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Original Article

Identification of a miRNA signature in neutrophils after traumatic injury

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Traumatic injury is the cause of significant mortality and morbidity. The molecular mechanisms underlying traumatic injury logically involve changes in gene expression that may be regulated through microRNAs (miRNAs). However, the association between miRNA deregulation and traumatic injury is largely unknown. The purpose of the present study is to address this issue. In this study, we used microarray profiling to evaluate the differential expressions of miRNAs in neutrophils obtained from patients with major trauma (injury severity scores >16), relative to healthy individuals. This neutrophilic miRNA signature was further validated using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Genes and signaling pathways related to trauma-induced deregulated miRNAs were investigated *in silico* using the ontology-based and network mapping algorithms of Gene Ontology and Kyoto Encyclopedia of Genes or Genomes. Results showed that 13 miRNAs in neutrophils of major trauma patients were significantly and differentially expressed compared with the miRNA profiles of healthy controls. The results of qRT-PCR and *in silico* analysis revealed that miR-23a-5p, miR-30e-3p, miR-223-5p, miR-3945, miR-155-5p, and miR-150-5p were likely participants in the traumatic pathogenesis of these patients. In conclusion, neutrophils associated with traumatic injury were found to have a unique miRNA signature. Changes in signaling pathways due to deregulated miRNAs may be involved in the pathological processes of traumatic injury.

Keywords microRNA; neutrophils; traumatic injury; bioinformatics

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Introduction

Trauma remains a common cause of death and disability worldwide, particularly in people under 50 years of age [1]. The traumatic insult initiates a systemic inflammatory response that may trigger a cascade of physiological and biochemical events that involve changes in gene expression [2]. To potentially reduce the mortality rate of traumatic injury, prognostic biomarkers that are easily measurable in clinic are important to indicate the need of immediate therapy. For decades, specific blood-based biomarkers such as C-reactive protein, interleukins (ILs), and heat shock protein have been investigated, but while these biomarkers do benefit early diagnosis of severe organ injury and inflammatory immune response [3–5], they have an unacceptable rate of false positives/negatives, poor sensitivity, and lack of tissue specificity. Therefore, the exploration for novel biomarkers with high sensitivity and specificity for the early diagnosis of trauma is urgently needed [6].

MicroRNAs (miRNAs) are evolutionarily conserved, small non-coding RNA molecules of 22 nucleotides that are involved in post-transcriptional regulation either by mRNA cleavage and degradation or by repressing the translation of mRNA into proteins [7]. Recent studies have revealed that miRNAs are present in various biological fluids and involved in many types of physiological and pathological processes. Specific miRNA signatures in different human diseases reflect changes in physiological and pathological conditions in patients [8–10]. The altered mRNA expression is associated with the diagnosis and prognosis of diseases [11,12]. There is increasing evidence that the expression of miRNAs in serum and trauma-related organs may be modulated by traumatic injury [13–15], such as miR-9 that was found more potent and sensitive than the current protein-based biomarkers in the early diagnosis of traumatic injury [6].

Within hours after injury, there is a significant increase in the number of leukocytes. Among these, 50%–60% is neutrophils which are the first cellular responders to severe trauma [16]. Several studies have found that during the early hours after injury, the association of neutrophils with clinical outcome was determined not only by cell numbers, but also by cell function [17,18]. As neutrophils are essential to traumatic injury, investigation of the altered miRNA expression in neutrophils after traumatic injury will improve our understanding to correlation between miRNA expression and traumatic injury. To this end, we hypothesized that altered

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miRNA expressions in neutrophils might be valuable biomarkers to detect and monitor the pathological developments associated with traumatic injury. Therefore, in order to indentify distinct miRNAs expression in neutrophils after traumatic injury, we performed a miRNA profiling using samples obtained from major trauma patients and normal volunteers.

Materials and Methods

Patients

This study was performed at the General Surgery Department, Shanghai Sixth People's Hospital, an affiliated hospital to the School of Medicine, Shanghai Jiao Tong University (Shanghai, China). The study conformed to the principles of the Declaration of Helsinki. All patients provided signed written informed consent. Five patients with major trauma but no serious pre-existing illnesses were enrolled in the study 12 ± 3 h after trauma. Samples from five normal volunteer controls were obtained during the same period at the same hospital. Following the microarray profiling, the above-mentioned samples together with another 10 samples of neutrophils, including 5 major trauma patients and 5 healthy controls were used for further qRT-PCR validation.

