ARTICLE
Journal of Cellular Biochemistry 112:2435–2442 (2011)

Journal of Cellular Biochemistry

Altered Expression Levels of miRNAs in Serum as Sensitive Biomarkers for Early Diagnosis of Traumatic Injury

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 22 nucleotides in length which regulate gene expression negatively and play important roles in many pathological processes. It has been demonstrated that circulating miRNAs hold promise to serve as practicable molecular markers for diverse physiological and pathological conditions. In this investigation, we chose partial hepatectomy (PH) as traumatic injury model. There were significantly differential expression of miRNAs in rat serum post-traumatic injury (21 miRNAs were more than twofold up-regulated). Especially, the expression of miR-9 showed the highest up-regulated (>70-fold), and it possessed the characteristics of biomarker that was more sensitive than aspartate aminotransferase and alanine aminotransferase and C-reactive protein for traumatic liver injury. There was also a prominent increase in the expression levels of miR-9 in different brain areas after traumatic injury. Our data suggest that serum miR-9 may serve as promising biomarker for traumatic injury with high sensitivity. Furthermore, these findings may help to elucidate the complex network which mediates stress response to traumatic injury. J. Cell. Biochem. 112: 2435–2442, 2011.

KEY WORDS: miRNA; BIOMARKER; SERUM; TRAUMATIC INJURY; PARTIAL HEPATECTOMY

Trauma is one of the leading causes of death in people under the age of 50 worldwide [MacKenzie, 2000; Evans, 2007]. Traumatic injury can trigger a multifaceted cascade of physiologic and biochemical events, which encompasses a wide range of endocrinological, immunological, and haematological effects, and it also involves many genes and proteins [Keel and Trentz, 2005; Menges et al., 2008]. When trauma occurred, specific clinical biomarkers have the potential to make an early and correct diagnosis about severe organ injury and inflammatory immune response, so as to warrant immediate therapy to potentially reduce the mortality rate. Also some biomarkers such as C-reactive protein (CRP), interleukin, and heat shock protein have been used to monitor and assess trauma [Maruszynski and Pojda, 1995; Pespeni et al., 2005; Neumaier et al., 2006a]. Further studies on new biomarkers with high sensitivity and specificity in early diagnosis of trauma are still warranted.

MicroRNAs (miRNAs) are an abundant class of highly conserved, approximately 22 nucleotides in length, non-coding RNA molecules which are able to induce mRNA degradation, translational repression, or both, via pairing with partially complementary sites in the 3′UTR of the targeted genes, and play a central role in many biological and pathological processes [Bartel, 2004; Kloosterman and Plasterk, 2006; O'Hara et al., 2009]. In general, miRNAs are regulated and transcribed like protein coding genes. Recently, studies provide evidence that trauma can induce changes in the expression of miRNAs in trauma-related organs [Lei et al., 2009; Liu et al., 2009; Yu et al., 2009]. It also has become clear that miRNAs are abundant and very stable in serum. Previous studies have demonstrated that serum miRNAs are little affected by severe conditions, such as RNase digestion, boiling, very low or high PH, extended storage, and freeze–thaw cycles [Chen et al., 2008; Mitchell et al., 2008]. Furthermore, serum miRNAs expression associate with different physiological stages and pathological conditions are significantly different [Gilad et al., 2008; Mitchell et al., 2008; Laterza et al., 2009; Wang et al., 2009, 2010a].

Therefore, we hypothesized that the circulating miRNAs might be used to detect and monitor the pathological development associated with traumatic injury. In this study, we used rat partial hepatectomy (PH) as traumatic injury model and detected the different expression profiles of miRNAs in rat serum after traumatic liver injury by miRNA microarray. Furthermore, we revealed that expression level of serum miR-9 was significantly up-regulated post-PH. The change of miR-9 expression was significantly positively correlated with serum aspartate aminotransferase (AST), alanine aminotransferase

Grant sponsor: Key Program of National Natural Science Foundation of China; Grant number: 81030010.
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Received 2 December 2010; Accepted 21 April 2011 • DOI 10.1002/jcb.23168 • © 2011 Wiley-Liss, Inc.
Published online 2 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

(ALT), and CRP levels, but more sensitive. Our results suggested that serum miR-9 could be as a novel non-invasive molecular marker for trauma.

