

Original Article

Serum miRNA expression and correlation with clinical characteristics in acute kidney injury

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Abstract: Acute kidney injury (AKI) is a common clinical emergency. Its fatality rate and mortality exhibit a rising trend following the increase of aging population. MiRNA participates in disease occurrence and development via targeting mRNA expression. Previous study indicated that miRNA expression was different in peripheral blood from AKI patients. However, there is still lack of related report about miRNA expression spectrum and correlation with clinical features. MiRNA microarray was applied to test miRNA expression in the serum of acute kidney injury patients. qRT-PCR was adopted to verify the differentially expressed miRNAs. Their correlation with the staging was analyzed. A total of 14 miRNAs exhibited upregulation, while 10 miRNAs presented downregulation in AKI patients compared with healthy control. Real-time PCR revealed that 14 selected miRNAs were obviously different. Correlation analysis demonstrated that miR-210, miR-21, miR-34 were positively correlated with AKI clinical staging ($r = 0.56, P < 0.05$; $r = 0.32, P < 0.05$; $r = 0.38, P < 0.05$, respectively). MiR-16 showed negative correlation with clinical staging ($r = -0.34, P < 0.05$). Multiple miRNAs were differentially expressed in the serum of AKI patients. MiR-210, miR-21, and miR-34 were correlated with AKI staging that could be treated as the biomarker of AKI.

Keywords: Acute kidney injury, miRNA, clinical correlation

Introduction

Acute kidney injury (AKI) is a type of disease with high clinical morbidity. It may induce renal function reduction in a short period, thus shows relatively high fatality rate [1]. It also can cause a variety of complications, resulting in chronic disease and to increase the damage of other organs [2]. AKI usually includes numerous types, such as drug induced, prerenal, and nephrotoxicity type. Various studies have been applied to investigate the mechanism of AKI, while renal tubular cell apoptosis is one of the main mechanisms of AKI. In addition, other mechanisms include renal ischemia or nephrotoxicity, further leading to renal epithelial cells degeneration. Specially, the possible mechanism includes the hemodynamic changes in the kidney, renal tubular damage, or most inflammatory cytokines release. However, its pathogenesis is still unknown. Traditional diagnosis is mainly upon blood urea nitrogen (BUN) and serum creatinine (SCr) [3]. In recent years, the diagnostic technique for AKI also improved fol-

lowing the development of molecular biology. For instance, neutrophil gelatinase associated lipocalin (NGAL) and KIM-1 are also used for the early diagnosis of AKI [4].

Epigenetic change involves in a wide variety of the occurrence of diseases, especially in the field of tumorigenesis and progression. MiRNAs are the important mechanism of epigenetics, which are a kind of small non-coding RNA existed in eukaryotes at about 22 nucleotides [5]. It affects mRNA expression via binding with the 3'UTR of the targeted genes, leading to degradation or transcription inhibition [6]. MiRNAs in the circulation blood and urine are thought to be potential biomarkers of a variety of diseases [7, 8]. Numerous studies showed that miRNAs played an important role in AKI. For example, miR-494 may induce the release of inflammatory mediators through targeted activating 3'UTR transcription. Furthermore, miR-494 level in the urine of AKI patients was about 60 times higher than normal control [9]. MiR-16 and miR-15a inhibited renal tubular cell apopto-

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Table 1. MiRNA sequences

miRNA	Primer sequences
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
hsa-miR-210	F: 5'-TTGACCTGTGCGTGTGACA-3'
hsa-miR-21	F: 5'-CGGTAGCTTATCAGACTGATGTTGA-3'
hsa-miR-30a	F: 5'-GCAGCTGCAAACATCCATCCGACT-3'
hsa-miR-489	F: 5'-GTAGCAGTTTAGCTGCCGTC-3'
hsa-miR-216	F: 5'-ATAATCCCAGAGCCACT-3'
hsa-miR-34	F: 5'-AGCAATCAGCAAGATACTGCC-3'
hsa-miR-150	F: 5'-CAGCCTGGTCAGGTGGGGACAG-3'
hsa-miR-1971	F: 5'-CTTCTGCTCCAGGAACAA-3'
hsa-miR-320	F: 5'-CGGGAGCTGGGGAGAGGGCG-3'
hsa-miR-122	F: 5'-CAAACGCCATTCACACT-3'
hsa-miR-16	F: 5'-CGCCAATAGGGACGT-3'
hsa-miR-192	F: 5'-CTGGCTGCTCAATTCATAGGT-3'
hsa-miR-329	F: 5'-GAGAAACAACCGAACCTCTTT-3'
hsa-miR-200a	F: 5'-CCAGCTTGACTCTAACAC-3'
Universal primer	R: 5'-GTGCAGGGTCCGAGGT-3'

sis through regulating Bcl2 gene expression [10]. Another study confirmed that miR-34 was involved in the renal tubular cell apoptosis by influencing p53 expression [11]. However, there is still lack of report about miRNA expression profile in AKI. This study tested miRNA expression spectrum in the peripheral blood of AKI patients and explored the possible mechanism.