Isolation of neutrophils using immunomagnetic beads

Whole blood samples from the subjects were processed immediately for isolation of neutrophils. Briefly, 3 ml of human peripheral blood were mixed with 10 ml red cell lysis (RCL) buffer (SLNco Lab, Austin, USA), for 3 min at room temperature. Cell pellets were collected through centrifugation at 400 *g*, then washed once with 1 ml RCL, and suspended in 1 ml suspension buffer (SLNco Lab). Fifty microliter aliquots of cell suspension were removed for other use. Twenty-five microliters of Dynabeads (CD15; Invitrogen, Carlsbad, USA) were added into the remaining cell suspension and mixed well for 30 min at 4°C. After separation using a Dynabead adsorption rack with a magnetic field, a neutrophil-rich preparation was collected. The purity of the preparation was determined using flow cytometry (BD Company, Franklin Lakes, USA).

RNA isolation

Total RNA was isolated using the SLNco total RNA Isolation Kit (SLNco) in accordance with the manufacturer's instructions. The quantity of RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). The concentration of RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA). Triplicate samples were used in the miRNA microarray.

Microarray profiling

Total RNA (100 ng) isolated from the samples and reference were each labeled with fluorescent tags using the Agilent miRNA labeling system (Agilent). After dephosphorylation and denaturation, the total RNA was labeled with cyanine 3-pCp and then hybridized to Agilent Human miRNA Microarray V2.0 for 20 h, using the miRNA Complete Labeling and Hyb Kit (Agilent), in accordance with the procedure described by the manufacturer. At the end of hybridization, the slides were washed two times using the Gene Expression Wash Buffer Kit (Agilent) and then scanned using an Agilent Scanner (G2565CA, Agilent). The images were processed and analyzed with Agilent Feature Extraction Software. Differentially expressed miRNAs were identified, according to $\log_2$ fold changes (fold change >2 or <0.5 and $P < 0.05$) in the ratio of the detected signals [$\log_2$(trauma/control)].

Validation of microarray data with quantitative real-time reverse transcription-polymerase chain reaction analysis

The differentially expressed miRNAs selected from the microarray were validated by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis. The isolated RNA was subjected to reverse transcription using miScript Reverse Transcription Kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. The synthesized cDNA was then amplified by quantitative PCR, using TOYOBO-SYBR Green Real-time PCR Master Mix (Toyobo). PCR was performed in FTC-3000 PCR Cycler (Funglyn, Ontario, Canada). All real-time reactions, including no-template controls, were run in duplicate. The small nuclear RNA U6 was used as the normalization control.

Algorithm analyses

To identify possible mRNA targets of the differentially expressed miRNAs, three different *in silico* analyses were performed: mapping with Gene Ontology (GO); pathway analysis with Kyoto Encyclopedia of Genes or Genomes (KEGG), Biocarta, and Reactome and the correlation of miRNAs and genes using the Sanger miRNA database. GO analysis: it is the key functional classification of NCBI, according to the classification of the GO category [19].

$$\chi^2 R_e = \frac{n_f/n}{N_f/N \; {}^{n}{}_{f} \; {}^{N}{}_{f} \; {}_{N}}$$

Pathway analysis was performed to determine the significant pathways that involved genes differentially expressed in neutrophils of trauma patients. Selection was based on Fisher's exact test and the χ^2 test. The threshold of significance was defined by the P value and the false discovery rate. The enrichment R_e was calculated as in the equation mentioned earlier [20].

To construct a miRNA–mRNA regulatory network, the correlation of miRNAs and genes was determined by their differential expression values, based on the interactions of

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Table 1 Clinical characteristics of study population

Patient	Group	Gender	Age	Disease	White blood count	Neutrophils ratio (%)	ISS scores
1	Controls	F	63	Normal	5.6×10^9	67	N/A
2	Controls	M	45	Normal	7.3×10^9	71	N/A
3	Controls	F	52	Normal	5.1×10^9	65	N/A
4	Controls	M	50	Normal	4.6×10^9	66	N/A
5	Controls	M	55	Normal	6.7×10^9	63	N/A
6	Major trauma	M	68	Traffic accident	14.2×10^9	82	17
7	Major trauma	F	56	Falling injuries	12.3×10^9	88	22
8	Major trauma	F	43	Falling injuries	15.4×10^9	81	19
9	Major trauma	M	51	Traffic accident	13.3×10^9	83	22
10	Major trauma	M	56	Traffic accident	12.5×10^9	85	17

miRNA and genes in the Sanger miRNA database. In the miRNA–gene network, the circle represented the gene and the square represented the miRNA, and the degree of their association was determined by one edge. The center of the network was represented by degree. Degree means the contribution of one miRNA to the genes around or the contribution of one gene to the miRNAs around. The target miRNA and the gene in the network always have the biggest degrees [21].