MATERIALS AND METHODS

ANIMALS

Male Sprague–Dawley rats of 7–10 weeks of age (body weight, 250 ± 20 g) were obtained from the Animal Research Center of Fourth Military Medical University, Xi'an, China. All animals were allowed a 2-week acclimation period until experiment. They were kept in groups of two in standard breeding cages and maintained in a temperature-controlled animal facility ($21 \pm 1^{\circ}$C), with a light/dark cycle of 12 h and ad libitum access to standard laboratory chow and water. All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85–23, revised 1996), and all procedures were approved by the Animal Research Center of Fourth Military Medical University.

PARTIAL HEPATECTOMY

2/3 PH was performed according to the technique described by Higgins and Anderson [1931]. After being anesthetized, the liver was exposed through a 2–3 cm longitudinal incision in the abdomen and the vascular pedicles were ligated. Then the left and middle lobes, totaling about two-thirds of the liver, were resected. We also used 1/3 PH (only medial lobe was removed), a procedure that causes less injury. Rats were killed at 6, 12, 24, and 48 h after PH.

HISTOLOGICAL ANALYSIS

Tissue samples from remaining liver were sectioned from each experimental group and immediately fixed in 10% neutral-buffered formalin, embedded in paraffin for histological examination. Tissue sections (4-mm thick) were stained with hematoxylin and eosin (HE) and examined under a light microscope.

SERUM COLLECTION

The blood samples were collected from rats in different time points. The blood was centrifuged at 1,500 rpm for 15 min at 4°C and then the supernatant (serum) was carefully transferred into 1.5 ml Eppendorf tubes for further experiment. Serum samples were stored at −80°C until use.

SERUM TRANSAMINASE AND C-PROTEIN ANALYSES

Serum AST and ALT levels were measured as markers of hepatocyte injury. The levels of ALT and AST were measured using an autoanalyzer (Cobas Integra 400 plus, Roche, Switzerland). Serum CRP was measured using rat CRP ELISA kit as per the manufacturer's instructions (R&D) and the results of optical density (OD) value were recorded at 450 nm by Microplate Reader (Bio-rad 550).

BLOOD CELL SEPARATION AND COLLECTION

Rat blood samples were collected into vacutainer tubes containing EDTA. All the samples were stored at room temperature and were immediately used as a source of leukocytes and polymorphonuclear leukocytes (PMN). Rat leukocytes and PMN were separated by animal white blood cell isolation kit and PMN isolation kit according to the protocol provided by the manufacturer (GenMed). The procedure of leukocyte separation as follow: Blood specimen was mixed with separation solution thoroughly at a ratio of 4:1 (Blood:Separation Solution) for 20 min at room temperature to lyse erythrocytes. The leukocyte-enriched solution was transferred to a 15 ml conical centrifuge tube and centrifuge at 300*g* for 10 min. Then we discarded supernatant and washed leukocytes for three times using sterile PBS. The procedure of PMN separation as follow: At first, 3 ml hyperbaric solution was added to a 15 ml conical centrifuge tube and 3 ml of hypobaric solution was carefully laid onto the hyperbaric solution. Then 6 ml of whole blood was carefully laid onto the upper gradient. The conical centrifuge tube was centrifuged at 700*g* for 30 min at room temperature to separate PMN. Then we carefully removed centrifuge tubes and transferred cells from the layer which contained PMN. Purity of the populations of leukocytes and PMN were routinely assessed by flow cytometry (BD Biosciences, Mountain View, CA).

miRNA MICROARRY ANALYSIS

The miRNA microarray was used to assess the level and composition of miRNA. Total RNA from serum, cells, and tissues were harvested using TRIzol (Inivitrogen) and RNeasy mini kit (Qiagen) according to manufacturer's instructions. After having passed RNA measurement on the Nanodrop instrument, the samples were labeled using the miRCURY^{TM} Hy3^{TM} Power labeling kit (Exiqon) and hybridized on the miRCURY^{TM} LNA Array (v.11.0). The miRCURY LNA^{TM} miRNA Array contained more than 1,700 probes for all organisms and viruses listed in miRBase, and have very high miRBase coverage. Scanning was performed with the Axon GenePix 4000B microarray scanner. GenePix pro V6.0 was used to read the raw intensity of the image. The intensity of green signal was calculated after background subtraction and four replicated spots of each probe on the same slide have been calculated the median. We used Median Normalization Method to obtain "Normalized Data," Normalized Data = (Foreground − Background)/median, the median was 50% quantile of miRNA intensity which was larger than 50 in all samples after background correction.

