Detection of apoptosis-associated microRNA in human apheresis platelets during storage by quantitative real-time polymerase chain reaction analysis

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Background. Platelet transfusion is an essential part of the treatment of a variety of conditions such as thrombocytopenia and qualitative platelet disorders. As indicated in previous reports, during *in vitro* storage, platelets undergo morphological and physiological changes collectively known as the platelet storage lesion. Apoptosis is a programmed process of cell death, which has been considered as an important cause of platelet storage lesion under the common storage conditions in standard blood banks. Platelets are anucleate blood cells, but contain significant amounts of microRNA (miRNA, miR), which may play an important role in the regulation of gene expression. Drawing on previously published reports on cell apoptosis, we selected 49 miRNA for analysis to explore whether miRNA are of importance during the storage of platelets.

Materials and methods. We used quantitative real-time polymerase chain reaction analysis to determine the levels of expression of miRNA in apheresis platelets at different times of storage. Bioinformatics analysis was applied to explore target genes and the main functions of the selected miRNA.

Results. Our observations suggest that apheresis platelets contain large amounts of apoptosis-associated miRNA. The levels of expression of 25 miRNA remained high and ten of these miRNA showed different expression from that at day 0. Of these ten miRNA, hsa-miR-326, hsa-miR-96, hsa-miR-16, hsa-miR-155 and hsa-miR-150 were up-regulated, while hsa-miR-7, hsa-miR-145, hsa-miR-24, hsa-miR-25 and hsa-miR-15a were down-regulated. The markedly increased expression of hsa-miR-326 in all platelets is noteworthy ($p<0.001$).

Discussion. Since Bcl-xl and Bak1, members of the Bcl-2 family, are the targets of hsa-miR-326, our findings suggest that hsa-miR-326 may be involved in platelet apoptosis during storage.

Keywords: miRNA, apheresis platelet, apoptosis, qRT-PCR.

Introduction

Human platelets are released into the circulation from the megakaryocytes of bone marrow as cytoplasmic fragments and play an important role in the development of cardiovascular diseases, inflammation and some tumours. Abnormalities in the number, adhesion, activation and aggregation of platelets are the key factors in thrombocytosis and haemorrhage, common conditions associated with high morbidity and mortality. Platelet transfusion is especially important in the treatment of a variety of conditions such as thrombocytopenia and qualitative platelet disorders. Platelets are anucleate cells and during *vitro* storage they undergo morphological and physiological changes collectively known as the platelet storage lesion[1,2]. The platelet storage lesion seriously affects the quality of stored platelets, and even causes them to be ineffective *in vivo* after transfusion. Investigations have, therefore, been focused on the mechanisms underlying the storage lesion[1,2].

Apoptosis is a programmed process of cell death, which has been considered as an important cause of the platelet storage lesion under normal storage conditions in standard blood banks[3,4]. Studies have revealed that although platelets are anucleate, they contain numerous messenger RNA (mRNA) and undergo signal-dependent translational regulation[5] and can synthesize some proteins[6,7]. Research on how mRNA participates in the regulation of gene expression and its function in platelets is the key to understanding the molecular mechanisms of platelets.

MicroRNA (miRNA, miR) are small, highly conserved, non-protein-coding RNA molecules. Studies have shown that they can regulate gene expression at the post-transcriptional level and play an important role in gene expression regulation, including cell differentiation, cell proliferation and metabolism. There is also recent evidence that miRNA plays an important role in the process of cell apoptosis[8]. MiRNA can regulate mRNA translation through recognition of binding sites of imperfect complementarity, and through

pairing of the miRNA. Recently, Ple *et al.*[9] reported results of high-throughput sequencing showing that human platelets express more than 492 miRNA. Kannan *et al.*[10] consider that platelets use miRNA as translational regulators and play a crucial role in platelet apoptosis during storage and it is known that platelets have a complex regulatory network involving miRNA[11]. On the basis of the e hypothesis that miRNA in platelets could act as translational regulators and play a crucial role in platelet apoptosis during storage, in this study we used quantitative real-time polymerase chain reaction (qRT-PCR) analysis to determine the expression of miRNA in stored apheresis platelets; we also predict the target genes of the miRNA and discuss the correlations between the miRNA and their target genes.

