RBC suspensions' storage and function as a tool of intercellular communication and molecular transfer. The generation of exosomes is thought to be related to TRIM in blood recipients. It was reported that exosomes from RBC suspensions were capable of promoting inflammatory cytokine secretion and mediating T-cell proliferation responses [12]. However, the mechanism of RBC suspension-derived exosomes involved in TRIM remains a challenge that requires further investigation. Previous researches on exosomes mainly focused on the proteins they contain. While recently, exosomal miRNAs have attracted wide attention because of their regulatory functions in gene expression. MiRNAs can be encapsulated in exosomes and stably exist in the circulatory system or can be used as endogenous miRNA through the fusion of exosomes and target cells [13]. MiRNAs act as crucial regulators in many biological processes, such as cell differentiation, signal transduction, and immunomodulation [14]. This study intends to analyze the dynamic alterations in the expression of exosomal miRNAs derived from RBC suspensions during different storage times. Furthermore, bioinformatic analysis is adopted to predict potential functions associated with TRIM of the miRNAs accumulated significantly.

Materials And Methods

Study samples

Twenty-five bags of randomly selected RBC suspensions with anticoagulants (ACD-A) were provided by the Central Blood Bank of Deyang City, Sichuan Province, China. Exosomes from five bags were used for gene chip, and the others were used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation of miRNA expressions. Meanwhile, the research protocol was approved by the ethics committee of the Institute of Blood Transfusion, Chinese Academy of Medical Sciences.

Storage of RBC suspensions and Isolation and Purification of Exosome

RBC suspensions were split into 100mL aliquots in100-mL transfer bags and stored at 4°C. The aliquots of RBC samples were used for exosome isolation after storing for 1, 3, and 5 weeks. The aliquots of RBC suspensions were centrifuged at 3000 g for 20 minutes to obtain supernatants. Exosomes were isolated from the supernatants using the ultracentrifugation method, with an initial speed at 300g for 10 min, followed by sequential centrifugation at 2000g for 10 min, 10,000g for 30min to separate supernatant,

then 100,000g for 70 min to separate exosome pellets. Exosome precipitation was resuspended in 200 μL of phosphate-buffered saline (PBS), and 0.22-μm centrifugal filter (Millipore, USA) was used to remove large extracellular vesicles (EVs).

Identification of Exosome

The isolated exosomes from RBC suspensions were verified by Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and Western Blot analysis (WB).

The particle size distribution was measured by NTA with NanoSight NS300 instrument (Malvern Instruments, UK). The size of the particles was analyzed using DTS v5.10 software (Malvern Panalytical, UK) and the results were given as particle size distribution.

For TEM analysis, copper grids were placed in the exosome suspensions fixed with 2% paraformaldehyde overnight. The morphology of the isolated exosomes was visualized by TEM (FEI Tecnai™ G2 Spirit, Czech Republic) at 80 kV.

Exosomes were treated with RIPA lysis buffer, and protein concentration was calculated using the method of bicinchoninic acid (BCA). Absorbance values were detected by Varioskan LUX (Thermo Fischer Scientific, USA). Exosome protein markers against TSG101 (Abcam, UK), CD9 (Abcam, UK), and CD63 (Santa Cruz, USA) were validated by WB analysis. Protein marker against Calnexin was also detected as a negative control. Lysates of Hela cells were tested as control samples. Signals of the membranes were captured and imaged by ChemiScope Mini 3000 (CLINX, Shanghai).

RNA isolation and cDNA preparation

Total RNA was extracted from exosomes using QRIzol reagent and miRNeasy Mini Kit (Qiagen, German). The quantity and quality of the total extracted RNA were measured by using Agilent 4200 platform and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA). Only high-quality RNA samples were used for subsequent experiments. The miScript II RT Kit with miScript hispec buffer (Qiagen, German) was used to prepare cDNA for subsequent gene chip and qRT-PCR validation.

MiRNA profiling

Microarray analysis was performed by Gminix Biotechnology Company (Shanghai, China) using Affymetrix GeneChip miRNA 4.0 array (Affymetrix, USA). Gene chips were then scanned using the GeneChip® Scanner 3000 7G (Affymetrix). CEL-files of the raw data were exported and then uploaded to the website, Gminix-Cloud Biotechnology Information (GCBI), of Genminix Informatics Co., Ltd. (Shanghai, China; http://www.gcbi.com.cn) for further analysis. The data were analyzed through the Robust Multichip Analysis algorithm (RMA) using Affymetrix default analysis settings and global scaling as a normalization method. Then we used the successive approximation method (SAM) for differentially expressed miRNAs analysis. According to the filter condition | Fold Change | >1.2 & *P* value <0.05, the final differential result was obtained.

Bioinformatics analysis of differentially expressed exosomal miRNAs

The target genes of differentially expressed exosomal miRNAs were predicted by miRBase and TargetScan. The gene ontology (GO) analysis of target genes was performed using a $P<0.05$ to define statistically enriched GO categories. Pathway analysis was used to determine the significant pathway of the differential genes according to the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/). The miRNAs–genes network was built to show the interactions of miRNAs and their target genes.