REAL-TIME RT-PCR ANALYSIS

Real-time RT-PCR was used to confirm the expression levels of miR-9 in rat serum. Total RNA was extracted from different groups of serum using Trizol reagent as per the manufacturer's instructions (Invitrogen, CA). The purity and concentration of the RNA was determined by measuring its absorbance at 260 and 280 nm. Reverse transcription PCR was performed according to the protocol of PrimeScript® RT reagent Kit (TaKaRa, Otsu, Japan), real-time PCR was performed with SYBR premix Ex Taq II (TaKaRa) on Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's instructions. The expression level of U6 was used as an internal control to normalize mir-9 expression in each sample. Primer sequences for PCR were as follow: rno-miR-9: 5′-GGGGGTCTTTGGTTATCTA-3′ and 5′-CAGTGCGTG-TCGTGGA-3′, U6: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ and 5′-CGCTTCACGAATTTGCGTGTCAT-3′. All reactions were run in

triplicate. The $\Delta\Delta C_t$ method determined miRNA expression level. Quantitative values were determined using the $2^{-\Delta\Delta Ct}$ equation.

STATISTICAL ANALYSIS

Data were expressed as mean + SE and compared between groups using the Student's *t*-test. All statistical analyses were carried out with the use of SPSS software Version 13.0 (SPSS, Chicago, IL). Pearson's correlation analysis was used to estimate the relationship between miRNA expression and traumatic injury. A P-value < 0.05 was considered significant.

RESULTS

PH CAUSE WIDESPREAD CHANGES IN HEPATIC TISSUE AND SERUM

Serum ALT and AST levels were quantified to determine the severity of injury in liver. Both 1/3 and 2/3 PH induced significant changes of ALT and AST levels in rat serum (Fig. 1A,B). The levels of ALT and AST could been found increased obviously at 12 h in group 1/3 and 2/3 PH, and reaching peak values at 24 h. For 2/3 PH group, the levels of ALT and AST were higher than that of 1/3 PH group. A significant increase of serum CRP level was also observed in the rats which subjected to PH. But there was on significant difference between 1/3 and 2/3 PH group (Fig. 1C).

To further evaluate the severity of injury which caused by PH, histological changes in liver tissues were examined. Both 1/3 and 2/3 PH caused significant hepatic damage, which displayed several morphological characteristics, including severe sinusoidal congestion, cytoplasmic vacuolization, and massive necrosis of parenchymal hepatocytes (Fig. 2). As expected, the serum, ALT, AST levels, and histological changes showed differences among 1/3 and 2/3 PH group; however, the variation of CRP measurements were not sensitive enough.

PH-INDUCED CHANGES IN THE EXPRESSION OF miRNAs IN RAT SERUM

Expression profiles of miRNA were examined using a commercial miRNA microarray that contained more than 1,700 capture probes, covering all miRNAs annotated in miRBase 11.0. The expression level of each miRNA was indicated as folds over U6 snRNA. Comparing the 2/3 PH groups with controls, the serum miRNAs expression pattern was found to be significantly different (Table I). Twenty-seven miRNAs were found to be expressed up-regulated more than twofold in 2/3 PH rats serum compared to controls. Furthermore, five of them, miR-9, miR-133a, miR-122, miR-133b, and miR-183, were found up-regulated more than 10-fold.

