Ingenuity Pathway Analysis of miRNAs and mRNAs in Stored Platelets Identifies the Potential of miRNAs in Regulating Platelet Functions Relevant to Storage Lesions

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Abstract

Under blood bank storage conditions platelets undergo physiological changes leading to storage lesions that affect platelet quality. Since small noncoding micrornas (miRNAs) are posttranslational regulators of cellular mRNA expression, we envisioned that miRNA targeting of mRNAs cause perturbations in stored platelets and affect their quality. In this report, differential miRNA and mRNA microarray data on day 0, day 5 and day 9 stored human platelet samples were analyzed. The analysis identified 7 upregulated miRNAs (miR-320b, miR-1-3p, miR-214-3p, miR-197-3p, miR-129-5p, miR-183-5p and miR-292b-5p) that are known to regulate 35 mRNAs. The analysis further identified 68 differentially expressed miRNAs common to day 5 and day 9 of storage (p-value <0.05). Ingenuity Pathway Analysis (IPA) based search for mRNAs that are potentially regulated by these miRNAs identified 17 mRNAs involved in platelet activation, 28 mRNAs associated with platelet aggregation, 12 mRNAs affecting platelet binding and 9 mRNAs involved in adhesion as well as 27 mRNAs implicated in cellular apoptosis. The IPA also assisted in identifying top 5 signaling pathways and network functions of platelets. The results clearly identify the potential of miRNAs in regulating functions relevant to the platelet storage lesions, which provides a basis for further experimental verification.

Keywords: Ingenuity pathway analysis; microarray; miRNA; Networks; Platelets; Signaling pathway

Introduction

Platelets (PLTs) are one of the most important life-saving transfusion products. During storage under standard blood bank conditions, platelets start to lose their viability with time and as a consequence, platelets do undergo morphological and physiological changes during storage collectively known as Platelet Storage Lesion (PSL), which negatively impact their performance following transfusion.

MicroRNAs (miRNAs) are a type of small non-coding RNAs involved in posttranscriptional regulation of the genes (mRNAs) that they target. Role of miRNAs in platelets biosynthesis and presence of an active post-transcriptional miRNA mediated mRNA regulation has already been reported [1]. In addition, a number of platelet originated miRNAs have also been identified as possible disease biomarkers [2,3]. Platelets also release microparticles packed with miRNA-Ago2 RISC machinery capable of regulating gene expression and phenotype of the recipient cells [4,5]. More recently, Rowley et al., [6] reported the effect of miRNA processing on platelet functions using a Dicer1 deficient murine model. All these studies suggest that miRNAs play a role in platelet biology. However, none of these studies address whether perturbations in miRNAs and Messenger RNAs (mRNAs) do occur in platelets stored under blood bank conditions for transfusion, and the consequences of these perturbations on the quality of stored platelets.

Due to PSL development, both structure and some of the functions of platelets are compromised during storage. Our goal is to identify potential miRNA:mRNA interactions that are consequential to these changes. We have generated both miRNA and mRNA differential profiles and part of the data was reported [7,8] and further analysis of this data constitutes present report. Here in this analysis, we performed a) IPA “MicroRNA target filter” tool to extract a list of potential miRNA-targeted mRNAs of stored platelets and, b) IPA “Core analysis” (In-silico analysis) to identify the potential functions of these mRNAs in stored platelets. Overall, this bioinformatics-based approach demonstrates that platelet miRNAs are capable of regulating miRNA functions relevant to PSL and provides confidence to the field to test each of these potential miRNA:mRNA interactions in the context of platelet storage quality towards improving the quality of platelets in storage.

Methods

Platelet samples and storage

Platelet samples collected from healthy donors at National Institutes of Health (NIH) blood bank were stored at 22°C in platelet shaker. The IRB approval for this study was exempted under FDA-RHSC approved protocol #03-120B. Samples were collected from the bag on day 0, day 5 and day 9 of storage and leukocyte-reduced by using CD45 conjugated beads (Dynabeads, Life Technologies, Carlsbad, CA, USA) and samples were stored at -80°C.

RNA extraction, quantification and quality assessment

Total RNA was extracted using TRIZOL as per manufacturer’s instruction (Life Technologies). Amount of RNA in each sample was
quantified using NanoVue GE (GE, Pittsburgh, PA, USA) and quality of RNA samples were determined by gel-on-chip analysis using Agilent bioanalyzer.

**Microarrays and RT-qPCR**

MicroRNA profiling was performed using Affymetrix Gene chip miRNA 3.0 arrays. Samples collected from 4 different donors at three different time points were used for microarray microarrays. For each array, 300 ng of total RNA was used for performing the experiment. The miRNA profiling was done using HumanRef-8 Expression BeadChip (Illumina, Inc., San Diego, CA, USA) microarrays. Total RNAs extracted from two independent donors at three different storage time points were used for the profiling. RT-qPCR was done using TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA, USA).