Identification of miRNA expression by qRT-PCR

According to the results of gene chip, there were 17 miRNAs upregulated more than 10 folds at 5weeks compared to 1week storage time. These miRNAs were selected and their expression changes were validated by qRT-PCR. Twenty bags of RBC suspension samples were used for validation. qRT-PCR was performed following the manufacturer's protocol of miScript SYBR PCR kit (Qiagen, German) and using different miRNA primers for each cDNA sample of reverse transcription. The miRNA qPCR primers were synthesized by RIBOBIO Biotech (Guangzhou, China). Cel-miRNA-39 (Qiagen, German) was used to normalize potential sample-to-sample variations and technical variations. Relative expression changes of miRNAs were analyzed through the method of $2^{-\Delta\Delta CT}$.

Results

Analysis and characterization of exosomes derived from RBC suspensions

NTA, TEM, and WB were used to identify and analyze the characterization of exosomes isolated from RBC suspensions. NTA for particle size distribution showed that the isolated vesicles from RBC suspensions at 5weeks storage had typical exosome size, with particle diameters ranging from 30 nm to 150 nm (Fig. 1a). The average diameter of the exosome particles was 76.07 ± 13.65 nm. The size distribution indicated that more than 95% of the exosome particles were less than 120 nm, and 99.98% of the particles had diameters less than 150 nm. TEM showed that the exosome's morphology was cup-shaped (Fig. 1b**)**. Moreover, the detection of exosomal surface markers (CD9, CD63, and TSG101) by WB demonstrated that all the specific protein markers were positive and the protein marker of Calnexin was negative (Fig. 1c).

Comparison of differentially expressed exosome-associated microRNAs during RBC suspension storage

With the use of gene chip, the differential expression analysis of miRNA in RBC suspension showed that 539 different miRNAs were identified in total from 1week to 5weeks during storage time. Figure 2a and Fig. 2b gave information about group-specific signal intensities of the exosomal miRNA profile and the volcano plot between 1week and 5weeks storage time. The statistical analysis revealed that 159 miRNAs expression (148 up-regulated and 11 down-regulated) were significantly different (fold change>|1.2| and $P< 0.05$). And there were 59 differential miRNAs that up-regulated more than 5 times at 5 weeks of

storage time. Table 1 listed the mean signal, fold changes, and *P*-value of miRNA expression in 1 and 5weeks' storage of RBC suspensions.

Table 1
List of differential exosomal miRNAs upregulated more than 5 folds at 5weeks vs. 1week storage time of RBC suspensions

miRNA	5weeks log_2 mean signal	1week log_2 mean signal	5weeks vs. 1week fold change	*p*-value
hsa-miR-6824-5p	6.3266	1.7901	23.2080	0.0019
hsa-miR-6716-5p	7.7924	3.3319	22.0159	0.0022
hsa-miR-1246	8.4004	3.9677	21.5962	0.0031
hsa-miR-939-5p	6.9238	2.6953	18.7462	0.0020
hsa-miR-4433-3p	7.4835	3.2970	18.2076	0.0021
hsa-miR-4701-3p	6.6046	2.5412	16.7191	0.0026
hsa-miR-6849-5p	5.9334	1.9205	16.1438	0.0021
hsa-miR-595	5.7246	1.9235	13.9395	0.0024
hsa-miR-150-3p	6.0808	2.2974	13.7697	0.0023
hsa-miR-7844-5p	5.6850	1.9356	13.4488	0.0023
hsa-miR-6798-5p	8.3634	4.6340	13.2639	0.0025
hsa-miR-4689	7.9570	4.2640	12.9339	0.0031
hsa-miR-1180-3p	6.1168	2.4509	12.6924	0.0028
hsa-miR-3064-5p	5.4298	1.8060	12.3269	0.0024
hsa-miR-1225-5p	6.5773	3.0099	11.8550	0.0029
hsa-miR-6819-5p	5.6768	2.2801	10.5317	0.0027
hsa-miR-1268b	7.3709	4.0387	10.0714	0.0037
hsa-miR-3148	5.0453	1.7497	9.8194	0.0030

miRNA	5weeks log_2 mean signal	1week log_2 mean signal	5weeks vs. 1week fold change	*p*-value
hsa-miR-1224-5p	4.9115	1.6434	9.6334	0.0028
hsa-miR-4417	4.7814	1.5850	9.1671	0.0026
hsa-miR-4433b-3p	8.7068	5.5427	8.9642	0.0034
hsa-miR-6782-5p	6.3763	3.3114	8.3684	0.0036
hsa-miR-6127	5.0514	2.0232	8.1578	0.0033
hsa-miR-5195-3p	4.7029	1.6900	8.0716	0.0044
hsa-miR-1268a	7.3690	4.4131	7.7597	0.0047
hsa-miR-4322	4.5703	1.6310	7.6704	0.0032
hsa-miR-1909-3p	6.4341	3.4984	7.6510	0.0043
hsa-miR-8089	5.7973	2.8943	7.4794	0.0045
hsa-miR-3648	4.8948	1.9933	7.4719	0.0033
hsa-miR-574-5p	4.4224	1.5850	7.1476	0.0039
hsa-miR-6802-5p	6.7645	3.9302	7.1321	0.0061
hsa-miR-3197	5.0004	2.1880	7.0244	0.0055
hsa-miR-4440	4.8204	2.0191	6.9707	0.0049
hsa-miR-3663-3p	4.9899	2.2004	6.9141	0.0045
hsa-miR-6808-5p	4.7275	1.9522	6.8462	0.0038
hsa-miR-4649-5p	7.4336	4.6594	6.8411	0.0042
hsa-miR-206	4.3141	1.5850	6.6306	0.0048
hsa-miR-297	5.1536	2.4291	6.6094	0.0056
hsa-miR-670-5p	4.3078	1.5850	6.6015	0.0039