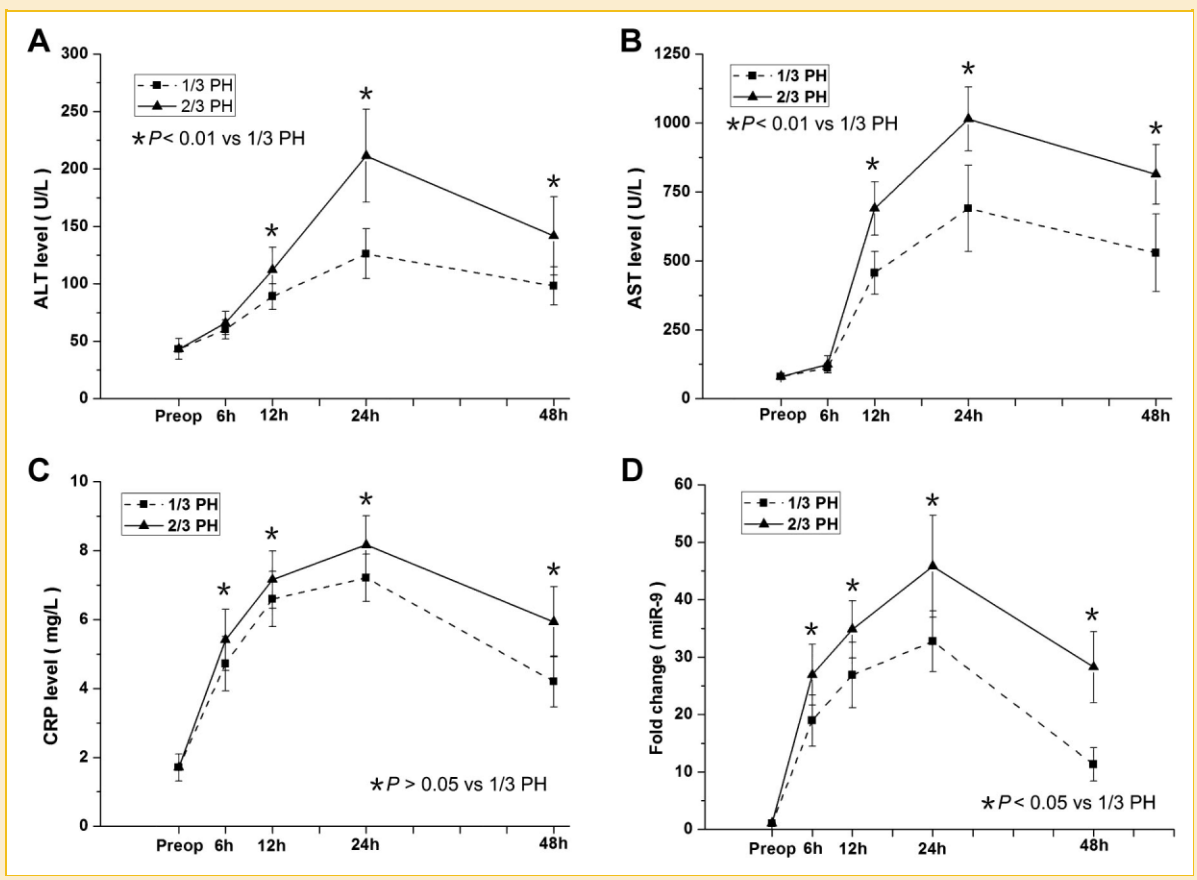

Fig. 1. Expression levels of ALT, AST, CRP, and miR-9 in rats' serum at different time points after PH. A: ALT. B: AST. C: CRP. D: miR-9. Data are mean ± SE; n = 6 in each group.

