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Expression and Bioinformatics Analysis of Key miRNAs in Stored Red Blood Cells

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Keywords
MicroRNAs · Erythrocyte · Bioinformatics analysis

Abstract
Introduction: Erythrocyte transfusion is the most common therapeutic procedure in hospitalized patients. Adding standard preservatives to red blood cells allows them to be stored for up to 42 days. However, whether storage has an effect on the erythrocyte transcriptome has not been well-studied. ***Objective:*** This study was designed to explore the change of key risk microRNA (miRNAs) in stored erythrocytes. ***Methods:*** We reanalyzed differentially expressed genes in the gene expression dataset GSE114990 and predicted their target genes, followed by experimental Gene Ontology (GO) analysis and (Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Furthermore, the PPI network of target genes was constructed by the STRING database, and the module analysis was carried out. ***Results:*** We found two differential miRNAs, which were hsa-miR-1245a and hsa-miR-381. Enrichment analysis of GO and KEGG pathways confirmed that these target genes were significantly enriched in organ and system development, anchoring junction, transcription factor binding, and pathways of cancer. ***Conclusion:*** The results suggest that the miRNAs hsa-miR-381 and hsa-miR-1245a may serve as biomarkers for storage products of erythrocytes.

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs with a length of about 21–23 nucleotides. It was first identified in *Caenorhabditis elegans* in 1993 and has been found in almost all animal model systems to date [1, 2]. miRNAs are involved in the pathogenesis of a variety of diseases and play roles in different biological processes, such as apoptosis, cell proliferation, inflammatory response, cancer, and neurological diseases, among others [3]. miRNAs are abundant in body fluids and blood, making miRNAs easier to detect in blood than in cells [4].

Blood transfusion is one of the most common clinical therapeutic procedures for acute or chronic blood loss, with about 85 million red blood cells transfused worldwide each year [5]. The addition of a standard preservative solution to the red blood cells during storage extended cell viability to 42 days. Erythrocytes undergo a variety of biochemical and morphological changes during storage which may have an adverse effect on clinical treatment [6–8]. However, whether storage has an effect on the erythrocyte transcriptome has not been well-studied. It has long been conventional wisdom that mature erythrocytes lack any DNA or RNA because they lack a nucleus. However, human erythrocytes have a rich variety of RNAs, among which miRNAs are an important component, and these miRNAs still exist after terminal differentiation [9, 10]. Multiple studies have shown that the transcriptome of erythrocytes changes significantly during storage [11]. For example, the expression of miR-96, miR-150, miR-196a, and miR-197 during erythrocytes storage in vitro