Materials and methods

Platelet preparation and storage for microRNA extraction

Apheresis platelets were collected from five healthy blood donors (3 males and 2 females) in Wenzhou Central Blood Station and Ningbo Central Blood Station, Zhejiang Province, China. The platelets were filtered through leucocyte-depletion filters for platelets (Nanjing Shuangwei Biotechnology Co. Ltd, Nanjing, China) within 12 hours to obtain samples with a volume of 15-20 mL, a platelet count ranging from $2.0\text{-}2.5\times10^9$/mL and a leucocyte count $<5\times10^6$/mL. Each sample was divided into four parts and stored in platelet storage bags at 22±2 °C. Fresh apheresis platelets were used as day 0 platelets. The units were also tested on days 1, 3 and 5 of storage. In order to separate white blood cells from the platelets further, the platelets were centrifuged at 400g for 10 minutes in 1.5 mL Eppendorf centrifuge tubes. Generally the white blood cell contamination in platelets was approximately 0.01%, which is negligible. The samples were then subjected to a haemogram analysis using an automated cell counter (Sysmex kx 2100, Sysmex Corporation, Hyogo, Japan) to determine that the sample contained sufficient numbers of platelets for RNA extraction and was free from red blood cells, white blood cells, and other cell debris, in order to ensure that the RNA analysed was truly from platelets. The sample was enriched using a platelet function centrifuge and the isolated RNA was subjected to miRNA profiling.

MicroRNA extraction

We used a miRNA isolation kit (Beijing CoWin Bioscience Co, Ltd, Beijing, China) for the purification of total platelet RNA, according to the manufacturer's instructions. The RNA concentration was estimated with a Nanodrop spectrophotometer (ND1000; Saveen & Werner, Limhamn, Sweden). The sample was kept at −70 °C until the reverse-transcription step.

Analysis of apoptosis-associated microRNA by quantitative real-time polymerase chain reaction

For reverse transcription of total RNA, including platelet miRNA, we used the miRNA cDNA Kit (Beijing CoWin Bioscience Co, Ltd) according to the manufacturer's instructions. Total RNA was treated with *Escherichia coli* poly-A polymerase to generate a poly-A tail at the 3'-end of each miRNA. Following polyadenylation, the miRNA first strand cDNA was synthesized using the poly (T) adapter (GCGAGCAC AGAATTAATACGACTCACTATAGGTTTTTTTTTT TTVN) at 42 °C for 1 hour. To measure the expression of mature miRNA, the miRNA-first strand cDNA was determined by qRT-PCR analysis, using the miRNA Real-Time PCR Assay Kit (Beijing CoWin Bioscience Co, Ltd) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Mature miRNA sense sequences (miRBase, http://microrna.sanger.ac.uk/) of tested miRNA were used as forward PCR primers (Table I). The universal reverse primer was 5'-GCGAGCACAGAATTAATACGACTC-3'. Results were normalized to 5s rRNA. The 5s RNA forward primer was 5'-TACGGCCATACCACCCTGAA-3' and the reverse primer was 5'-TAACCAGGCCCGACCCTGCT-3'. PCR cycling conditions were 95 °C for 10 minutes followed by 40 cycles of the following steps: 95 °C for 15 seconds and 60 °C for 1 minute. The qRT-PCR data were normalized using the $2\text{-}\Delta\Delta^{Ct}$ method [$\Delta\Delta^{Ct}=(Ct^{target}–Ct^{5s})$ days 1, 3, 5−$(Ct^{target}–Ct^{5s})$ day 0]. Melting curve analysis was performed to test the specificity and quality of the qRT-PCR amplifications. The data were processed using StepOne™ software v2.2.2 (Applied Biosystems).