**miRNA target filter analysis**

The detailed protocol for total RNA extraction from platelets stored for up to 9 days, miRNA and mRNA arrays and RT-qPCR are all as described previously [8]. Using microarrays, the differentially expressed miRNAs (fold cut off >1.5, p-value <0.05) and mRNA (fold cut off >1.5, p-value <0.05) during storage were identified. "MicroRNA target filter" function was applied in our ingenuity pathway analysis tool to determine potential targets of miRNAs differentially expressed on day 5 and day 9 compared to day 0. The list of potential miRNA targets (i.e., mRNAs) compiled by IPA includes target genes predicted by different algorithms such as mirRecords, target scan and experimental targets listed in tarbase (a database of experimental targets) and identified by Ingenuity Knowledge Base. The "MicroRNA target filter" tools provide a unique opportunity to find miRNA targets relevant to the biological context of interest (by performing miRNA and mRNAs profiling of the same samples) which can be very helpful in overcoming the complexity of miRNA target genes.

**Network functions and pathway analyses**

We populated a list of miRNAs differentially expressed on both day 5 and day 9 and extracted their potential target mRNAs using IPA tool. To find the mRNAs relevant to platelets in storage, mRNAs showing significant differential expression in platelets during storage at 22°C (p-value <0.05). In our previous report [8] we found differential expression of 302 miRNAs in platelet samples during storage at 22°C (p-value <0.05). In this report, the principal component analysis of these significant miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 mRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). Of the 302 miRNAs, 68 miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1).

**Results**

In our previous report [8] we found differential expression of 302 miRNAs in platelet samples during storage at 22°C (p-value <0.05). In this report, the principal component analysis of these significant miRNAs showed clear separation of samples into three distinct groups based on the days in storage (Figure 1). One of the 302 miRNAs, 68 mRNAs have shown similar expression pattern on day 5 and day 9, however the changes are more pronounced on day 9 compared to day 5 (Table 1). In order to understand the role of these differentially expressed miRNAs during storage, the mRNAs targeted for regulation by these miRNAs were determined. The mRNA profiling of two sets of the same RNA samples that were subjected to microRNA microarray profiling showed differential expression of 54 and 864 mRNAs on day 5 and day 9, respectively (Supplementary Table 1). To determine which miRNAs serve as potential targets of the differentially expressed common miRNAs, the Ingenuity Pathway Analysis tool was used. We searched for miRNA:mRNA pairs showing opposite expression pattern on different days of storage. The analysis resulted in identification of 1788 miRNA:mRNA pairs involving 59 miRNAs and 605 mRNAs showing negative correlation (Supplementary Table 2).

![Figure 1: Principal Component Analysis (PCA) of platelet miRNAs differentially expressed (p<0.05) on day 5 and day 9 of storage, compared to day 0.](image-url)
In the next step, we analyzed these 605 potential mRNAs using IPA “core analysis” to dissect their functional role in platelets. Interestingly, the mRNAs were found to be associated with several different signaling pathways, of which two pathways, ‘actin cytoskeleton signaling’ and ‘integrin signaling’ were already known to have important role in platelet biology. Ephrin receptor signaling, VEGF signaling and molecular mechanisms of cancer pathway were also among the top most affected pathways in platelets during storage. Table 2 lists top 5 signaling pathways involving these 605 mRNAs. With regards to the functional significance, in “molecular and cellular functions” category, hundreds of genes were associated with cell morphology, cellular assembly and organization, cellular movement, cellular growth and proliferation and cell death and survival (Table 3). Again, these represent all major morphological, physiological and functional changes that occur in platelets during storage.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Adj p-value</th>
<th>Number of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin Cytoskeleton Signaling</td>
<td>&lt;0.05</td>
<td>29</td>
</tr>
<tr>
<td>Integrin Signaling</td>
<td>&lt;0.05</td>
<td>28</td>
</tr>
<tr>
<td>Molecular Mechanisms of Cancer</td>
<td>&lt;0.05</td>
<td>36</td>
</tr>
<tr>
<td>Ephrin Receptor Signaling</td>
<td>&lt;0.05</td>
<td>23</td>
</tr>
<tr>
<td>VEGF Signaling</td>
<td>&lt;0.05</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2: IPA analysis of platelet miRNA-targeted mRNAs revealed top 5 cellular signaling pathways.

<table>
<thead>
<tr>
<th>Top Network Functions</th>
<th>Adj p-value</th>
<th>Number of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Morphology</td>
<td>&lt;0.05</td>
<td>239</td>
</tr>
<tr>
<td>Cellular Movement</td>
<td>&lt;0.05</td>
<td>184</td>
</tr>
<tr>
<td>Cellular Assembly and Organization</td>
<td>&lt;0.05</td>
<td>187</td>
</tr>
<tr>
<td>Cellular Growth and Proliferation</td>
<td>&lt;0.05</td>
<td>282</td>
</tr>
<tr>
<td>Cellular Function and Maintenance</td>
<td>&lt;0.05</td>
<td>244</td>
</tr>
</tbody>
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Table 3: IPA analysis of platelet miRNA-targeted mRNAs revealed top 5 network functions.

