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Urinary miR-26b as a potential biomarker for patients with sepsis-associated acute kidney injury: a Chinese population-based study

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Abstract. – OBJECTIVE: Acute kidney injury (AKI) is common in critically ill patients, and sepsis patients with AKI had a higher mortality rate. The aim of the present study was to determine the potential value of urinary miR-26b in the diagnosis of sepsis-associated AKI.

PATIENTS AND METHODS: Urinary samples were collected from a cohort of 155 sepsis patients (68 AKI patients and 87 non-AKI patients) and 57 patients with non-infectious systemic inflammatory response syndrome (SIRS). The expression levels of urinary miR-26b were measured by RT-qPCR analysis. ROC curve analysis was performed to determine the diagnostic value. Pearson correlation analysis was performed to assess the levels of urinary miR-26b and several clinical parameters. Kaplan-Meier curves were plotted to show the impact of urinary miR-26b on the 28-day survival.

RESULTS: Significantly increased urinary miR-26b levels were found in patients with sepsis-associated AKI. Urinary miR-26b had a sensitivity of 90.8% and specificity of 75.0% for distinguishing between AKI sepsis and non-AKI sepsis. Urinary miR-26b levels were closely correlated with clinical parameters reflecting the severity of the disease. Kaplan-Meier analysis revealed that sepsis patients with high urinary miR-26b levels had an elevated mortality rate.

CONCLUSIONS: Taken together, these findings suggested that urinary miR-26b might be utilized as a potential biomarker for sepsis-associated AKI.

Key Words:
Sepsis, Acute kidney injury, Biomarker, Circulating microRNA, Prognosis.

Introduction

Sepsis represents a major cause of death for critically ill patients during Intensive Care Unit (ICU) treatment[1]. In the USA alone, there are annually 750,000 cases of severe sepsis, causing over 200,000 deaths[2]. Sepsis often results in multiple organ dysfunctions, including acute kidney injury (AKI)[3]. Sepsis patients with AKI have a longer length of hospital stay, consume more healthcare resources, and have increased morbidity and mortality[4,5]. Therefore, developing reliable biomarkers for the early diagnosis of sepsis-associated AKI is of critical importance. Recently, attention has focused on the application of circulating microRNAs (miRNAs) as a novel class of promising biomarkers in the diagnosis and prognosis of human diseases.

MicroRNAs (miRNAs), a naturally-occurring class of short, non-coding RNA molecules between 19 and 21 nucleotides in length, serve as negative regulators of gene expression through directly binding to the 3′-untranslational region (UTR) of their target mRNAs, leading to the degradation of target mRNAs or inhibition of translation[6,7]. It is estimated that approximately 60-70% of the human coding genes are modulated by miRNAs[8]. While the majority of miRNAs remain intracellular, there is a small population of miRNAs termed circulating miRNAs that are ejected into the circulation. Increasing studies have indicated that circulating miRNAs may function as disease fingerprints and novel molecular biomarkers owing to their high abundance and stability in various body fluids, including blood and urine samples[9,10]. In humans, urinary miRNA has been detected in patients with kidney damage[11].

However, to date, miRNAs as urine-based biomarkers for detection of sepsis-associated AKI have not been extensively investigated. Upregula-

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tion of serum miR-26b has recently been reported in a CLP (cecal ligation and puncture)-induced sepsis mouse model[12]. In the present study, we aimed to examine the urinary levels of miR-26b to identify and analyze its diagnostic accuracy as a robust and non-invasive biomarker for sepsis-associated AKI in humans.

Patients and Methods

Patient Selections

From January 2016 to March 2017, we recruited a total of 155 sepsis patients with positive microbiological culture results that were consecutively admitted to Department of Intensive Care Unit (ICU) at 3201 Hospital (Hanzhong, China), Chang'an District Hospital (Xi'an, China) and Fengcheng Branch, The Ninth People's Hospital Affiliated to Shanghai Jiao Tong University (Shanghai, China). Besides, 57 patients with systemic inflammatory response syndrome (SIRS) but not organ dysfunction and sepsis were recruited as controls. The characteristics of these participants were recorded in Table I. The sepsis patients were then divided into two groups: an AKI sepsis group (n=68) comprised sepsis patients who developed AKI during the first 7-day observation period, and a non-AKI sepsis group (n = 87). The diagnosis of sepsis was performed according to the International Sepsis Definition Conference criteria[13]. AKI was defined and staged according to the KDIGO and AKIN criteria[14]. The study protocol was approved by the Ethics Committee of 3201 Hospital, Chang'an District Hospital and Fengcheng Branch, The Ninth People's Hospital Affiliated to Shanghai Jiao Tong University. Written informed consent was obtained from each individual or their first-degree relatives before participation. Demographic information and medical history were obtained after informed consent. Acute Physiology and Chronic Health Evaluation (APACHE) II scores[15] were calculated after the onset of sepsis. The enrolled sepsis patients were followed for 28 days or until death.