MicroRNA target gene prediction

Bioinformatic analysis was applied to explore target genes and the main functions of the miRNA identified. To obtain a higher degree of prediction verification, the targets were predicted by at least three prediction programmes for further data analysis (TargetScan 5.2, Miranda, PicTar, miRGen). The software provided information regarding miRNA sequences and target genes. The genes were then reorganized on the basis of statistical significance.

Statistical analyses

Results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad prism 5 software (GraphPad Software, La Jolla, CA, USA). Comparisons between groups were assessed using the Student's t-test. Given the non-Gaussian distribution of the results, non-parametric Kruskal-Wallis ANOVA was used to compare the data from groups. Expression levels that differed by more than 2-fold between day 0 and day 5 were regarded as significant. For all results, p values <0.05 were considered statistically significant.

Table I - The upstream primers of 49 apoptosis-associated miRNA.

Sanger Registry ID	Sanger Accession #	Entrez Gene ID	Primer
hsa-let-7a	MIMAT0000062	406883	TGAGGTAGTAGGTTGTATAGTT
hsa-let-7b	MIMAT0000063	406884	TGAGGTAGTAGGTTGTGTGGTT
hsa-let-7c	MIMAT0000064	406885	TGAGGTAGTAGGTTGTATGGTT
hsa-let-7d	MIMAT0000065	406886	AGAGGTAGTAGGTTGCATAGTT
hsa-let-7e	MIMAT0000066	406887	TGAGGTAGGAGGTTGTATAGTT
hsa-let-7f	MIMAT0000067	406888	TGAGGTAGTAGATTGTATAGTT
hsa-let-7g	MIMAT0000414	406890	TGAGGTAGTAGTTTGTACAGTT
hsa-let-7i	MIMAT0000415	406891	TGAGGTAGTAGTTTGTGCTGTT
hsa-miR-1	MIMAT0000437	406905	TGGAATGTAAAGAAGTATGTAT
hsa-miR-101	MIMAT0000099	406893	TACAGTACTGTGATAACTGAA
hsa-miR-10a	MIMAT0000253	406902	TACCCTGTAGATCCGAATTTGTG
hsa-miR-133a	MIMAT0000427	406922	TTTGGTCCCCTTCAACCAGCTG
hsa-miR-133b	MIMAT0000770	442890	TTTGGTCCCCTTCAACCAGCTA
hsa-miR-142-3p	MIMAT0000434	406934	TGTAGTGTTTCCTACTTTATGGA
hsa-miR-142-5p	MIMAT0000433	406934	CATAAAGTAGAAAGCACTACT
hsa-miR-144	MIMAT0000436	406936	TACAGTATAGATGATGTACT
hsa-miR-145	MIMAT0000437	406937	GTCCAGTTTTCCCAGGAATCCCT
hsa-miR-148a	MIMAT0000243	406940	TCAGTGCACTACAGAACTTTGT
hsa-miR-150	MIMAT0000451	406942	TCTCCCAACCCTTGTACCAGTG
hsa-miR-151-5p	MIMAT0004697	442893	TCGAGGAGCTCACAGTCTAGT
hsa-miR-152	MIMAT0000438	406943	TCAGTGCATGACAGAACTTGG
hsa-miR-153	MIMAT0000439	406944	TTGCATAGTCACAAAAGTGATC
hsa-miR-155	MIMAT0000646	406947	TTAATGCTAATCGTGATAGGGGT
hsa-miR-15a	MIMAT0000068	406948	TAGCAGCACATAATGGTTTGTG
hsa-miR-15b	MIMAT0000417	406949	TAGCAGCACATCATGGTTTACA
hsa-miR-16	MIMAT0000069	406950	TAGCAGCACGTAAATATTGGCG
hsa-miR-182	MIMAT0000259	406958	TTTGGCAATGGTAGAACTCACACT
hsa-miR-184	MIMAT0000454	406960	TGGACGGAGAACTGATAAGGGT
hsa-miR-188	MIMAT0004613	406964	CTCCCACATGCAGGGTTTGCA
hsa-miR-193a	MIMAT0000459	406968	AACTGGCCTACAAAGTCCCAGT
hsa-miR-193b	MIMAT0002819	574455	AACTGGCCCTCAAAGTCCCGCT
hsa-miR-196a	MIMAT0000226	406972	TAGGTAGTTTCATGTTGTTGGG
hsa-miR-197	MIMAT0000227	406974	TTCACCACCTTCTCCACCCAGC
hsa-miR-21	MIMAT0000076	406991	TAGCTTATCAGACTGATGTTGA
hsa-miR-210	MIMAT0000267	406992	CTGTGCGTGTGACAGCGGCTGA
hsa-miR-214	MIMAT0000271	406996	ACAGCAGGCACAGACAGGCAGT
hsa-miR-216b	MIMAT0004959	100126319	AAATCTCTGCAGGCAAATGTGA
hsa-miR-218	MIMAT0000275	407000	TTGTGCTTGATCTAACCATGT
hsa-miR-224	MIMAT0000281	407009	CAAGTCACTAGTGGTTCCGTT
hsa-miR-24	MIMAT0000080	407012	TGGCTCAGTTCAGCAGGAACAG
hsa-miR-25	MIMAT0000081	407014	CATTGCACTTGTCTCGGTCTGA
hsa-miR-28	MIMAT0004502	407020	CACTAGATTGTGAGCTCCTGGA
hsa-miR-326	MIMAT0000756	442900	CCTCTGGGCCCTTCCTCCAG
hsa-miR-337	MIMAT0000754	442905	CTCCTATATGATGCCTTTCTTC
hsa-miR-338	MIMAT0000763	442906	TCCAGCATCAGTGATTTTGTTG
hsa-miR-342	MIMAT0000753	442909	TCTCACACAGAAATCGCACCCGT
hsa-miR-371	MIMAT0000723	442916	AAGTGCCGCCATCTTTTGAGTGT
hsa-miR-7	MIMAT0000252	407045	TGGAAGACTAGTGATTTTGTTGT
hsa-miR-96	MIMAT0000095	407053	TTTGGCACTAGCACATTTTTGCT