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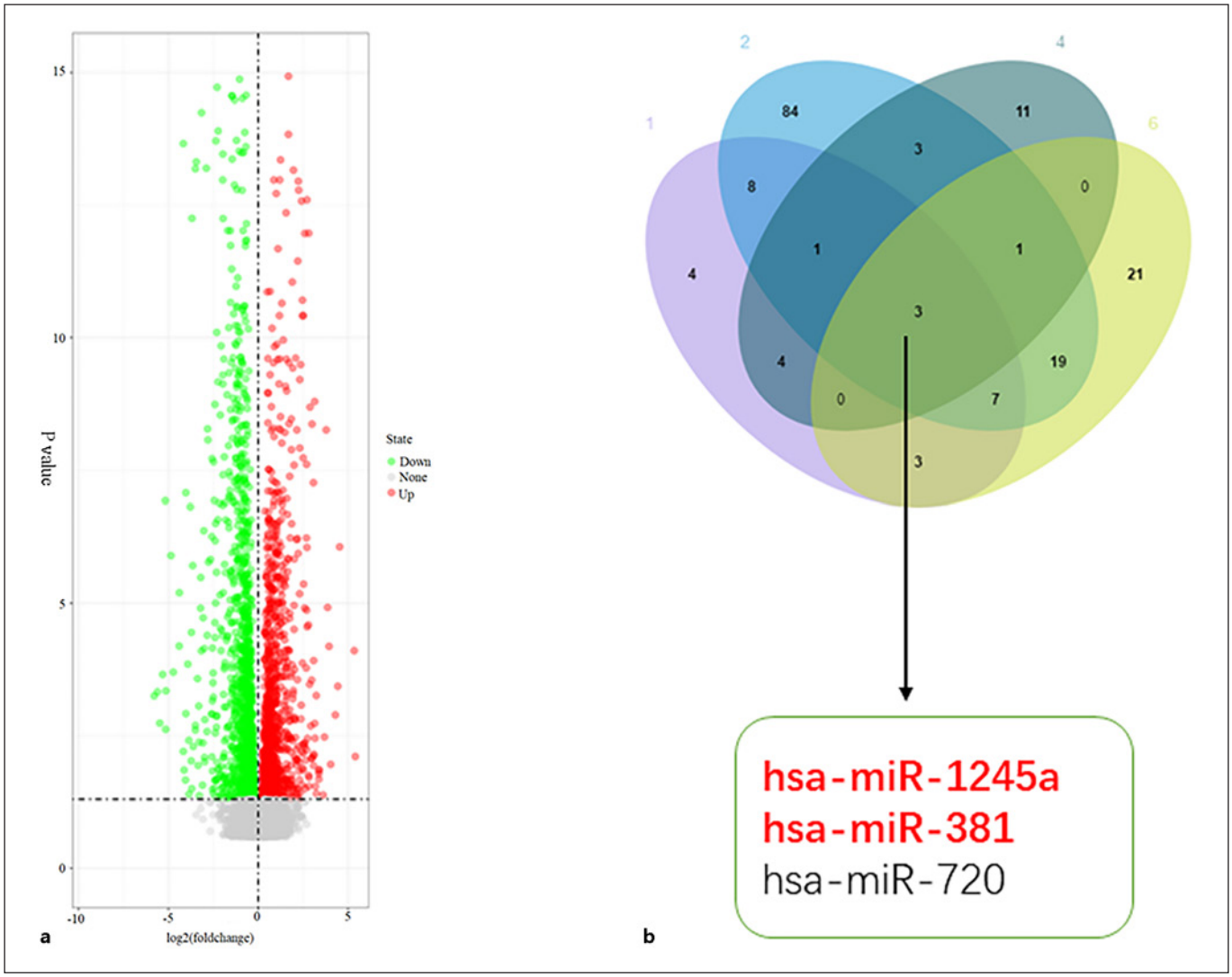

Fig. 1. DEGs of GSE114990. **a** Volcano map of the DEGs. **b** Venn diagram analyzing the intersection of DEGs.

increased over time until the 20th day of storage and subsequently decreased [11]. Changes in these miRNAs have a protective effect on cell death and ATP loss and are associated with certain storage lesion [12]. Therefore, the alteration of miRNA in stored erythrocytes has the potential to serve as a marker to determine the quality and safety of stored erythrocytes.

At present study, we reanalyzed the gene expression dataset GSE114990 to evaluate key risk miRNAs in stored erythrocytes. Based on bioinformatics methods, we obtained the differentially expressed miRNA genes and predicted their target genes, followed by experimental Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Through the construction of protein-protein interaction (PPI) network and the screening of hub genes, the molecular mechanism of stored erythrocytes transcriptome changes can be understood to provide guarantee for the quality of stored erythrocytes and the safety of clinical treatment.

Materials and Methods

Data Resources

We obtained all information on the miRNA expression profile of the transcriptome during erythrocytes storage in vitro from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) [13], which contains 24 samples, platform information (GPL25042), and the GEO accession number (GSE114990).