Statistical analysis

Data were expressed as mean ± standard error. Student's *t*-test was used to determine the significance of differences in miRNA expression between groups. All statistical analyses were performed using SPSS software Version 13.0 (SPSS, Chicago, USA). $P < 0.05$ was considered significant. GeneSpring GX software (zcomSilicon Genetics, Redwood City, USA) was used to normalize data for the selection of differentially expressed miRNAs.

Results

Baseline population characteristics

There were no significant age or gender differences between the trauma patients and volunteer controls enrolled for microarray ($P > 0.05$; **Table 1**). The injury severity scores (ISS) [22] were assigned based on the first clinical examination on admission.

miRNA signature in neutrophils after traumatic injury

To investigate whether the inflammatory response after major trauma resulted in a miRNA signature profile in neutrophils, miRNA microarray analysis was performed with Agilent Human miRNA Microarray V2, using samples isolated from major trauma patients and age-, gender-matched healthy volunteers. A total of 15,744 probes representing 723 human miRNAs were included in the version of miRNA microarray that we used. We found 13 differentially expressed miRNAs, among which 8 were up-regulated and 5 were down-regulated (with a fold change cutoff of 2.0 and $P < 0.05$; **Table 2**). The differential miRNA expression patterns of the patients with major trauma were accurately separated from that of the healthy controls by hierarchical cluster analysis (**Fig. 1**).

Table 2 Differentially expressed miRNAs in neutrophils following traumatic injury

Systematic name	*P* value	Fold change[a]
hsa-miR-146b-5p	0.0354	−5.0864
hsa-miR-146a-5p	0.012125	−4.9334
hsa-miR-363-3p	0.002551	−4.7802
hsa-miR-155-5p	0.034924	−4.509
hsa-miR-150-5p	0.046829	−4.5078
hsa-miR-29b-1-5p	0.044707	3.1274
hsa-miR-23a-5p	0.011894	3.552
hsa-miR-125a-5p	0.027391	3.7162
hsa-miR-30e-3p	0.048935	4.1188
hsa-miR-3188	0.030202	4.1256
hsa-miR-193a-5p	0.012173	4.8542
hsa-miR-223-5p	0.03642	5.1876
hsa-miR-3945	0.009757	5.7524

[a]Positive $\log_2$ scale indicating up-regulation and negative $\log_2$ scale indicating down-regulation.

Validation of the miRNA candidates by qRT-PCR

To confirm the miRNA signature found in the microarray study, we used qRT-PCR to validate 13 miRNA candidates that were selected. Our data revealed that the expression levels of miR-3945 [**Fig. 2(A)**, $P = 0.0313$], miR-125a-5p [**Fig. 2(B)**, $P = 0.0276$], miR-363-3p [**Fig. 2(C)**, $P = 0.036$], and miR-150-5p [**Fig. 2(D)**, $P = 0.0107$] were significantly altered in the neutrophils of the patients with major trauma, as compared with those in the controls. Those data supported the

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expression pattern of miRNAs that were found in miRNA microarray. However, the rest miRNAs did not show the differentially expression pattern in the validation.

Functional categories of altered miRNAs in the neutrophils

The differentially expressed miRNAs were classified into different functional categories (**Fig. 3**), according to GO analysis of biological processes. The top six GO functional categories for up-regulated miRNAs were: sodium ion transport; negative regulation of transcription from RNA polymerase II promote; protein transport; small GTPase mediated signal transduction; interspecies interaction between organisms; and ubiquitin-dependent protein catabolism [**Fig. 3(A)**]. The six primary GO categories for down-regulated miRNAs were: cell cycle; protein amino acid phosphorylation; ubiquitin-dependent protein catabolism; cell adhesion; cell differentiation; and cell cycle arrest [**Fig. 3(B)**].