Urine samples were collected daily during the first week of the ICU admission and centrifuged at 3000 rpm for 10 min to remove cellular debris. The supernatants were frozen, shipped on dry ice, stored at -80°C, and thawed immediately prior to miRNAs analysis. All urine samples were processed within 24 h after they were obtained.

RNA Extraction and RT-qPCR Analysis

Total RNA was extracted from 1000 µl of urine samples using the mirVana® miRNA isolation Kit (Ambion, Austin, TX, USA). Total RNA concentration and integrity were measured by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). During RNA purification, synthetic cel-miR-39 (Life Technologies, Carlsbad, CA, USA) was added exogenously to each sample. 2 µg of total RNA were reverse transcribed by Transcript First-strand cDNA synthesis superMix (TransGen Biotech, Beijing, China). Thereafter, quantitative PCR (qPCR) analysis was performed using the miScript SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) on the LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Primers of human miR-26b were purchased from GenePharma (Shanghai, China). The levels of miRNA were calculated relative to that of cel-miR-39 using the $2^{-\Delta Ct}$ method[16]. Each reaction was run in duplicate and performed at least twice.

Statistical Analysis

Quantitative data were expressed as mean ± SD or median range. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences between

Table I. Demographic and clinical characteristics of the study subjects on ICU admission.

Characteristic	SIRS patients	Sepsis patients
Number	56	155
Age (yr)	54.5 ± 12.9	56.1 ± 14.2
Gender (M/F)	32/24	94/61
APACHE II score	9.1 ± 5.3	18.0 ± 6.5
PCT (ng/ml)	0.39 (0.04-21.65)	3.12 (0.46-54.33)
CRP (mg/dl)	3.8 (0.4-6.1)	11.5 (2.6-25.5)
WBC ($\times 10^9$/L)	13 ± 6	15 ± 7

APACHE: Acute Physiology and Chronic Health Evaluation; PCT: Procalcitonin; CRP: C-reactive protein; WBC: White blood cell.

groups were assessed by Mann-Whitney U test or Kruskal-Wallis test. Receiver operating characteristic (ROC) curve analysis was used to evaluate whether urinary miR-26b levels could distinguish patients with sepsis-associated AKI, and the Youden's index (sensitivity+specificity−1) was used to determine the optimal cutoff thresholds[17,18]. Pearson correlation analysis was performed to assess the association between two quantitative variables. Kaplan-Meier curves were plotted to show the impact of urinary miR-26b on the 28-day survival and the differences were assessed using the log-rank test. All *p*-values were 2-sided, and those less than 0.05 were accepted as statistically significant.

Results

For the analysis, 212 adult participants were enrolled. The clinical characteristics of these subjects are provided in Table I. 68 patients with sepsis (43.9%) developed AKI within 1 week after ICU admission based on the KDIGO and AKIN criteria. Using RT-qPCR assay, we determined the urinary levels of miR-26b in these participants at various time points after ICU admission. As shown in Figure 1A, urinary miR-26b levels were significantly higher in sepsis patients compared with SIRS patients on ICU admission day.

Furthermore, during the first 7-day observation period, we found that in non-AKI sepsis patients, urinary miR-26b levels were maintained slightly elevated or declined without evident variation; however, in sepsis patients who eventually developed AKI, urinary miR-26b levels exhibited a remarkable elevation at 1 day before AKI onset (Figure 1B). Furthermore, sepsis patients suffering AKI had been further subdivided and graded (stage 1, n=38; stage 2, n=11; stage 3, n=19), and we found that sepsis patients with AKI of higher stages were more likely to have higher urinary miR-26b levels measured at 24 hours before AKI development (Figure 1C).