Identification of Differentially Expressed Genes

We first divided the erythrocytes into 4 groups according to the storage time according to the information of data GSE114990, which were stored for 1, 2, 4, and 6 weeks, respectively. Probe sets

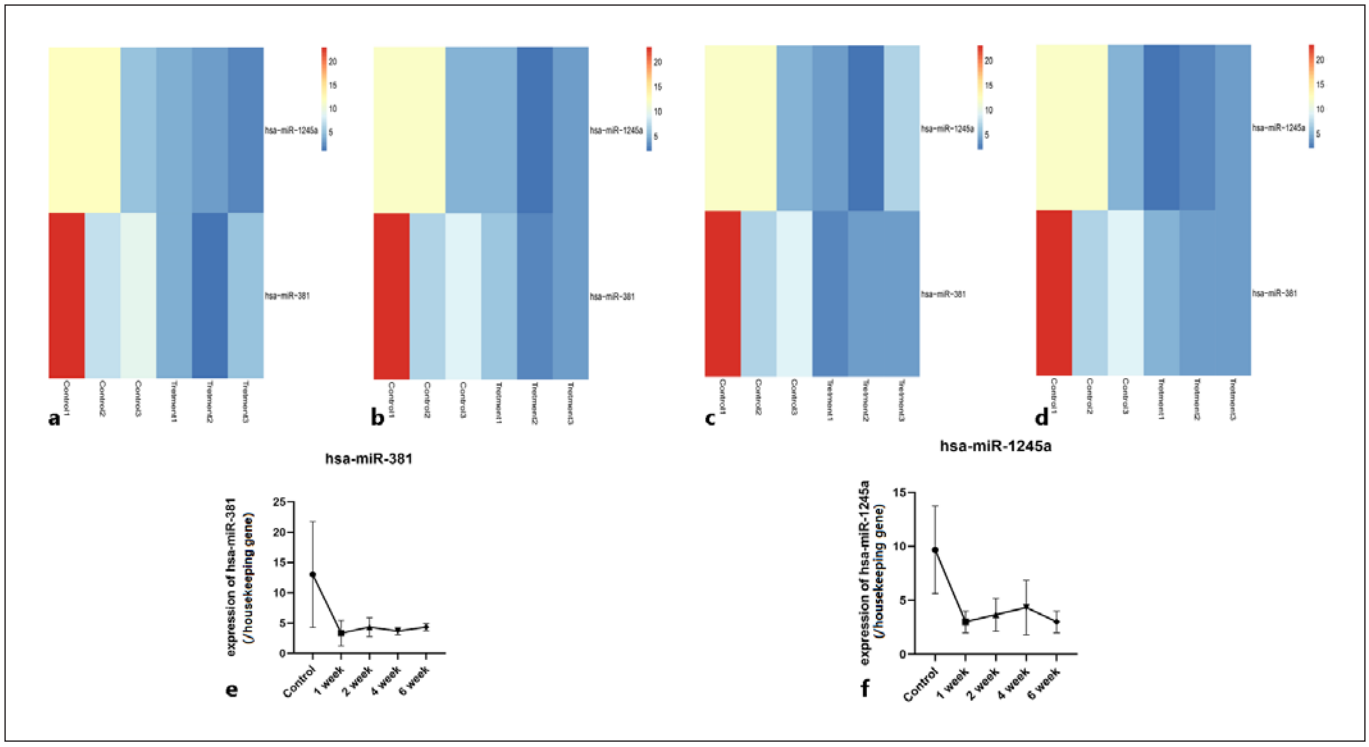

Fig. 2. Expression of miR-1245a and miR-381 after 1, 2, 4, and 6 weeks of storage. **a** Storage of 1 week. **b** Storage of 2 weeks. **c** Storage of 4 weeks. **d** Storage of 6 weeks. **e**, **f** Expression of hsa-miR-381 and hsa-miR-1245a in erythrocytes with different storage times.

mapping multiple genes were removed, and expression value of those mapping on one gene was calculated by the mean value. The data were normalized, and differentially expressed genes (DEGs) were subsequently identified using the "limma" package in R software (version 3.6.1). DEGs were selected with the commonly used thresholds of |log2fold change (FC)|> 1 and p value <0.05. The heatmap and volcano plot of the DEGs were constructed using the "ggplot2" package in R [14].