Results

Comparison of microRNA expression in apheresis platelets after different periods of storage qRT-PCR was used to identify 49 miRNA in apheresis platelets after different periods of storage under standard blood bank conditions. We analysed miRNA at baseline (day 0) and after 1, 3 and 5 days of storage. We found that platelets contain abundant amounts of apoptosis-associated miRNA (Table I). The expression of 25 miRNA consistently remained at high levels (Table II) in all samples at all time points, and ten miRNA had a more than two-fold difference in expression between day 0 and day 5 (Table II). In detail, hsa-miR-326, hsa-miR-96, hsa-miR-16, hsa-miR-155 and hsa-miR-150 were up-regulated, while hsa-miR-7, hsa-miR-145, hsa-miR-24, hsa-miR-25 and hsa-miR-15a were down-regulated (Figure 1). The increase of hsa-miR-326 was particularly marked.

Potential target mRNA of microRNA with significant expression changes

The miRNA whose expression changed significantly in all platelets under blood bank storage conditions were selected for target prediction analysis. Bioinformatics analysis indicated that hsa-miR-15a and hsa-miR-16 target Bcl-2, hsa-miR-24 and hsa-miR-25 target Bim, and hsa-miR-326 targets Bcl-xl and Bak. Bcl-2 and Bcl-xl belong to an anti-apoptotic family of proteins, while Bax, Bak and Bim are members of a pro-apoptotic family and are, therefore, relevant to apoptosis in platelets (Table III).