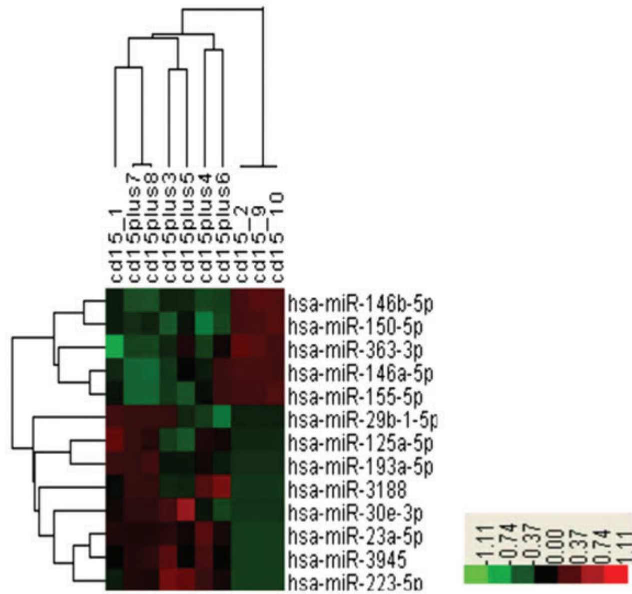

Figure 1 Hierarchical clustering analysis of 13 differentially expressed miRNAs.

Altered miRNA-associated regulatory networks

The pathways associated with the differentially expressed miRNAs were determined by KEGG pathway analysis. The most significant pathways of the up-regulated miRNAs concerned: axon guidance; Wnt; insulin; mitogen-activated protein kinase (MAPK); and cell adhesion molecules [**Fig. 4(A)**]. Down-regulated miRNAs were related to pathways that regard: MAPK; ErbB; calcium; chronic myeloid leukemia; and cell cycle [**Fig. 4(B)**].

The up-regulated miRNAs, including miR-23a-5p, miR-30e-3p, miR-223-5p, and miR-3945 have a variety of potential targets (mRNAs; **Fig. 5**). The miRNAs miR-155-5p and miR-150-5p showed the highest degree of underexpression (with degrees of 6 and 4, respectively).

Discussion

Trauma remains the leading cause of death for children, adolescents, and young adults and the fourth leading cause of death over all age groups in the USA [1]. Although significant progress has been made to improve the prognosis of major trauma, early diagnosis and evaluation are still the highest priority in saving the patient's life. Several

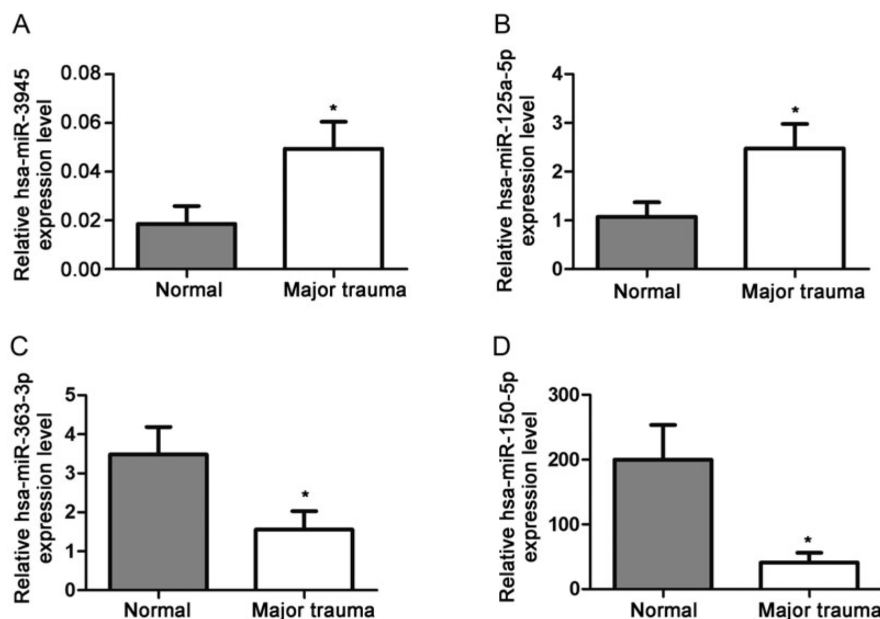

Figure 2 Validation of the differentially expressed miRNAs by qRT-PCR. (A) hsa-miR-3945; (B) hsa-miR-125a-5p; (C) hsa-miR-363-3p; (D) hsa-miR-150-5p. $^{*}P < 0.05$.