Identification of Target Genes and Functional Analysis

The public databases miRDB (http://mirdb.org/) and TargetScan (http://www.targetscan.org/mamm_31/) were used to obtain miRNA target genes [15, 16]. Then, GO and KEGG analysis of target genes were conducted by Metascape (https://metascape.org/gp/index.html#/main/step1) database [17]. The significance criterion to screen the significantly enriched GO terms and KEGG pathways was set at $p < 0.05$.

PPI Network Construction, Module Analysis, and Hub Gene Selection

The PPI network of target genes were predicted by an online database STRING (http://string-db.org) and subsequently visualized in Cytoscape (version 3.7.2). Then we used the Molecular Complex Detection plug-in in Cytoscape to excavate the densely connected nodes of the network in the target genes and identify and visualize the important modules. The key genes in PPI network were identified by cytoHubba plug-in in Cytoscape. Six methods of EPC, MCC, Closeness, BottleNeck, Degree, and MNC are used to identify the first 20 genes and to conduct overlapping identification.

Results

Identification of Key miRNAs in the GEO Dataset

As shown in Figure 1a, we first analyzed the volcano map of the DEGs in the GEO data (GSE114990). We then divided the erythrocytes into 4 groups according to their storage time, i.e., 1, 2, 4, and 6 weeks, respectively. Further, the differentially expressed miRNAs were analyzed, respectively, to find the overlapping miRNAs. Three differential miRNAs were found, which were hsa-miR-1245a, hsa-miR-381, and hsa-miR-720 (Fig. 1b).

Expression of hsa-miR-1245a and hsa-miR-381 in Erythrocytes with Different Storage Times

We analyzed the expression levels of hsa-miR-1245a and hsa-miR-381 after 1, 2, 4, and 6 weeks of erythrocytes storage, and the results showed that compared with 1 day of storage, the expressions of hsa-miR-1245a and hsa-miR-381 were decreased (Fig. 2a–d). The expression of hsa-miR-381 was not significantly changed after 1, 2, 4, and 6 weeks of storage (Fig. 2e). The expression of hsa-miR-1245a increased slowly during 1, 2, and 4 weeks of storage and decreased from the 4th week to the 6th week and returned to the expression level of the 1st week (Fig. 2f).