Table II - List of expression levels of miRNA in apheresis platelets by qRT-PCR (ranked according to their abundance in apheresis platelets) and the change trend of expression levels (cut-off was 2-fold change compared with day 0).

miR (expression in platelets)	Trend in level changes
hsa-miR-21	No change
hsa-miR-142-3p	No change
hsa-miR-16	Up
hsa-let-7f	No change
hsa-miR-15b	No change
hsa-let-7a	No change
hsa-miR-24	Down
hsa-miR-15a	Down
hsa-let-7i	No change
hsa-let-7g	No change
hsa-miR-142-5p	No change
hsa-miR-151-5p	No change
hsa-miR-326	Up
hsa-let-7d	No change
hsa-miR-150	Up
hsa-miR-155	Up
hsa-miR-96	Up
hsa-miR-342	No change
hsa-miR-337	No change
hsa-miR-145	Down
hsa-miR-133a	No change
hsa-miR-133b	No change
hsa-miR-101	No change
hsa-miR-25	Down
hsa-miR-7	Down

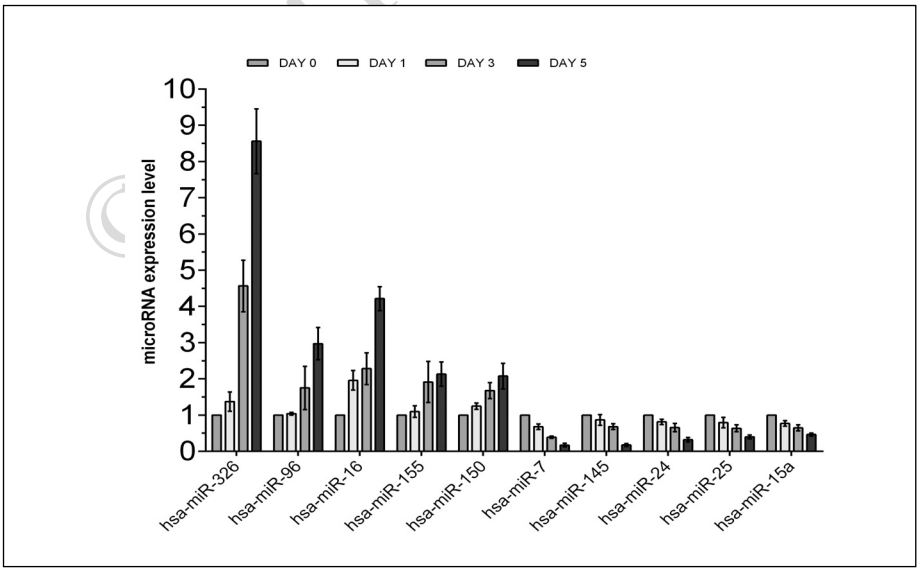

Figure 1 - Relative expression levels of selected miRNA during storage.
The miRNA in apheresis platelets are differentially expressed on days 1, 3 and 5 compared with on day 0, as determined by qRT-PCR. MiRNA whose expression changed at least 2-fold change are listed. Statistical analyses were performed using GraphPad prism 5 software. Results identified as significant were those that were more than 2-fold different between day 0 and day 5. The change for each miRNA represents the average found in five independent donors. Results are expressed as mean±standard deviation (SD) (*$p<0.05$ *vs* day 0).

Table III - Predicted target mRNA of selected miRNA which regulate apoptosis.