A number of genes altered during storage are known to have important functions in major physiological functions of platelets such as activation, adhesion, aggregation and binding (Figure 2). In addition to deregulation of genes important for maintaining the structure and function of platelets, we identified 27 genes (AKT1, BCL2L1, BCR, CASP2, CASP3, CBL, CD44, CEBPA, CEBPB, DIABLO, EPOR, ETV6, FADD, FOXO3, HCLS1, IL7, IRS1, MAP2K1, NUMB, PRK-CQ, PTPN6, RAF1, RAC1, SHC1, SYK, TAL1 and YY1) associated with apoptosis suggesting that platelets might be undergoing apoptosis during storage. A phenomenon until recently confirmed to occur only in nucleated cells (Figure 3).

In this study, we found down regulation of 35 mRNAs which are known targets (based on experimental targets in the IPA data analysis tool) for 7 miRNAs (miR-1-3p, miR-129-5p, miR-214-3p, miR-292b-5p and miR-320b), which are increased in platelets during storage (Figure 4).
Discussion

Since mRNAs are subjected to regulation by miRNAs, it is important to assess the changes both in mRNA and miRNA expression levels of platelets during storage. The functional analysis of potential miRNA targets showing inverse correlation with miRNA expression identified several signaling pathways. The actin mediated cytoskeleton signaling and integrin signaling were the top most signaling pathways implicated in platelet functions such as aggregation, granule secretion and activation. Flaumenhaft et al., [9] also reported regulatory effect of actin cytoskeleton signaling on platelet granule secretion. In a previous study, 12 members of integrin signaling pathway were identified as contributors for storage lesion development [10]. Other significant pathways implicated in platelets during storage were focal adhesion pathways, IGF-1 signaling pathway and ephrin receptor signaling pathway. Integrin and focal adhesion pathways have been reported to be involved in early storage lesion in apheresis platelets [11]. IGF-1 signaling pathway has been reported to regulate platelet activation [12-13] and the ephrin receptor signaling pathway has been reported to regulate platelet granule secretion, Rap1 activation, platelet adhesion and aggregation [14-16].

The morphology of platelets do change during storage and platelet movement following transfusion into patients is critical as platelets are supposed to migrate rapidly to the site of damage to initiate formation of “platelet plug” to seal the damaged blood vessel and subsequent wound healing [17,18]. The change in expression levels of different genes identified in our study is able to reflect the changes in platelets morphology and physiological functions during storage. Since these molecular changes in terms of their ability to activate, aggregate and adhere as well as interact with each other are representative of the quality of platelets in storage, the differences in the expression levels of these genes may help develop platelet storage quality biomarkers predictive of their performance in platelet-transfused patients.

Apoptosis is an important process implicated in platelet storage lesion [19-20]. Our IPA analysis found a list of 27 apoptosis related genes. The list included genes such as CASP3 and BCL2L1 which are important players of apoptosis [21-23]. These candidate genes and the miRNAs that target these genes could be important in assessing the platelet quality during storage. The treatment of platelet concentrate with caspase inhibitor has been shown to increase the survival of the platelets [21]. Similar results were reported with use of antibodies against caspases [24]. The existence of apoptotic machinery in platelets and its alteration during storage provides another alternative for intervention to improve the quality of platelets during storage. The miRNAs targeting the genes of these apoptotic molecules could also serve as potential quality biomarkers of stored platelets.

In conclusion, the results reported here clearly identify the potential of miRNAs in regulating functions relevant to the platelet storage lesions. Our analyses of the data we generated demonstrates that in fact there are several platelet signaling and functional pathways that are potentially subject to miRNA:mRNA interactions and 7 miRNAs that we identified to be upregulated (miR-320b, miR-1-3p, miR-214-3p, miR-197-3p, miR-129-5p, miR-183-5p and miR-292b-5p) could serve as potential platelets storage biomarkers. There is very limited information on functional role miRNAs in platelets. Of the seven miRNAs identified in our study only miR-320b has been studied in relation to...
its specific function in platelets. Activated platelets have been reported to release miR-320b in circulation where it has been found to regulate expression of Intercellular Adhesion Molecule-1 (ICAM-1) in endothelial cell [25]. Further experimental studies are required to assess the role of these miRNA:mRNA pairs.

Acknowledgement

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Authorship Contributions

ND designed the study, performed experiments, analyzed data and wrote manuscript. CDA provided training to ND and also participated in designing the study and writing the manuscript. SK assisted ND in obtaining samples from NIH Blood Bank and ordering laboratory supplies and reagents needed for all experiments.

Disclosure of Conflicts of Interest

The authors declare that they have no conflicts of interest.

References