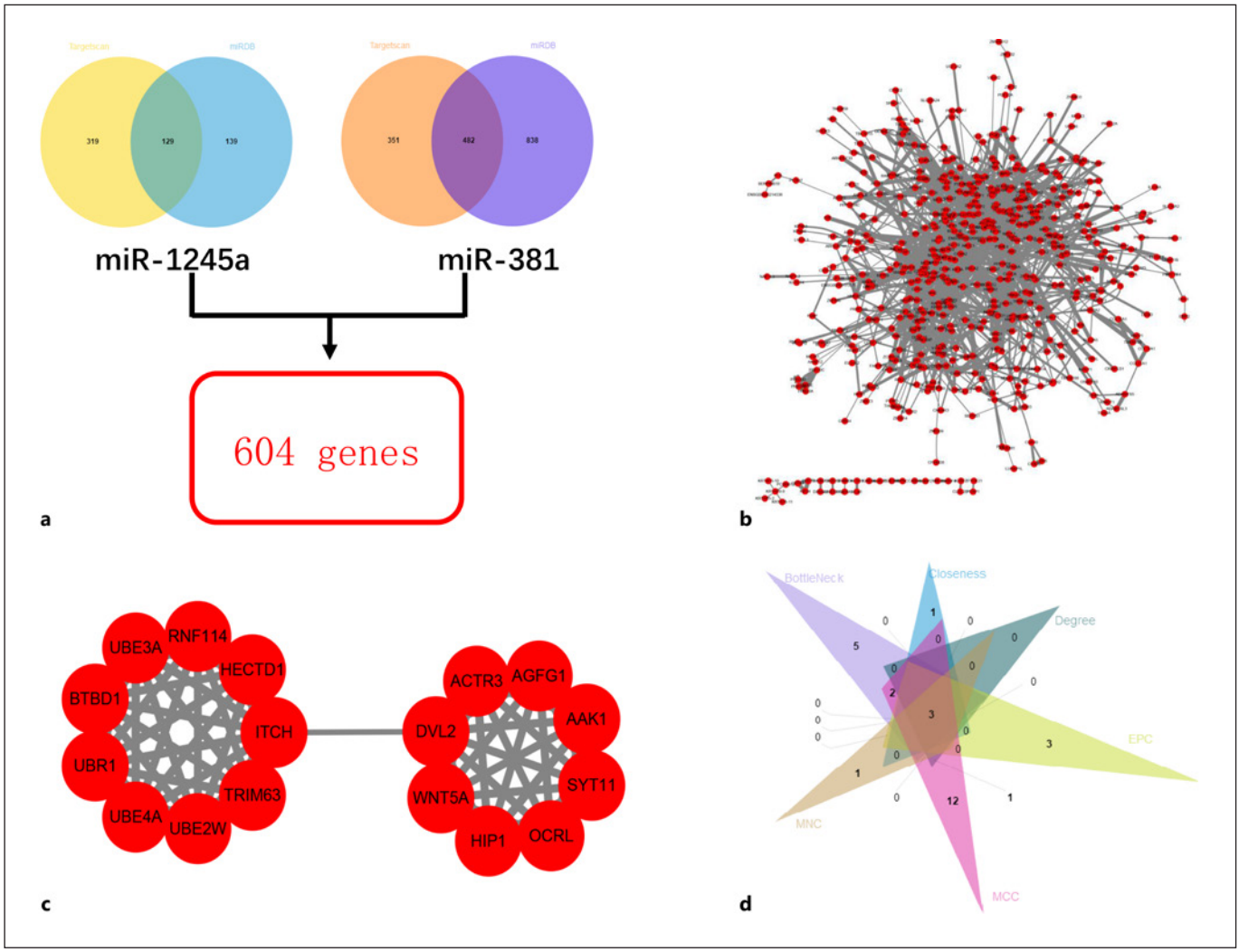

Fig. 3. Target genes and PPI network analyzed. **a** Identification of hsa-miR-381 and hsa-miR-1245a target genes. **b** PPI network was visualized in Cytoscape. **c** Identified the PPI network by cluster analysis. **d** Hub genes were identified by the overlapping of the six methods in cytoHubba.

Identification of hsa-miR-381 and hsa-miR-1245a Target Genes and PPI Network Construction

Since hsa-miR-720 was considered to be a tRNA fragment, there was no consensus on the naming, and it was removed from the miRBase database. Therefore, we excluded hsa-miR-720 as a follow-up study object in this study [9, 18]. To determine the interaction of hsa-miR-381 and hsa-miR-1245a in the post-storage RBC transcriptome, we used public databases to predict their target genes. A total of 604 target genes were predicted by miRDB and TargetScan database (Fig. 3a). Then we used the STRING database and Cytoscape to construct a PPI network. The PPI network consisted of 540 nodes and 1,651 edges (Fig. 3b). One significant module was identified by cluster analyzing the PPI network in Molecular Complex Detection (Fig. 3c).

Next, we identified the top 20 hub genes in Cytoscape using the 6 most commonly used classification methods in cytoHubba (Table 1). By overlapping the 20 genes, 3 central genes (estrogen receptors alpha [ESR1], brain-derived neurotrophic factor [BDNF], and Wnt family member 5A [WNT5A]) were consequently identified as shown in Figure 3d.