miRNA	5weeks log_2 mean signal	1week log_2 mean signal	5weeks vs. 1week fold change	*p*-value
hsa-miR-3149	4.3011	1.5850	6.5711	0.0041
hsa-miR-6763-5p	5.1502	2.4480	6.5080	0.0040
hsa-miR-483-5p	4.4704	1.7748	6.4784	0.0046
hsa-miR-6752-5p	9.9830	7.2890	6.4716	0.0047
hsa-miR-5196-5p	4.7928	2.1296	6.3346	0.0041
hsa-miR-6871-5p	4.3557	1.7197	6.2161	0.0034
hsa-miR-7114-5p	4.1999	1.5850	6.1260	0.0048
hsa-miR-6856-5p	4.3709	1.7587	6.1144	0.0060
hsa-miR-6861-5p	4.3169	1.7292	6.0116	0.0036
hsa-miR-7107-5p	6.6870	4.1313	5.8794	0.0087
hsa-miR-6781-5p	4.9498	2.4063	5.8303	0.0064
hsa-miR-3679-5p	4.9067	2.3821	5.7543	0.0065
hsa-miR-3188	4.7797	2.2552	5.7534	0.0065
hsa-miR-4793-3p	4.7511	2.2808	5.5415	0.0071
hsa-miR-1290	4.0241	1.5850	5.4232	0.0053
hsa-miR-572	6.4109	3.9902	5.3546	0.0138
hsa-miR-6870-5p	4.2777	1.8887	5.2382	0.0057
hsa-miR-3180	4.8989	2.5145	5.2213	0.0070
hsa-miR-3180-3p	5.6635	3.3025	5.1374	0.0089
hsa-miR-7109-5p	4.0591	1.7284	5.0304	0.0058

Bioinformatic analysis-GO enrichment and KEGG pathway of predicted target genes of the significantly differentially up-regulated miRNAs stored for 5 weeks

To further determine the function of the significantly up-regulated miRNAs within 5 weeks of storage time, we used miRanda and Targetscan database to predict target genes of these miRNAs and found out 5538 non-repeated genes.

GO analysis described the function of predicted target genes, and the GO database mainly includes biological pathways, cellular components, and molecular functions. The results of GO enrichment analysis indicated that the target genes of the significantly increased miRNAs were primarily involved in RNA polymerase II transcription cofactor activity, chromatin DNA binding, transcription factor activity, RNA polymerase II transcription factor binding, etc. (biological pathways), presynaptic active zone, presynaptic membrane, phosphatase complex, etc. (cellular components), and negative regulation of neurogenesis, negative regulation of nervous system development, negative regulation of cell development, etc. (molecular functions) (Fig. 3a). KEGG pathway analysis showed that the most significant pathway was the thyroid hormone signaling pathway (Channel ID: 04919), which was correlated with growth, development, and metabolism. However, the target genes of differential miRNAs were mainly enriched in the MAPK signaling pathway (Path ID: 04010), which played important roles in various cellular functions (Fig. 3b). Other pathways significantly enriched in the miRNAs included focal adhesion (Path ID: 04510) and Ras signaling pathway (Path ID: 04014). Figure 4 described the network of the top 17 miRNAs that up-regulated more than 10 times in 5 weeks of storage time, together with their target genes.

Verification of miRNAs expression by qRT-PCR

The top 17 miRNAs which accumulated more than 10 folds at 5weeks of storage time compared to 1week were selected for validation and 20 RBC suspension samples were used for each miRNA identification. The results were expressed as relative expression changes, showing that all the 17 miRNAs expression were increased as storage time goes on and the accumulation of miRNAs at 5weeks compared to 1week were all statistically significant ($P < 0.01$). According to the validation of qRT-PCR, the average changes of 6 miRNAs were more than 10 folds at 5weeks storage time, which were miRNA-1246, miRNA-150-3p, miRNA-3064-5p, miRNA-1225-5p, miRNA-6849-5p, and miRNA-4701-3p (Fig. 5). Among these miRNAs, there were two miRNAs accumulated more than 150 times at 5weeks compared to 1week, with average data 275.94 times of miRNA-1246 and 150.93 times of miRNA-150-3p.