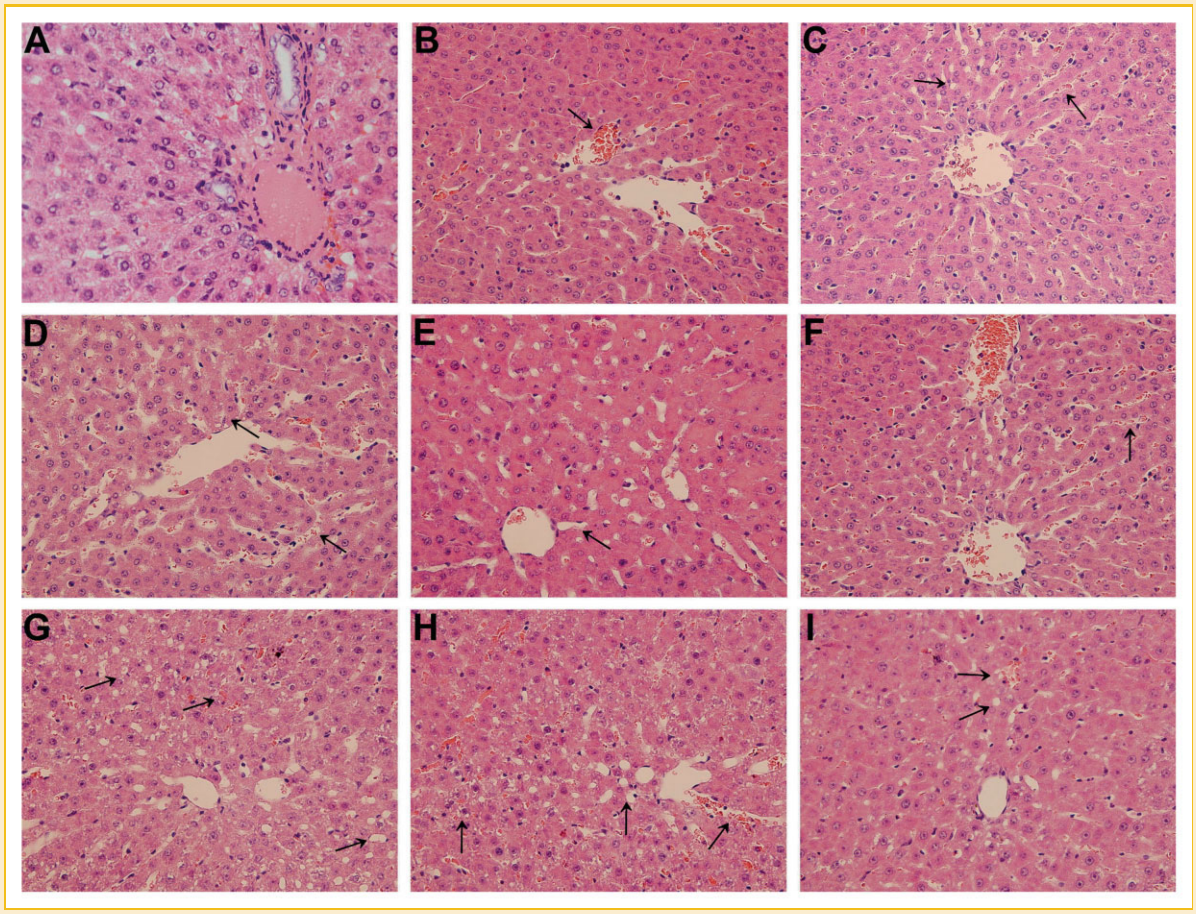

Fig. 2. Hematoxylin–eosin staining revealed histological change of liver sections. A: Normal group. B: 6 h after 1/3 PH. C: 12 h after 1/3 PH. D: 24 h after 1/3 PH. E: 48 h after 1/3 PH. F: 6 h after 2/3 PH. G: 12 h after 2/3 PH. H: 24 h after 2/3 PH. I: 48 h after 2/3 PH. Original magnification 400×.

TABLE I. List of All Significantly Changed miRNAs (24 h After 2/3 PH)

miRNA name	Fold change (up-regulated)
rno-miR-22	2.01
rno-miR-340-5p	7.71
rno-miR-9	74.08
rno-miR-151	3.14
rno-miR-133a	18.57
rno-miR-99a	2.27
rno-miR-206	6.99
rno-miR-378	2.04
rno-miR-34a	2.18
rno-miR-17-3p	2.30
rno-miR-148b-3p	2.02
rno-miR-23a	4.99
rno-miR-122	10.13
rno-miR-181a	2.12
rno-miR-193	3.08
rno-miR-133b	11.93
rno-miR-374	3.15
rno-miR-22	3.05
rno-miR-542-3p	2.28
rno-miR-365	2.66
rno-miR-877	2.35
rno-miR-183	16.16
rno-let-7f	2.12
rno-miR-685	6.32
rno-miR-7a	3.14
rno-miR-667	2.39
rno-miR-138	3.69

Especially, the expression of miR-9 demonstrated the highest up-regulated (70-fold overexpressed).