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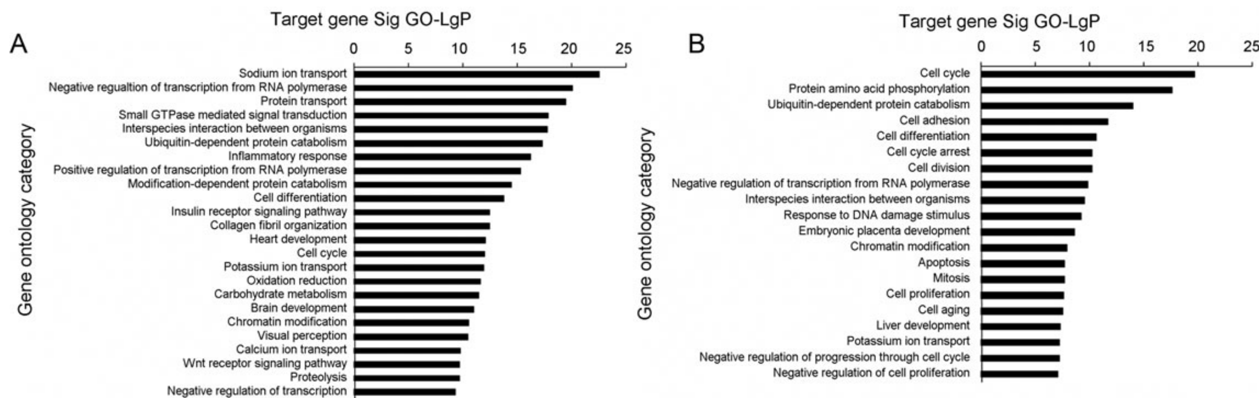

Figure 3 GO functional categories of altered miRNAs in the neutrophils Eight up-regulated (A) and five down-regulated (B) miRNAs, respectively. All of these GOs show an increased enrichment in each category by these miRNAs. The vertical axis is the GO category, and the horizontal axis is the enrichment of GO.

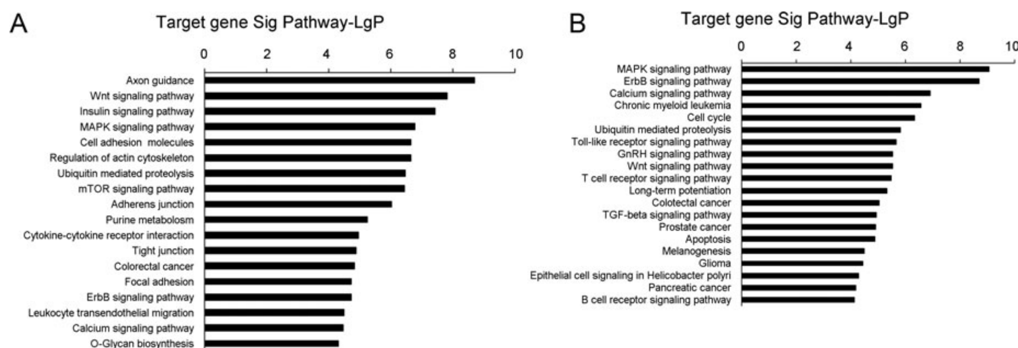

Figure 4 Pathway analysis based on miRNA-targeted genes Main pathways targeted by up-regulated (A) and down-regulated miRNAs (B), respectively. The vertical axis is the pathway category and the horizontal axis is the degree of enrichment of the pathway.

commonly used systems such as the ISS and Trauma and Injury Severity Score have been developed to classify the severity of injuries of trauma patients [23]. In spite of their simplicity, some known and inherent limitations actually affect their effectiveness [24]. Thus, many studies have been conducted to identify an ideal biomarker for trauma diagnosis. C-reactive protein, ILs, and heat shock protein are potential biomarkers that help to make an early and correct diagnosis for the severity of organ injury and the level of inflammatory immune response [3–5]. However, as biomarkers, these proteins are limited by low sensitivity and specificity during examinations in the clinic. Therefore, more accessible biomarkers remained to be identified.