Discussion

Although RBC transfusion can help improve hypoxic symptoms, it may bring some adverse reactions to patients, such as acute hemolytic transfusion reactions, delayed hemolytic (or serologic) transfusion reactions [15]. Loads of evidence support that RBC transfusion has connections with immunomodulation

through various mechanisms or RBC-derived mediators. Recently, interest in the relationship between extracellular vehicles from blood components and TRIM has emerged from the academic circle [16, 17]. Some studies have found that exosomes in the blood may promote TRIM through the production of pro-inflammatory cytokines and immune activation [11, 12]. However, this mechanism is still an open issue that needs further exploration. As a potential functional molecule in exosomes, miRNA was investigated in this study. Exosomal miRNA profiles of RBC suspension at different storage times were analyzed, and the functions of the predicted target genes of the significantly accumulated miRNAs were annotated.

RBC suspensions-derived exosomes were isolated from RBC supernatant by Ultracentrifugation and the characteristics of exosomes were identified based on NTA, TEM, and WB analysis. According to the results of the gene chip, the exosome-associated miRNAs were listed, which were expressed at different storage times of the RBC suspensions and their expressions were different. Compared with the RBC stored for 1week, the RBC stored for 5weeks had 159 miRNAs with significantly different expressions. And 148 of those 159 miRNAs were up-regulated. Perhaps these accumulated miRNAs were related to the adverse clinical outcomes of aged blood transfusions, requiring further investigation.

GO and KEGG were used to predict the potential functions of the target genes of those abundant miRNAs within 5 weeks' storage time. Results found out that the encoded proteins mainly came from synapse-related proteins and phosphatase complexes (presynaptic active zone, presynaptic membrane, phosphatase complex, protein serine/threonine phosphatase complex), which had transcription factor activities and DNA binding functions that were involved in different system development, hormone secretion, and transportation. We made use of the KEGG database to further annotate the target genes and revealed significant enrichment in the thyroid hormone signaling pathway. Thyroid hormone is closely correlated with immunoregulation and plays an essential role in several inflammatory-related processes, such as the production of cytokines and chemokines [18]. MAPK signaling pathway took the first when it comes to the enrichment of target genes. MAPK signaling pathway contains three main kinases, namely MAPK kinase kinase, MAPK kinase, and MAPK. They activate and phosphorylate downstream proteins to regulate and control various physiological and pathological functions, including innate immunity, inflammation, apoptosis, cell growth, cell differentiation, tumor invasion and metastasis, etc [19, 20]. Besides, Focal adhesion and Ras signaling pathway were also significant and enriched signaling pathways, and both of them played important roles in immunomodulation. Focal adhesion participates in multiple biological processes including cell proliferation, regulation of gene expression, etc., and serves as a scaffold for many signaling pathways [21]. Ras protein with GTPase activity acts as a switch protein in the cell signaling pathway, regulating cell differentiation, proliferation, apoptosis, survival, etc. [22]. Recent researches also clarified that Ras played a vital role in the formation of exosomes, and may play a signaling role through exosome secretion [23]. The target genes of miRNAs accumulated during storage were significantly enriched in the above signaling pathways, and these signaling pathways played important roles in the immune system. It can be deduced that by regulating these signaling pathways, exosomal miRNAs up-regulated in the old RBC suspensions were involved in TRIM.

RBC suspensions stored for 5weeks had 17 significantly different miRNAs upregulated more than 10 times compared with those of RBC suspensions stored for 1week. Afterward, qRT-PCR was conducted to validate those miRNA expressions in exosomes from RBC suspensions. The results identified 6 miRNAs accumulated more than 10 folds at 5weeks storage time. According to the upregulated expression level from high to low, they were miRNA-1246, miRNA-150-3p, miRNA-3064-5p, miRNA-1225-5p, miRNA-6849-5p, and miRNA-4701-3p, respectively. These miRNAs have been reported to play essential roles in immuno-regulation and tumorigenesis. Such as in dilated cardiomyopathy (DCM), miRNA-3064-5p, miRNA-6849-5p and miRNA-4701-3p were upregulated in abnormal activated CD4 + T cells which may be associated with the proliferation of CD4 + T cells [24]. MiRNA-1225-5p displays various impacts on the growth, invasion and metastasis of different malignancies by targeting specific proteins [25, 26, 27]. MiRNA-3064-5p, which upregulated more than 20 times during RBC suspension storage helped to inhibit the development of hepatocellular carcinoma through angiogenesis suppression, which might provide potential molecular therapy for tumors [28].

MiRNA-1246 and miRNA-150-3p were the two miRNAs that significantly accumulated more than 150 folds at 5weeks compared to 1week storage of RBC suspensions. Exosome-derived miRNA-1246 in hypoxic glioma was shown to be related to the induction of M2 macrophage polarization by targeting TERF2IP [29]. In addition, miRNA-1246 also mediates lipopolysaccharide (LPS)-induced lung injury and neutrophil activation by targeting angiotensin-converting enzyme 2 (ACE2) [30]. MiRNA-150-3p was reported to exert important roles in anti-apoptosis process [31] and also involved in inflammation pathways [32]. By inhibiting miRNA-150-3p expression, sacubitril/valsartan can effectively alleviate cyclophosphamide-induced lung inflammation [32]. Thus, it can be seen that these two miRNAs were all associated with pro-inflammatory responses and might promote acute lung injury. Transfusion of aged RBC suspensions with these two obvious enriched miRNAs may exert essential roles in individuals' immuno-regulatory responses. The effect and mechanism of miRNA-1246 and miRNA-150-3p in TRIM or transfusion-related adverse reactions still need further investigation.