EXPRESSION LEVELS OF SERUM MIR-9 CAN BE USED TO EVALUATE THE SEVERITY OF TRAUMATIC INJURY

The miR-9 was chosen as the candidate miRNA for validation according to the results from the array analysis by RT-PCR analysis, and investigated the character of it as potential serum biomarker of traumatic injury. Expression level of miR-9 in rats' serum, which was very low under normal condition, was significantly increased at 6 h after PH, and remained significantly increased until 24 h after PH (Fig. 1D). To better understand the correlation between the severity of traumatic injury and the expression of miR-9, we tested the expression levels of serum miR-9 after 2/3 PH as compared with 1/3 PH. The data showed that PH-induced miR-9 change was significantly different between 2/3 PH and 1/3 PH (2/3 PH induced higher up-regulation of miR-9 than 1/3 PH) (Fig. 1D). Pearson's correlation analysis was performed to estimate the potential relationship between miR-9 expression level and severity of 2/3PH-induced traumatic injury. Scatter plots illustrated that serum miR-9 expression level was significantly positively correlated with serum AST, ALT, and CRP levels (Fig. 3). Taken together, serum

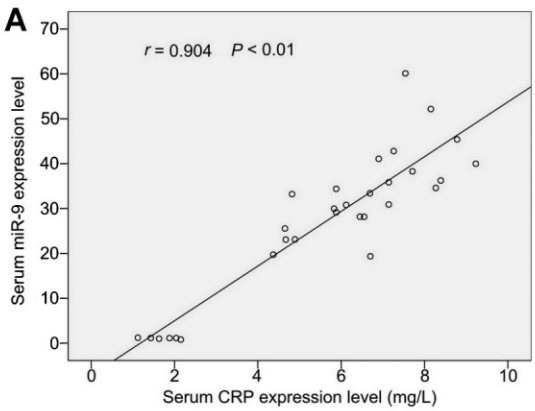

Fig. 3. Correlation of serum miR-9 expression levels with the severity of traumatic injury. A: The expression level of miR-9 was significantly positively correlated with serum CRP levels. B: The expression level of miR-9 was significantly positively correlated with serum ALT levels. C: The expression level of miR-9 was significantly positively correlated with serum AST levels. n = 6 in each group.

miR-9 may be a potentially far more sensitive and reliable biomarker for PH-induced injury.

THE ORIGIN OF SERUM MIR-9 AND ITS EXPRESSION IN DIFFERENT TISSUES

To further investigate the origin of serum miR-9, we used real-time RT-PCR to detect the expression of miR-9 in leukocytes and several important organs such as liver, lung, brain, and heart. Our data showed that miR-9 was produced at low level in rat serum under normal condition. 2/3 PH resulted in significantly up-regulated of miR-9 expression in leukocytes (Fig. 4). The expression levels of miR-9 in leukocytes which induced by 2/3 PH, could been found up-regulated obviously after 6 h and steadily increasing over the time period assessed. The change of miR-9 expression in PMN was similar to leukocytes.

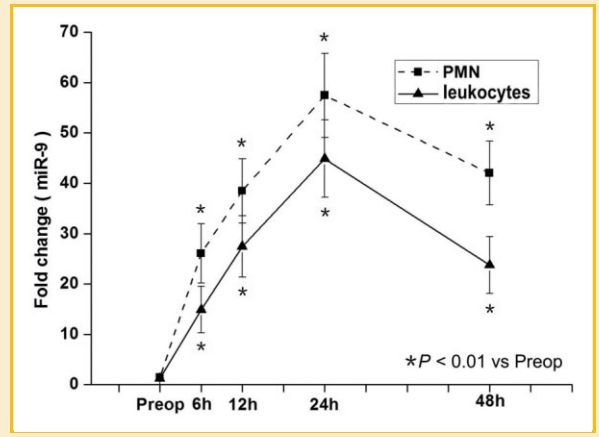

Fig. 4. 2/3 PH induced up-regulation of miR-9 in leukocytes and PMN at different time points. Data are mean ± SE; n = 6 in each group.