miRNA	Symbol	Gene Description	Function
hsa-miR-7	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	apoptosis
	EGFR	epidermal growth factor receptor	anti-apoptosis
	Raf1	v-raf-1 murine leukemia viral oncogene homolog 1	apoptosis
	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	anti-apoptosis
	TNK2	tyrosine kinase, non-receptor, 2	anti-apoptosis
	IGF1R	insulin-like growth factor 1 receptor	anti-apoptosis
hsa-miR-15	Bcl2	B-cell CLL/lymphoma 2	anti-apoptosis
	CCND1	cyclin D1	anti-apoptosis
	CCNE	cyclin E1	anti-apoptosis
	DEDD	death effector domain containing	apoptosis
	MYB	v-myb myeloblastosis viral oncogene homolog	anti-apoptosis
	AKT3	v-akt murine thymoma viral oncogene homolog 3	anti-apoptosis
	RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	ant-apoptosis
hsa-miR-16	Bcl2	B-cell CLL/lymphoma 2	anti-apoptosis
	CCND1	cyclin D1	anti-apoptosis
	CCNE	cyclin E1	anti-apoptosis
	DEDD	death effector domain containing	apoptosis
	RECK	reversion-inducing-cysteine-rich protein with kazal motifs	apoptosis
	ZYX	zyxin	apoptosis
	MYB	v-myb myeloblastosis viral oncogene homolog	anti-apoptosis
	AKT3	v-akt murine thymoma viral oncogene homolog 3	anti-apoptosis
	RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	ant-apoptosis
hsa-miR-24	E2F2	E2F transcription factor 2	apoptosis
	Myc	v-myc myelocytomatosis viral oncogene homolog	anti-apoptosis
	Net1A	neuroepithelial cell transforming 1	anti-apoptosis
	Bim	BCL2-like 11 (apoptosis facilitator)	apoptosis
	DHFR	dihydrofolate reductase	anti-apoptosis
hsa-miR-25	Bim	BCL2-like 11 (apoptosis facilitator)	apoptosis
	TRAIL	TNF-related apoptosis-inducing ligand	apoptosis
	FASLG	Fas ligand (TNF superfamily, member 6)	apoptosis
	TP53	tumor protein p53	apoptosis
hsa-miR-96	GPC3	glypican 3	apoptosis
	FOXO1	forkhead box O1	apoptosis
	CASP2	caspase 2, apoptosis-related cysteine peptidase	apoptosis
	BIRC4	X-linked inhibitor of apoptosis	anti-apoptosis
	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	anti-apoptosis
hsa-miR-150	MUC4	mucin 4, cell surface associated	anti-apoptosis
	EGR2	early growth response 2	apoptosis
hsa-miR-155	TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein 2	anti-apoptosis
	FADD	Fas-associated protein with death domain	apoptosis
	RIP-1	small subunit (SSU) processome component, homolog	apoptosis
	IKK	inhibitor of kappa light polypeptide gene enhancer in B-cells	anti-apoptosis
	ET-1	Endothelin-1	anti-apoptosis
	SMAD5	SMAD family member 5	apoptosis
	FOXO3	forkhead box O3	apoptosis
	SOCS1	suppressor of cytokine signaling 1	anti-apoptosis
hsa-miR-145	c-MYC	V-Myc Myelocytomatosis Viral Oncogene Homolog	apoptosis
	CDK4	cyclin-dependent kinase 4	anti-apoptosis
	FSCN1	fascin homolog 1, actin-bundling protein	anti-apoptosis
	SOX2	SRY (sex determining region Y)-box 2	anti-apoptosis
hsa-miR-326	Bcl2L1	BCL2-like 1	anti-apoptosis
	Notch1	notch 1	apoptosis
	BAK1	BCL2-antagonist/killer 1	apoptosis
	PKM2	pyruvate kinase, muscle	anti-apoptosis

Discussion

Platelet lacks nuclear DNA, but do contain various mRNA and can synthesize some apoptosis proteins given the existence of mitochondria and ribosomes, which come from macrophages. In recent years, research on the functions of platelet miRNA has provided new perspectives regarding the mechanism of platelet apoptosis. Platelets contain numerous miRNA; the process of maturation of miRNA is different in platelets than in nucleated cells. In platelets, the maturation of miRNA begins with the unspliced pre-miRNA, which mostly originates from the cytoplasm of macrophages. Platelets contain related regulatory proteins, Dicer and Argonaute 2 (Ago2), and can process pre-miRNA into mature miRNA. These miRNA may be relevant for post-transcriptional gene regulation in platelets, which are anucleate cells. In this study, we found ten miRNA whose levels of expression were significantly different (>2-fold increase or decrease) at day 5 from baseline (day 0) (Table II and Figure 1). A possible explanation for the differential expression of these miRNA might be that platelets simultaneously contain both pre-miRNA that are processed to mature miRNA and miRNA-degrading enzymes and/or an miRNA partitioning mechanism that promotes miRNA degradation[11].