Conclusions

This study discovered that many exosome-associated miRNAs isolated from RBC suspensions could be accumulated during storage. Analysis of these miRNAs illustrated that they had multiple functions, and some certain miRNAs, such as the obvious upregulated miRNA-1246 and miRNA-150-3p might be essential mediators of immunoregulation. Further researches focus on the relationship between these miRNAs and TRIM will be conducted.

Abbreviations

TRIM: Transfusion-related Immunomodulation

RBC: Red Blood Cell

miRNA: microRNA

NTA: Nanoparticle Tracking Analysis

TEM: Transmission Electron Microscopy

WB: Western Blot

qRT-PCR: Quantitative Reverse Transcription-polymerase Chain Reaction

MAPK: Mitogen-activated Protein Kinase

ACD-A: A-form of Acid-citrate-dextrose

SAG-M: Saline Adenine Glucose Mannitol

PBS: Phosphate-buffered Saline

EV: Extracellular Vesicle

BCA: Bicinchoninic Acid

RMA: Robust Multichip Analysis

SAM: successive approximation method

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

DCM: Dilated Cardiomyopathy

LPS: Lipopolysaccharide

ACE2: Angiotensin-converting Enzyme 2

Declarations

Ethics approval

The research was approved by the ethics committee of the Institute of Blood Transfusion, Chinese Academy of Medical Sciences.

Consent for publication

All authors have read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have disclosed no conflicts of interest.

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Authors' contributions

Yujie Kong was responsible for Experiment performance, data collection and analysis, and original draft written. Xue Tian, Rui He, Chenyue Li and Haixia Xu were responsible for data curation and analysis; Li Tian and Zhong Liu contributed to the study design and manuscript revision. All authors read and approved the final manuscript.

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Figures

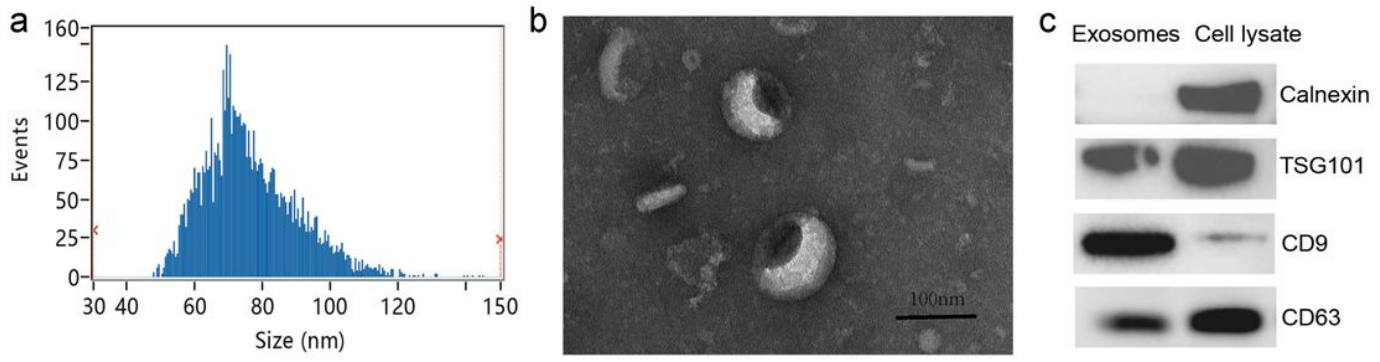

Figure 1

Characterization of exosomes isolated from RBC suspensions at 5weeks storage time. (a) NTA for particle size distribution of exosomes. (b) TEM for morphology of exosomes. Scale bar,100nm. (c) WB identified the specific immunological markers TSG101, CD9, and CD63 of exosomes.