GO Functional and KEGG Pathway Enrichment Analyses

To determine the functional roles of the above target genes, GO function and KEGG pathway enrichment analyses were performed using Metascape database. The Biological Processes category of the GO analysis results showed that target genes were significantly enriched in sensory organ and system development (Fig. 4a). For the Cellular Components category, the target genes were en-

Table 1. List of the top 20 hub genes selected by EPC, MCC, Closeness, BottleNeck, Degree, and MNC methods in cytoHubba

BottleNeck	Closeness	Degree	EPC	MCC	MNC
ESR1	ESR1	ESR1	ESR1	UBE3A	ESR1
APP	APP	APP	KRAS	ITCH	APP
TP53BP1	KRAS	SOX2	SOX2	UBE4A	SOX2
SOX2	BDNF	KRAS	BDNF	BTBD1	KRAS
CCNA2	SOX2	BDNF	APP	UBR1	BDNF
BDNF	LEF1	TP53BP1	LEF1	HECTD1	WNT5A
FYN	CCNA2	LEF1	WNT5A	TRIM63	LEF1
SRSF1	TP53BP1	WNT5A	BMI1	RNF114	SMARCA5
SMARCA5	NR3C1	CCNA2	HGF	UBE2W	CCNA2
NRXN1	FYN	SRSF1	FGFR2	WNT5A	CRK
RHOT1	WNT5A	SMARCA5	CCNA2	DVL2	YY1
WNT5A	SMARCA5	YY1	TP53BP1	AGFG1	NRXN1
PRPF8	YY1	CRK	YY1	OCRL	HGF
SP1	BMI1	NRXN1	COMMD3-BMI1	ACTR3	SRSF1
VAPA	FGFR2	HGF	NR3C1	SYT11	BMI1
SYT11	HGF	FYN	FYN	AAK1	TP53BP1
ITCH	CRK	SP1	CRK	HIP1	NR3C1
NFKBIA	SP1	UBE3A	SP1	KRAS	FYN
YY1	UBE3A	NR3C1	JAG1	ESR1	GRM5
CACNA1C	JAK2	DVL2	BRCA2	BDNF	FGFR2

riched in the anchoring junction (Fig. 4b). In the category of Molecular Functions, the target genes were enriched in histone binding and transcription factor binding (Fig. 4c). According to KEGG pathway enrichment analysis, the target genes were significantly enriched in cancer pathways and the Hippo signaling pathway (Fig. 4d).

Discussion

Erythrocyte is a special organ that provides sufficient oxygen to cells that cannot obtain oxygen directly from the surrounding air, thus facilitating oxidative phosphorylation, which in turn enables efficient energy production, thus facilitating the evolution of multicellular organisms [19]. Erythrocyte transfusion is the most common therapeutic procedure in hospitalized patients. Isolated erythrocytes with an acidic additive solution can be stored in cold storage (at 1–6°C) for 3–7 weeks. The transfusion of erythrocytes stored for extended durations remains common but is risky [12]. The various components of erythrocytes undergo a series of changes over time during storage, collectively called "storage lesion" [19].

It has long been widely believed that mature erythrocytes lose their nuclei during differentiation and therefore do not contain any nucleic acid. However, contrary to conventional belief, several research groups have found that human erythrocytes contain a rich diversity of miRNAs that persist beyond terminal differentiation [10, 11, 20–22]. Thus, changes in the erythrocyte transcriptome may provide additional insights into identifying potential sources of storage lesion and potential impact on erythrocytes. In fact, multiple studies have found significant changes in the erythrocyte transcriptome during storage, particularly miRNAs. Kannan et al. [11] analyzed miRNA expression profiles of erythrocytes stored at 0, 20, and 40 days by using a membrane-based array. The results showed that the expression of four miRNAs, miR-96, miR-150, miR-196a, and miR-197, were elevated, and miR-96 still remains the function of targeting the apoptotic erythrocyte calpain small subunit-1 during storage [11]. Sarachana et al. [21] demonstrated that miR-196a had a protective effect against cell death and ATP loss in stored erythrocyte. These studies have shown that miRNA changes during storage of erythrocytes are associated with certain storage lesion. Therefore, miRNA has the potential to serve as a biomarker to determine the quality and safety of stored erythrocytes.