Except for expression in leukocytes, miR-9 was also detected up-regulated in brain compare to other tissues from rat that was injured by PH (Fig. 5). RT-PCR analysis showed that miR-9 basal expression levels vary considerably among different areas in rat brain (cerebellum showed the highest expression level). The trauma imposed on rat rapidly induced up-regulation of miR-9 expression in cerebral cortex, hypothalamus and pituitary gland. But the expression levels of miR-9 were not significantly altered in cerebellum and hippocampus (Fig. 5). Furthermore, the expression

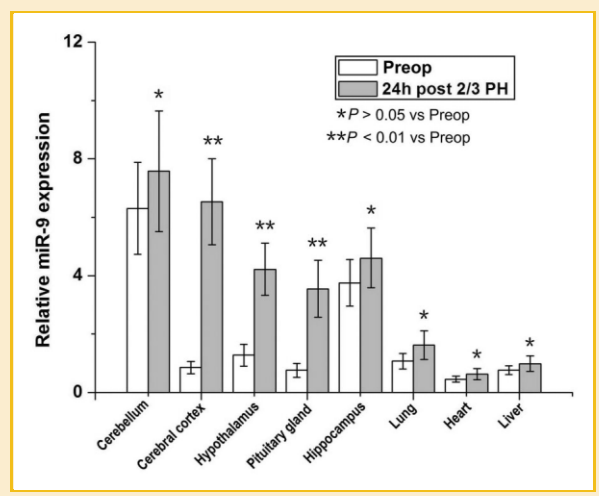

Fig. 5. The miR-9 expression in different tissues at 24 h after 2/3 PH. Data are mean ± SE; n = 6 in each group.

levels of miR-9 in lung, liver, and heart were low, and 2/3 PH could not alter their miR-9 expression obviously even the traumatic liver.

DISCUSSION

Early diagnosis and evaluation of trauma are crucial for saving the patient's life. Accumulating evidence suggested that miRNAs not only play a central role in physiological and pathologic processes, the spectra and levels of some miRNAs could also reflect altered physiological and pathological conditions [Chen et al., 2008; Gilad et al., 2008; Bartels and Tsongalis, 2009; Wang et al., 2010]. To our knowledge, protein-based biomarker must be translated by mRNA to have a biological effect whereas miRNA′s characteristics make it inherently as a biomarker that reflects altered physiology more directly. Considering recent studies of circulating miRNAs in serum, the serum miRNAs may be novel biomarkers for the diagnosis and evaluation of trauma.

PH is a complex pathologic process which is associated with the interaction of many genes and proteins, can cause inflammatory response and severely harmful to the body [Li et al., 2009]. So we chose PH as traumatic model to carry out our research. As shown in Figure 1, the levels of ALT and AST were elevated in serum samples from PH-treated rats. Furthermore, the alterations of ALT and AST expression in rats serum after 2/3 PH were greater than that of 1/3 PH rats. ALT and AST are members of the transaminase family of enzymes. Hepatocellular damage with the subsequent disruption of the plasma membrane allows leakage of intracellular enzymes such as ALT and AST into the bloodstream, and elevated levels of ALT and AST in serum usually imply hepatic injuries [Giannini et al., 2005; Moreno et al., 2007]. Even though ALT and AST could evaluate the severity of trauma indirectly, the obvious elevated levels in the serum were not rapid enough (at least 12 h after PH). CRP is an acute-phase serum protein which is synthesized by hepatocyte, displayed rapid and pronounced rise of its serum concentration in response to infection or tissue injury [Neumaier et al., 2006b]. The levels of CRP in rat serum begun to rise obviously at 6 h after PH, but there was no significant difference between 1/3 and 2/3 PH group. Because of the disadvantages of ALT, AST, and CRP, it is difficult to make an accurate diagnosis for the severity of trauma.