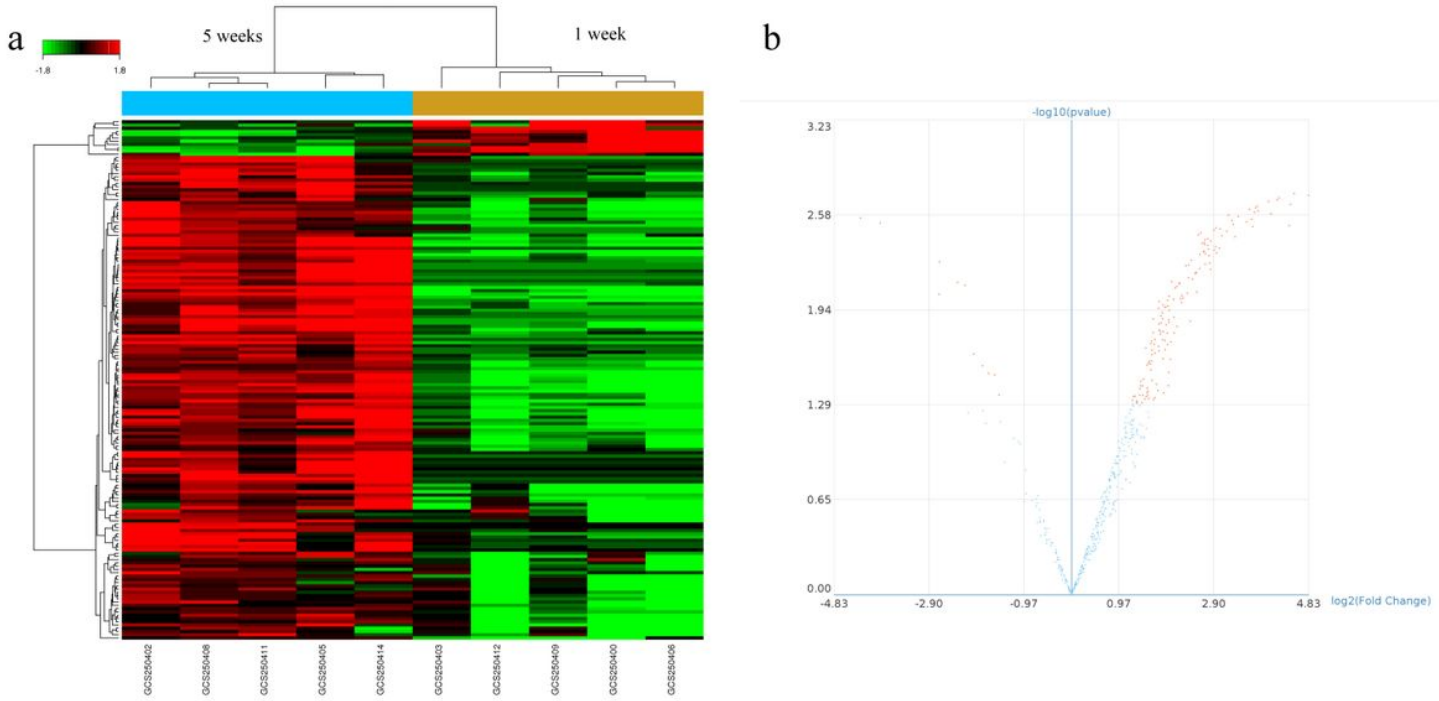

Figure 2

Microarray assay of differential miRNAs in exosomes derived from RBC suspensions. (a) Heatmap of differential miRNAs expressed in exosomes stored for 5 weeks vs. 1 week. Each column represents an RBC suspension sample and each row represents a kind of miRNA. Color describes the expression level of each miRNA in each sample and red indicates high level expression of the differential miRNA, whereas green indicates low expression. (b) Volcano plot shows the distribute expression of differential miRNAs.

Different color shows the expression level and red represents the significantly differential miRNAs (P<0.05).