Materials and methods

Reagents and instruments

Trizol reagent was from Invitrogen. qRT-PCR kit was from Takara. ABI 7500 real time PCR amplifier was from Life Tech (Applied Biosystem). High speed freezing centrifuge was from Eppendorf. RNA reverse transcription kit was from ABI. MicroRNA expression microarray was from Exiqon. MiRNA microarray related reagents (miRCURY™ Array Power labeling kit) were from Shanghai Kangchen. Total RNA purification kit was from QIAGEN.

Clinical cases

There were 28 cases of AKI patients diagnosed in the Second Affiliated Hospital of Zhejiang Chinese Medicine University between Oct 2014 and May 2016. The patients were diagnosed according to the criteria published by the first time meeting of international acute kidney inju-

ry network (Sep 2005). There were 19 males and 9 females with mean age at 53.2±21.6 (47-75) years old. Another 58 health volunteers without renal dysfunction were selected as normal control. There were 39 males and 19 females with average age at 52.5±23.6 years old. No statistical difference was observed on basic information including gender, age, and blood pressure between two groups (P > 0.05).

This study was approved by ethics committee in the Second Affiliated Hospital of Zhejiang Chinese Medicine University and all the enrolled objects had signed informed consent.

Clinical indicator detection

Urine volume and serum creatinine (Scr) were recorded. The blood sample was centrifuged at 1000 r/min for 10 min to obtain serum. Clinical staging was performed according to RIFLE.

RNA extraction

The serum was treated by Trizol reagent and total RNA was extracted using chloroform and isopropanol. The RNA was solved in DEPC water and purified. Ultraviolet spectrometry and 1% agarose gel electrophoresis were used to detect RNA concentration and purification. Absorbance value A260 nm/A280 nm between 1.8-2.0 and three binds in the electrophoresis were confirmed as good purification. The RNA was stored at -80°C.

TaqMan low density microarray

miRCURY™ Array Power labeling kit was used to fluorescence label miRNA in purified RNA. Fluorescence probe and microarray were hybridized according to the manual under the standard condition. Next, the fluorescence intensity was tested on the scanner and the signal was transformed for normalization processing. Difference between two groups was calculated as follows: AKI group/normal control = fold change.

Real time PCR

Screened miRNAs were tested by PCR. MiRNAs were reverse transcribed to cDNA using the

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Table 2. Upregulated miRNA in serum from AKI patients

miRNA ID	AKI signal intensity	Control signal intensity	Fold change
hsa-miR-210	17130	7324	2.34
hsa-miR-92a	9175	4572	2.01
hsa-miR-21	12673	10312	1.23
hsa-miR-204	1657	264	6.28
hsa-miR-30a	1343	532	2.52
hsa-miR-489	978	321	3.05
hsa-miR-155	856	245	3.49
hsa-miR-216	11321	896	12.64
hsa-miR-28	2451	1342	1.83
hsa-miR-494	11212	456	24.59
hsa-miR-34	2341	234	10.00
hsa-miR-698	4389	1435	3.06
hsa-miR-150	10974	3560	3.08
hsa-miR-1971	1789	345	5.19

Table 3. Upregulated miRNA in serum from AKI patients

miRNA ID	AKI signal intensity	Control signal intensity	Fold change
hsa-miR-2146	2569	5856	0.44
hsa-miR-320	647	891	0.73
hsa-miR-122	3190	3570	0.89
hsa-miR-197	3567	3987	0.89
hsa-miR-122	34310	53435	0.64
hsa-miR-16	2736	3812	0.72
hsa-miR-192	1567	3645	0.43
hsa-miR-329	923	3114	0.30
hsa-miR-1324	2789	3564	0.78
hsa-miR-200a	2736	3812	0.59

Takara kit according to the manual. The PCR reaction system was composed of 9 μ l 2 \times SYBR Green Mixture, Primer 1 (5 μ M) 2 μ l, 2 μ l Primer 2 (5 μ M), 2 μ l DNA, and 5 μ l dd H₂O. PCR reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Each experiment was repeated for three times. MiRNA sequence was inquired on miRBase and the primers were designed on Primer premier 5.0 software (**Table 1**). U6 was selected as internal reference.