According to our data, the expression of hsa-miR-326 significantly increased in all platelets, while the level of expression of hsa-let-7b was low and there was no statistically significant difference during the storage process. These findings differ somewhat from those of Kannan *et al.*[10] who used membrane arrays and found that let-7b remained at high levels, with a tendency to increase, during storage. There were no related reports of further study. In 2011, Barrey *et al.*[12] verified the existence of pre-miRNA and mature miRNA in the mitochondria of human muscle cells. In the same year, Bandiera *et al.*[13] found 13 miRNA expressed in the mitochondria of Hela cells, which they named mitomiR, and a large number of let-7b. We, therefore, speculate that let-7b is abundant in the mitochondria of platelets. Perhaps some mitomiR, including hsa-let-7b, were lost during our extraction process and influenced our findings.

The intrinsic apoptosis pathway is regulated by the Bcl-2 family of proteins, which are divided into two groups[14-16], the pro-apoptotic family and anti-apoptotic family. The pro-apoptotic family, including Bax and Bak, play an essential role in mediating the release of cytochrome c and trigger the apoptotic cascade[17,18]. Research shows that the anti-apoptotic family comprises five members: Bcl-2, Bcl-xl, Mcl-1, A1 and Bcl-w, and maintains cellular viability by preventing the activation of Bax and/or Bak[14-16,19,20]. Mason *et al.*[19] found that older platelet contain less Bcl-xl than younger platelets; decreasing levels of Bcl-xl lead to a reduction in Bcl-xl-mediated inhibition of Bak and, therefore, induce platelet apoptosis. Their research identified Bcl-xl as a major regulator of platelet survival. Our data show that the expression levels of hsa-miR-326 increase significantly during storage and target the genes *Bcl-xl* and *Bak1*. We hypothesized that the increase of hsa-miR-326 may be related to the down-regulation of *Bcl-xl* gene expression and then restraint of Bak and/or Bax proteins, which have an important role in the onset and progress of platelet apoptosis.

Platelets not only play a central role in the maintenance of haemostasis and thrombotic disorders, but also contribute to diverse functions and conditions, such as inflammatory and immune responses, acute lung injury, tumour progression and metastasis[21,22]. Recently, Benoit Laffont *et al.*[23] demonstrated that platelet microparticle-derived miR-223 can be delivered to endothelial cells and possibly other recipient cells of the circulatory system and regulate genes at both mRNA and protein levels; this provides a new research direction for the regulatory model of miRNA in platelets.

Platelet transfusion is important in the treatment of multiple conditions such as thrombocytopenia and qualitative platelet disorders. During storage, platelets undergo apoptosis, and finally, lose their viability, and even become ineffective *in vivo* after transfusion. Careful research on the regulatory network of miRNA in platelets during storage will provide some information to help the understanding of the regulatory mechanisms of platelet apoptosis and function.

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Authorship contributions

Shifang Yu and Gang Deng contributed equally to this work. Shifang Yu, Qiang Li and Gang Deng conceived and designed the experiments; Shifang Yu, Gang Deng, Qiang Li and He Sun performed the experiments and evaluated the results; Dingliang Qian, Zuoting Xie and Dandan Huang contributed reagents, materials or analysis tools; Shifang Yu and Qiang Li wrote the manuscript. All authors read and approved the final version of the manuscript.

The Authors declare no conflict of interest.

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