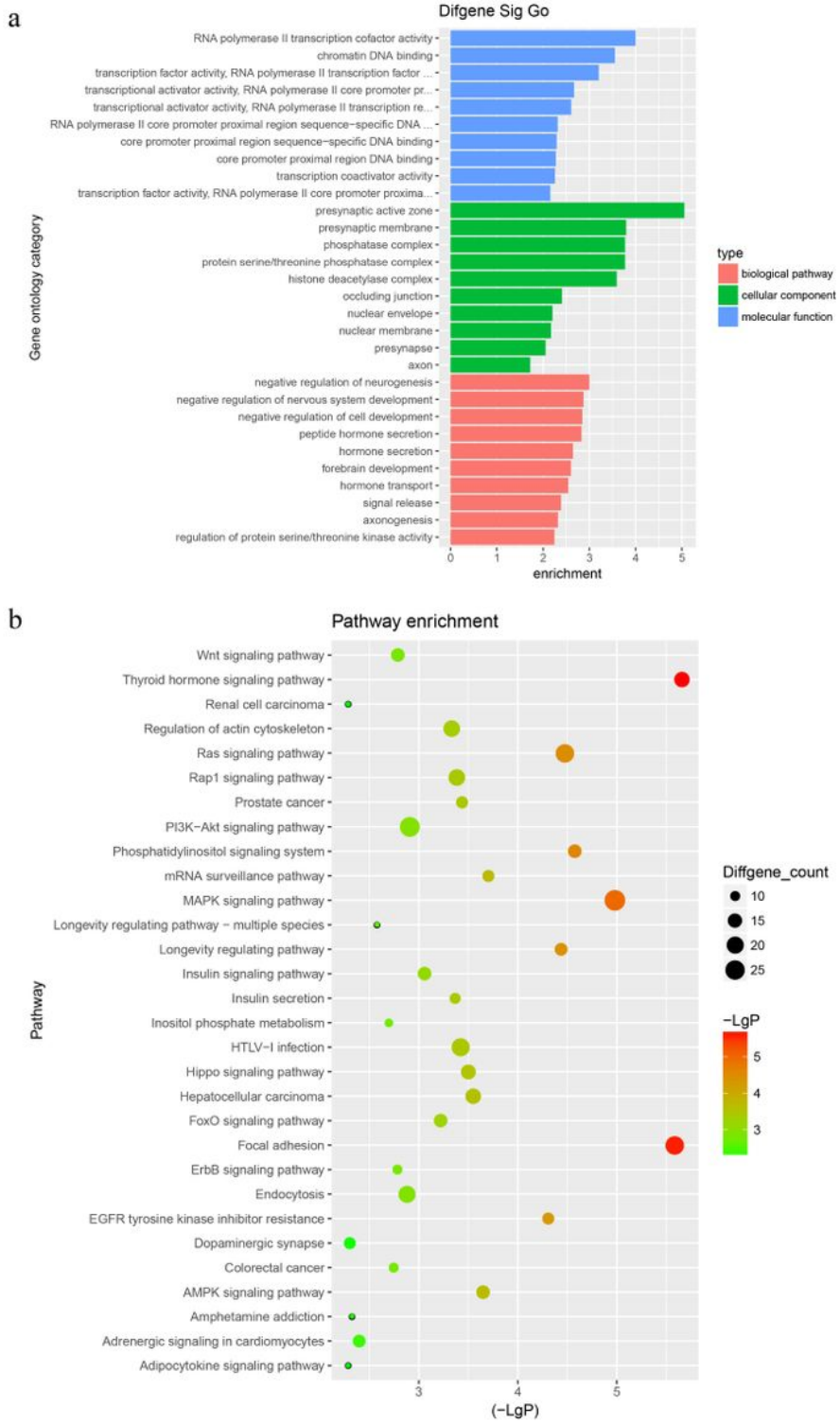

Figure 3

Prediction of functions and signal pathways of target genes for significant upregulated exosomal miRNAs by GO and KEGG pathway analysis. (a) The GO function enrichment terms of target genes for the significant upregulated miRNAs in 5weeks vs. 1week storage time; The function terms were sorted

from high to low by enrichment scores. (b) The KEGG pathway analysis of target genes for the significant upregulated miRNAs in 5weeks vs. 1week storage time. The dot color represents -log (p value) and the dot size represents the number of target genes included in the pathway.