Statistical analysis

SPSS 16.0 software was used for data analysis. Measurement data was exhibited as mean

\pm SD and compared by t test or ANOVA. $\alpha = 0.05$ on bilateral test was applied in all statistical analysis.

Results

Microarray analysis

MiRNA expression microarray was applied to test miRNA expression between AKI group and normal control. The criteria for miRNA upregulation were the signal intensity ratio between AKI patients and healthy control > 1 and $P < 0.05$. On the contrary, miRNA downregulation was defined as the signal intensity ratio between AKI patients and healthy control < 1 and $P < 0.05$. A total of 24 miRNAs were found dysregulated, including 14 upregulated (**Table 2**) and 10 downregulated (**Table 3**). Hsa-miR-210, hsa-miR-21, and hsa-miR-494 upregulation had been reported before [9, 12, 13]. On the other side, hsa-miR-320 and miR-16 were also confirmed to be downregulated [12].

Real time PCR verification

Since real time PCR presented higher sensitivity and specificity than PCR, and microarray may produce false positive result, qRT-PCR was applied to test screened miRNA expression in 20 typical AKI patients and 20 healthy controls. U6 was selected as internal reference. Upregulation fold change ≥ 1.5 and $P < 0.05$ were considered as upregulation, whereas downregulation fold change ≤ 0.75 and $P < 0.05$ were treated as downregulation. Eight upregulated miRNAs including miR-210, miR-21, miR-30a, miR-489, miR-216, miR-34, miR-150, and miR-1971 were screened out. Six downregulated miRNAs, such as miR-320, miR-122, miR-16, miR-192, miR-329, and miR-200a were confirmed (**Figure 1**).

Correlation analysis between miRNAs and clinical characteristics

The patients were classified into three stages according to the Scr level and urine volume. Stage I was Scr elevation > 0.3 mg/dl or 50%, urine volume < 0.5 ml/kg/h for more than 6 h. Stage II was Scr elevation > 200 -300%, urine volume < 0.5 ml/kg/h for more than 12 h. Stage III was Scr elevation $> 300\%$ or 4.0 mg/dl (acute elevation > 0.5 mg/dl), oliguria (< 0.3 ml/kg/h) for 24 h or anuria > 12 h. The staging

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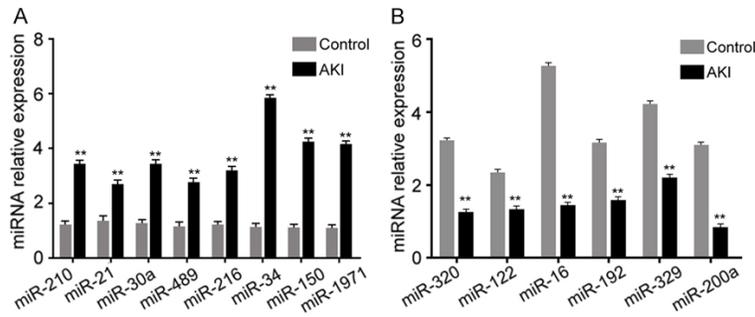


Figure 1. qRT-PCR detection of miRNAs. A. Upregulated miRNAs. B. Down-regulated miRNAs. ** $P < 0.05$.

Table 4. Correlation analysis between AKI staging and upregulated miRNAs

miR ID	Stage I	Stage II	Stage III	r value	P value
miR-210	2.12±0.45	4.21±0.23	5.15±0.23	0.56	0.031
miR-21	1.45±0.23	2.35±0.16	3.45±0.45	0.32	0.0012
miR-192	2.43±0.35	2.49±0.19	2.78±0.27	0.13	0.34
miR-489	2.13±0.25	2.39±0.14	2.38±0.25	0.16	0.42
miR-216	2.15±0.32	2.42±0.12	2.58±0.17	0.15	0.36
miR-34	1.42±0.32	3.22±0.33	4.53±0.25	0.38	0.001
miR-150	2.16±0.28	1.38±0.23	2.25±0.17	0.16	0.21
miR-1971	2.23±0.35	2.50±0.13	2.09±0.24	0.13	0.37