miRNAs that regulate protein expression by acting on the 3′-untranslated regions of target genes [25] have a central role in physiological and pathological processes. Deregulation of miRNA expression reflects altered physiological and pathological conditions in human diseases [8–10]. Compared with normal healthy controls, a miRNA signature in the disease that is comprised of differentially expressed miRNAs may be used as potential biomarkers for the diagnosis of various human diseases [26–28]. Recent studies have shown that circulating miRNAs in serum also could be taken as novel biomarkers for trauma diagnosis and evaluation. For example, a report by Laterza *et al*. [29] has shown that liver-, muscle-, and brain-specific miRNAs are used as biomarkers associated with tissue injury. Redell *et al*. [30] also have demonstrated that miR-16, miR-92a, and miR-765 are reliable indicators of severe traumatic brain injury. Furthermore, Zhang's report [6] has suggested that serum miR-9 may serve as a promising biomarker for traumatic injury with high sensitivity. In that report, the authors have shown that leukocytes might be the main source of serum miR-9 in rats with traumatic injury caused by partial hepatectomy, which is consistent with other findings that circulating miRNAs are usually released from cells [31,32]. As is well known, systemic activation of neutrophils occurs shortly after injury, leading to leukocytosis and an increased number of neutrophils [16]. Several studies have revealed that a high number of neutrophils during the first hours after injury is correlated with clinical outcome of trauma patients [17,18]. Activated neutrophils have been found to have a pivotal role in the inflammatory response to

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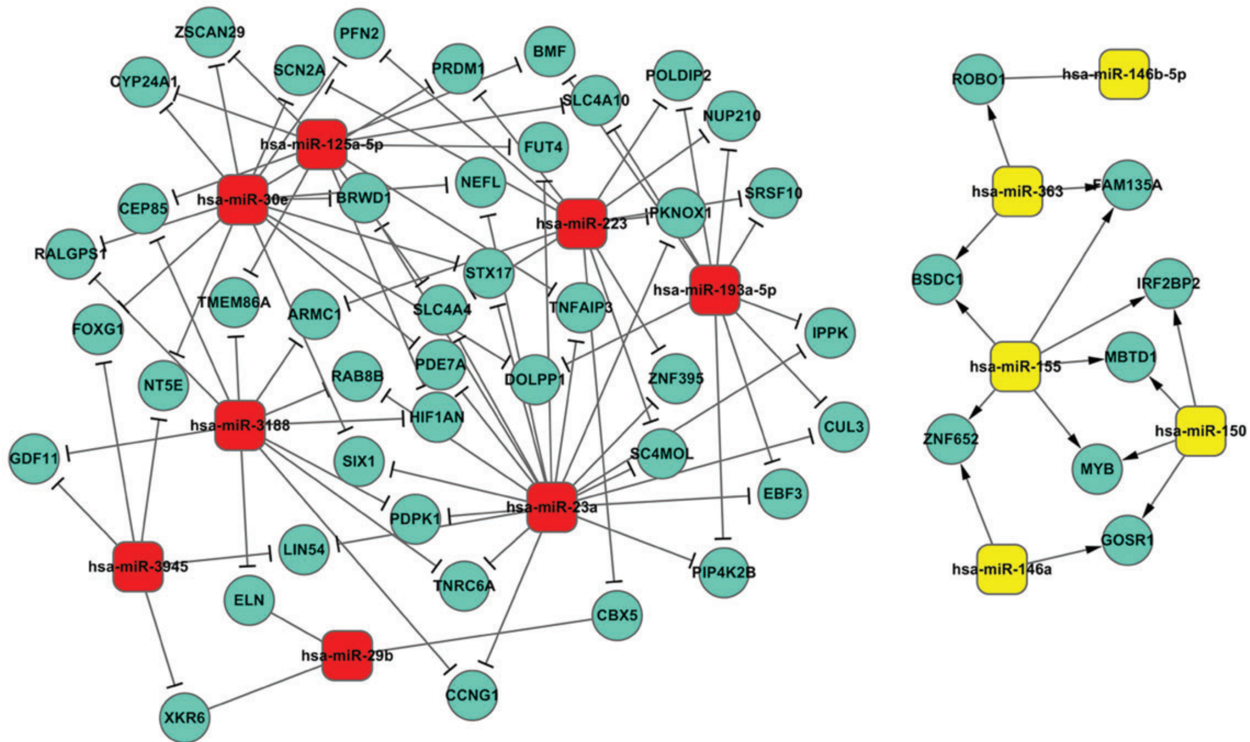

Figure 5 miRNA–mRNA network Red and yellow box nodes represent miRNA, and blue cycle nodes represent mRNA. Edges show the inhibitory effect of miRNA on mRNA. The red box shows the overexpression of miRNA–mRNA network and the yellow box is the underexpression of miRNA–mRNA network.

injury [33,34]. In a recent study, miR-9 in human monocytes and neutrophils has been recognized as a novel miRNA induced by proinflammatory signals [35].