It has already been reported that pathological conditions such as cancers and drug-induced tissue injury, could induce a significant change in the expression levels of a number of miRNAs in serum [Wang et al., 2009]. The results of our present study clearly showed that 27 miRNAs with significant increased in 2/3 PH rats serum compared to controls (a $>$2-fold change), which was consistent with previous reports. Then we chose miR-9 for a more detailed analysis, given that it is the highest up-regulated miRNA in response to 2/3 PH and that it has not been reported before. Previous study demonstrated that miR-9 is highly brain-enriched and play an important role in brain development such as patterning, neurogenesis, and differentiation [Leucht et al., 2008; Coolen and Bally-Cuif, 2009]. Recently, miR-9 has been found take part in the activation of innate immune response though regulating the pro-inflammatory transcription factor nuclear factor NF-κB [Bazzoni et al., 2009; Tsitsiou and Lindsay, 2009]. RT-PCR analysis showed that miR-9 in normal rats′ serum keeping low level and PH could significantly up-regulate the level of serum miR-9 rapidly. Even at 6 h post-2/3 PH, there was a more than 26-fold change of miR-9 expression compared to normal controls, and the expression of serum miR-9 could reached its peak at 24 h post-2/3 PH. Pearson's correlation analysis also showed that the level of serum miR-9 had positive correlation with severity of trauma, which was similar to the ALT, AST, and CRP. Such observations support the concept that serum miR-9 possess the characteristics of biomarker for traumatic injury.

To further explore the function of serum miRNAs, studies are needed to gain greater insight into the origin of circulating miRNAs. Currently, the main viewpoints about the origin of serum miRNAs are as follows: exosomes that are secreted from cells, circulating RNAs from cell apoptosis or necrosis, and fragments of circulating cells [Valadi et al., 2007a; Hunter et al., 2008; Rosell et al., 2009]. The PH could induce intensive stress responses containing inflammatory response and hepatic cells apoptosis, and also take harmful effects on other important organs such as brain, lung, and heart. Previous study showed that miR-9 was up-regulated significantly in leukocytes which were induced by LPS [Bazzoni et al., 2009]. Leukocytes are essential in the first line of defence to traumatic injury, and the severity of injury and the inflammatory response are positively correlated [Pasquale et al., 1996]. In addition, PMN represents 50–60% of total circulating leukocytes and are the leading cells in the first response to severe trauma [Botha et al., 1995]. So it is important to detect the expression levels of miR-9 in leukocytes, PMN and other important organs before and after PH. Like its serum level, expression of miR-9 tested in leukocytes and PMN were both low levels under normal condition. The expression levels of miR-9 in leukocytes and PMN began to rise largely after 6 h of PH. We speculated that one of the main causes which up-regulated the expression of miR-9 in leukocytes and PMN were intensive inflammatory response and leukocytes might be the main source of circulating miR-9 post-PH. Hypothalamic-pituitary-adrenal axis (HTPA axis), a major part of the neuroendocrine system that controls reactions to stress [Desborough, 2000]. According to previous study, brain express higher level of miR-9 than other organs [Rinaldi et al., 2010]. It should be noted that PH rapidly induced up-regulation of miR-9 expression in brain, and its expression levels vary considerably among the different areas. We found low levels of miR-9 in lung, liver, and heart. PH could not alter their expression levels obviously even the traumatic liver (Fig. 5). These finding suggested that leukocytes might be the main source of serum miR-9 in rat which imposed traumatic injury by PH. Because each miRNA can affect the translation of multiple protein-coding genes, the alteration of expression of miR-9 in rat brain indicated that miR-9 should take part in mediation of traumatic stress which executed by neuroendocrine system. Previous study showed that circulating miRNAs could make genetic exchange between cells (e.g., exosome) [Valadi et al., 2007b]. We speculate that miR-9 in serum that could circulate through the body, and affect other organs through transfer its activities in an encapsulated membrane structure to other cell types of the body.

In conclusion, we provided evidence that injury caused by PH could make significant change in the spectra and levels of serum miRNAs; and the level of specific serum miRNAs such as miR-9, could be used as a novel serum-based biomarker potentially offering more sensitive tests than those current protein-based biomarkers for early diagnosis of traumatic injury. Furthermore, based on our study, we tentatively proposed that miR-9 may also be involved in regulating the stress response post-trauma. However, the molecular pathways that serum miR-9 play a role in these events are not completely understood at this time. We will take future studies in animals and humans to enrich our understanding in the mechanism of miR-9 in traumatic process, and may offer a novel approach in trauma therapeutics.

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