At present study, we reanalyzed data of GSE114990 and identified 2 differentially expressed miRNAs (hsa-miR-381 and hsa-miR-1245a). So far, several literatures have confirmed that hsa-miR-381 plays an important regulatory role in promoting apoptosis [23–25], and erythrocyte apoptosis is one of the important characteristics of "storage lesion" [12, 26]. In this study, we found that the expression level of hsa-miR-381 in red blood cells decreased and remained at a relatively low level after 1 week of storage, which indicates to some extent that hsa-miR-381 can resist the adverse effects of "storage lesion."

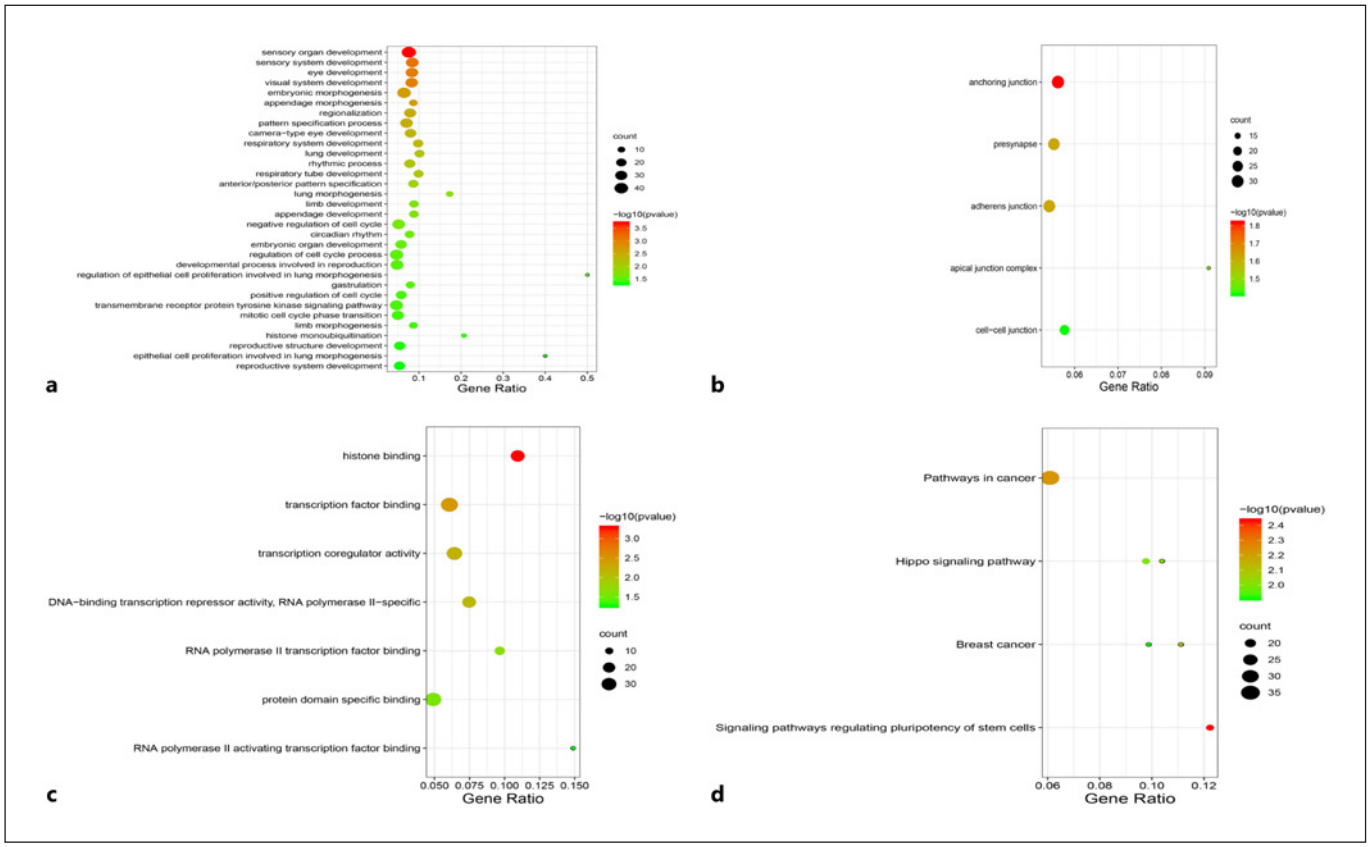

Fig. 4. GO and KEGG pathway enrichment analysis of target genes. **a** BP category of the GO analysis. **b** CC category of the GO analysis. **c** MF category of the GO analysis. **d** KEGG pathway enrichment analyses. BP, Biological Processes; CC, Cellular Components; MF, Molecular Functions.

However, this observation needs to be validated in future experiments.

Furthermore, we predicted the target genes of hsa-miR-381 and hsa-miR-1245a. The PPI network of target genes was constructed by the STRING database, and the module analysis was carried out. Subsequently, we performed GO enrichment analysis on these target genes, and the results showed that these genes were mainly involved in anchoring junction, adherens junction, and cell-cell junction. The adhesion between red blood cells is one of the important factors of thrombosis [27]. A growing body of mechanistic studies has shown that red blood cells can promote thrombosis and enhance the stability of thrombosis [28]. One of the main reasons for the increased risk of thrombosis after transfusion is that the transfused red blood cells suffer storage damage, resulting in changes in shape, function, and intracellular communication [29]. Ghazi et al. [30] and Jiang et al. [31] reported that red blood cells transfusion is associated with increased risk of deep vein thrombosis after cardiac surgery in a dose-dependent fashion, and blood transfusion increases the incidence of postoperative deep-vein thrombosis in total knee and hip arthroplasty. This means that these target genes may influence whether transfusions of red blood cells lead to thrombosis in patients with certain diseases. Then, in order to determine how hsa-miR-381 and hsa-miR-1245a regulate the possible mechanism of target genes leading to thrombosis, we finally filtered