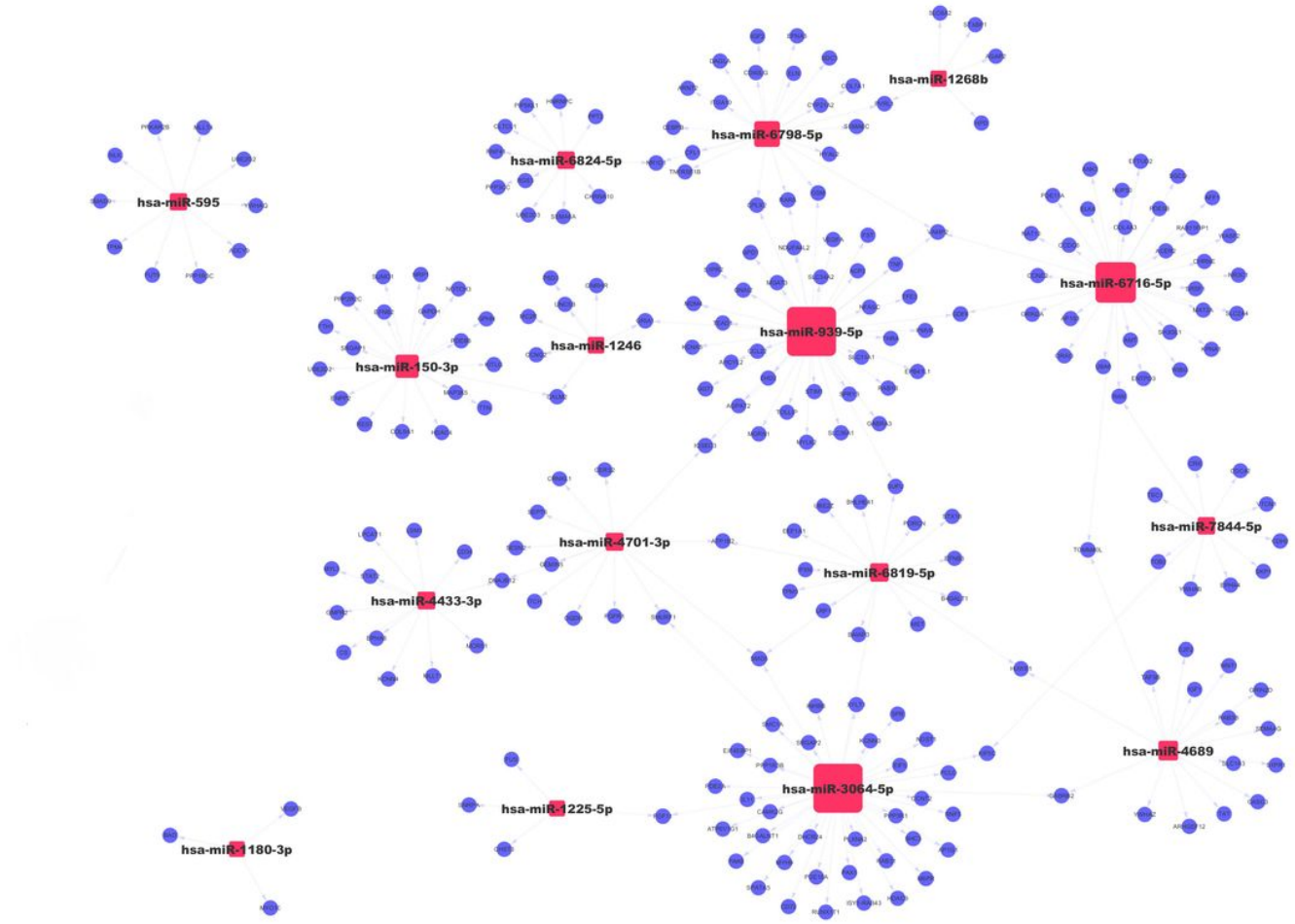

Figure 4

The networks of the top 17 exosomal miRNAs that upregulated more than 10 folds in 5weeks vs. 1week storage time with the target genes.

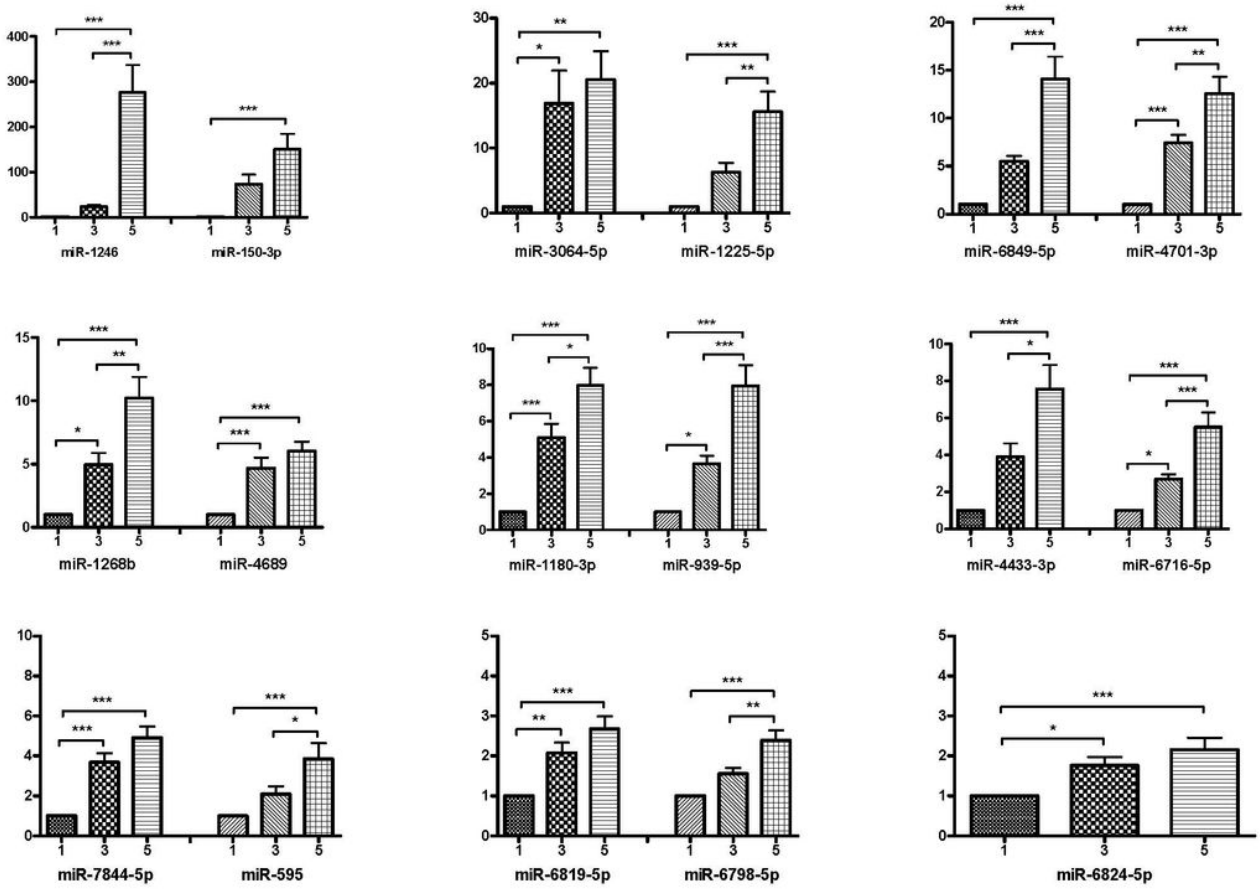

Figure 5

Validation of exosomal miRNAs upregulated more than 10 folds in 5weeks vs. 1week storage time (*P<0.05, **P<0.01, ***P<0.001). The horizontal axis represents storage time, with 1week at 1, 3weeks at 3 and 5weeks at 5. The vertical axis represents relative expression changes.