Towards a better understanding of the molecular mechanisms underlying traumatic injury, our study focused on a miRNA signature in neutrophils following traumatic injury using miRNA microarray analysis. In total, 13 differentially expressed miRNAs in neutrophils of trauma patients were identified, including 8 up-regulated and 5 down-regulated miRNAs. These altered miRNAs were further validated by qRT-PCR. To determine the signaling pathways and genes related to the deregulated miRNAs, *in silico* analysis tools including GO and KEGG pathway annotation were used. GO analysis showed a correlation between the down-regulated miRNAs and ubiquitin-dependent protein, catabolism, and cell adhesions. This result implies that the potential targets of the altered miRNAs may be involved in various pathological processes that promote the development of traumatic injury. Among those associated genes, one of the most interesting molecules is ubiquitin, which has an important role in the regulation of immunodepression in critical illness [36]. Furthermore, intracellular ubiquitin has been implicated in the regulation of inflammatory responses, membrane protein trafficking, transcription, and protein–protein interactions [37]. The effect of exogenous ubiquitin on resuscitation during severe trauma has also been investigated [38]. On the other hand, the expression of adhesion molecules in circulating leukocytes during traumatic injury could be regulated for a close contact between leukocytes and the endothelium at the site of injury [22].

KEGG annotation showed that the MAPK and toll-like receptor (TLR) signaling pathways are associated with the down-regulated miRNAs in our study. MAPK has been reported as an important player in the response to extracellular stimuli including injury [39]. In an ischemic liver injury model, inhibition of p38 MAPK activation can attenuate ischemia-reperfusion injury of the liver [40]. p38 MAPK can be activated by neutrophil elastase, leading to enhanced activities of nuclear factor-kappa B and activator protein 1, thereby inducing IL-8 mRNA expression and protein synthesis [41]. Since macrophage prostaglandin E2 (PGE_2) production in macrophages after trauma is induced by active MAPK, pharmacological inhibition of MAPK could block trauma-induced cyclooxygenase-2 expression, and PGE_2 production [42]. TLR is another contributor in inflammatory immune responses during trauma-induced infections [43]. Aberrant activation of the TLR/CD14 pathway is related to the risk of infectious complications in severely injured trauma patients [44]. Genetic variation in TLR1 is also associated with increased mortality in patients with sepsis after traumatic injury. Therefore, TLR may be a novel biomarker of risk of death in critically injured patients [45].

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In the present study, to identify more potential targets of the altered miRNAs in neutrophils, we also conducted a miRNA–mRNA regulatory network analysis. In the analysis, we found up-regulated miR-23a-5p, miR-30e-3p, miR-223-5p, and miR-3945 with 20, 14, 12, and 5 candidate targets, respectively, while down-regulated miR-155-5p and miR-150-5p containing 6 and 4 candidate targets. Of the differentially expressed miRNAs (relative to healthy controls) that were identified by microarray from RNA samples isolated from neutrophils of patients with major trauma, both miR-3945 and miR-150-5p were confirmed by qRT-PCR. Although the role of miR-3945 in traumatic injury is unclear, it has been reported that miR-150 is related to critical illness and sepsis. Schmidt *et al*. [46] have found a decrease in the expression of miR-150 in leukocytes collected from human volunteers upon treatment with lipopolysaccharide. Another study has shown that the expression level of serum miR-150 is related to either intensive care unit survival or long-term survival rates [47]. It appears that lower miR-150 expression may be linked to an unfavorable prognosis.

Trauma is a complex process caused by multiple pathological pathways, involving many factors, genes, and cellular signaling pathways. miRNA profiling or establishing a miRNA signature could provide a basis for further investigation of the molecular mechanisms underlying development of traumatic injury. It is also a useful approach to identify novel biomarkers for the diagnosis and prognosis of traumatic injury. We identified a unique miRNA signature in neutrophils after traumatic injury, which included up-regulated miR-3945 and miR-125a-5p and down-regulated miR-363-3p and miR-150-5p. These findings highlight the important roles of miRNAs in the development of traumatic processes and may improve our understanding of the molecular mechanisms related to trauma. Utilization of this miRNA signature as a biomarker has the potential to improve current methods for the diagnosis and prognosis of traumatic injury.

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