out three hub genes, including ESR1, BDNF, and WNT5A, from the whole network. ESR1 is a member of the nuclear receptor superfamily and one of the main factors that mediate the biological effects of estrogen. Aléssio et al. [32] confirmed that ESR1 gene polymorphisms increase fibrinogen levels and thus increase the risk of deep-vein thrombosis. BDNF encodes a member of the nerve growth factor family of proteins and may play a role in the regulation of the stress response and in the biology of mood disorders. Recent studies have confirmed that the alteration of single-nucleotide polymorphism of BDNF gene (BDNF Val66Met) is a major regulator of arterial thrombosis in mice [33, 34]. In addition, the results of the KEGG pathway analysis showed that these target genes chiefly participated in the pathways of cancer. Many authors have indicated that ESR1, BDNF, and WNT5A are correlated with cancer malignant features [35–37].

In conclusion, our study documented changes in hsa-miR-381 and hsa-miR-1245a expression in erythrocytes at different storage times. Moreover, by analyzing the functions of target genes of hsa-miR-381 and hsa-miR-1245a, it was suggested that hsa-miR-381 and hsa-miR-1245a might be involved in the regulation of thrombosis and tumor development, reminding us to pay attention to some possible problems in these patients after blood transfusion. However, the specific mechanism of these regulatory effects remains to be further studied. Nevertheless, these findings enhance our understanding of the changes in miRNAs expression profile of red blood cells during storage and their impact on disease and contribute to better clinical use of red blood cells.

Acknowledgments

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Statement of Ethics

This study was determined by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University that ethical approval was not required.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F.J. analyzed the experimental data and drew up the manuscript. Y.P. provided technical assistance in the acquisition of data. M.B. designed and supervised the study and was a major contributor in modifying the manuscript. All the authors read and approved the final version.

Data Availability Statement

The data that support the findings of this study are openly available in GEO database at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114990.

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