Table 5. Correlation analysis between AKI staging and downregulated miRNAs

miR ID	Stage I	Stage II	Stage III	r value	P value
miR-320	2.13±0.47	3.21±0.23	2.15±0.23	0.56	0.32
miR-122	4.42±0.24	2.34±0.19	3.39±0.38	0.34	0.31
miR-16	4.43±0.35	3.49±0.19	2.78±0.27	-0.34	0.01
miR-192	3.23±0.25	2.39±0.14	2.38±0.25	-0.16	0.32
miR-329	4.32±0.32	3.22±0.33	4.54±0.23	-0.43	0.21
miR-200a	2.16±0.28	3.38±0.23	4.45±0.17	-0.46	0.0012

criteria referred to RIFLE [14]. Correlation analysis demonstrated that miR-210, miR-21, miR-34 were positively correlated with AKI clinical staging ($r = 0.56$, $P < 0.05$; $r = 0.32$, $P < 0.05$; $r = 0.38$, $P < 0.05$, respectively) (Table 4). miR-16 showed negative correlation with clinical staging ($r = -0.34$, $P < 0.05$) (Table 5). It suggested that miRNA could be used as biomarker for AKI diagnosis.

Discussion

AKI is a clinical syndrome featured as glomerular filtration function decrease sustained for 24 h within 7 days, together with urine volume

reduce and Scr elevation. It is a type of critical disease in clinic. Early diagnosis is of great significance for the diagnosis and treatment of the disease [15]. MiRNAs dysregulation is one of the important changes of epigenetics and has been applied for auxiliary diagnosis in a variety of diseases. At present, miRNA can only be used for the diagnosis of some disease, while most diseases still need further diagnosis [16]. MiRNA dysregulation has been widely investigated in kidney related diseases. However, there is still lack of report about miRNA expression profile in AKI.

Our miRNA microarray and qRT-PCR results confirmed miR-210 and miR-21 upregulation in AKI patients. Previous report revealed that miR-210 can enhance endothelial cell growth factor expression to participate in the microvessel formation of AKI patients [9]. It was also found that miR-21 level in the urine of AKI patients was higher than normal control and related to AKI process [17, 18]. A preliminary study for the biomarker of AKI showed numerous differentially expressed miRNAs in the serum, while miR-210 was similar with our results [19]. Receiver operating characteristics curve revealed that miR-210 can predict the 28-day survival rate [20]. This study also found that miR-30a increased in AKI. There is still lack of report about miR-30a dysregulation in AKI. It was found that serum miR-30a-5p, miR-151-3p, miR-150, miR-191, and miR-19b elevated, while urine miR-30a-5p increased in children with nephrotic syndrome [21]. However, further investigation is needed to confirm whether miR-30 participated in the process of AKI. Urine miRNA detection in rat AKI model induced by gentamicin demonstrated that mmu-miR-138-5p, mmu-miR-1971, mmu-miR-218-1-3p, and rno-miR-489 sustained elevation and appeared

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before Scr and BUN changes [22]. MiR-150 overexpression in endothelial cells decreased IGF-1R, suggesting that miR-150 played a role in AKI animal model via inhibiting IGF-1R [23]. MiR-150 was found obviously upregulated in AKI in our results.

Correlation analysis between dysregulated miRNAs and AKI staging showed that miR-210, miR-21, miR-34 were positively associated with AKI clinical staging, indicating that they may be correlated with the severity of AKI and could be treated as biomarker for AKI diagnosis. However, the mechanism of differentially expressed miRNAs without correlation with staging still needs further investigation. MiR-210 is widely studied in AKI, and most researches showed similar conclusion with our results. Other miRNAs still needed further exploration.

In addition to differentially expressed miRNAs screened in this study, there are many other miRNAs have been reported. For instance, miR-NA-146b may be used as new biomarker of AKI and indicator of mesenchymal cells treatment [24]. Multiple dysregulated miRNAs in the serum of AKI patients revealed that miRNA could be treated as biomarker of AKI. This study only screened part of miRNAs in AKI. More in-depth research is urgently needed to explore the correlation between other miRNAs and AKI staging or occurrence.

Conclusion

AKI is a severe clinical syndrome. MiRNA dysregulation may provide reference for the investigation of the pathogenesis of AKI and more sensitive biomarker. This study showed multiple miRNAs expression was correlated with clinical characteristics. Further study could focus on the dysregulated miRNAs.

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Disclosure of conflict of interest

None.

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