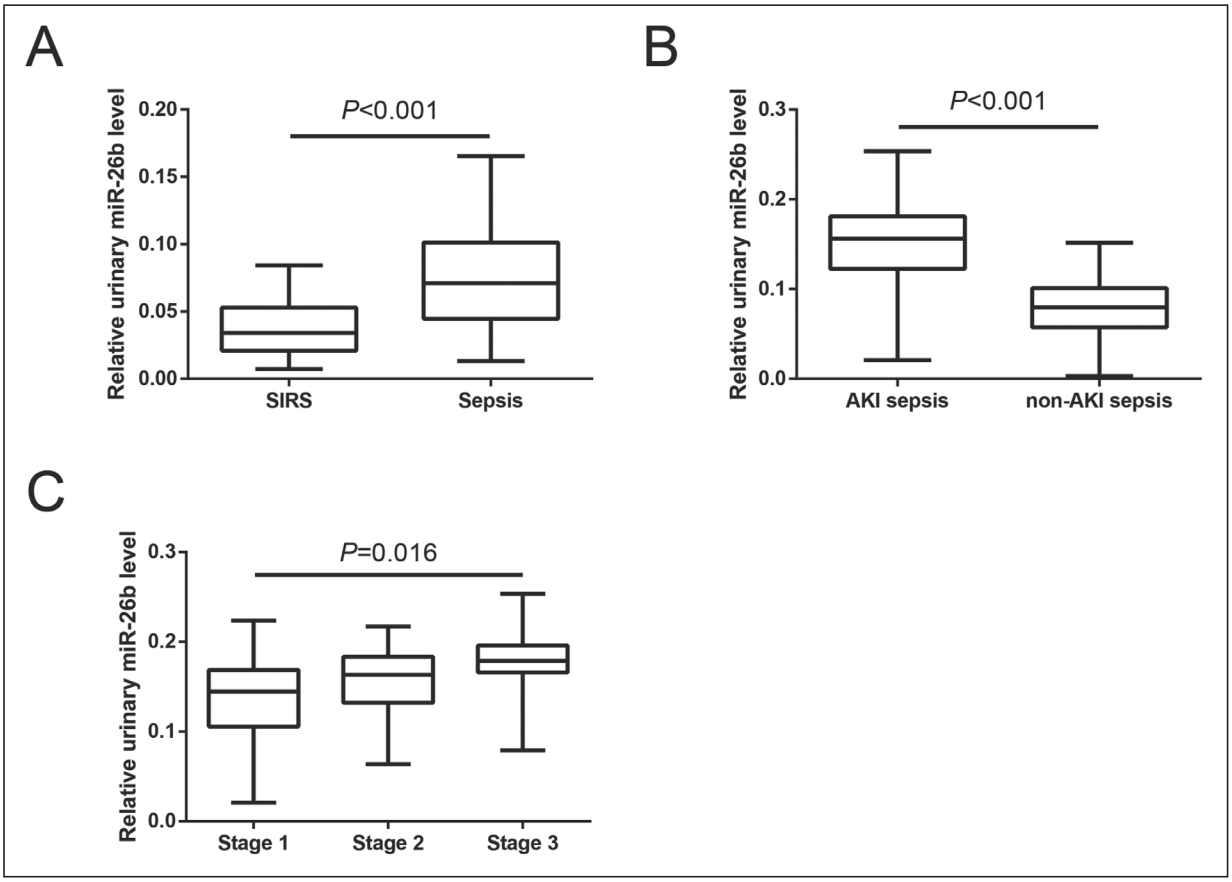

Figure 1. Urinary miR-26b levels in the validation population. ***(A)*** Urinary miR-26b levels were higher in sepsis patients on ICU admission. ***(B)*** Urinary miR-26b levels were higher at 1 day before AKI onset. ***(C)*** Urinary miR-26b levels were higher in sepsis patients with AKI of higher stages.

ROC curve analysis was then performed in order to determine the cut-off point for the prediction of AKI 24 hours before its development. As shown in Figure 2A, the area under the ROC curve was found to be 0.886 (95% confidence interval, 0.831-0.942), with a sensitivity of 90.8% and specificity of 75.0% at the cutoff value of 0.124. Intriguingly, when classified according to sepsis *vs.* SIRS, the discriminative value of urinary miR-26b measured at 24 hours after ICU admission was reduced (Figure 2B).

Pearson correlation coefficients between the levels of urinary miR-26b and several clinical parameters were calculated in 30 randomly selected sepsis patients. As shown in Figure 3, higher serum C-reactive protein levels ($r = 0.382$, $p = 0.037$), higher serum procalcitonin levels ($r = 0.566$, $p = 0.001$), and higher APACHE II score ($r = 0.394$, $p = 0.031$) were found in sepsis patients with high urinary miR-26b levels on ICU admission day.

Furthermore, the sepsis patients were grouped according to the urinary miR-26b levels: the high level group (n = 72, fold change ≥ mean ratio) and the low level (n = 83, fold change ≤ mean ratio). There were 155 sepsis patients enrolled in this study, and 56 sepsis patients (36.1%) died within 28 days. Kaplan-Meier analysis showed that the sepsis patients with high urinary miR-26b levels on ICU admission had a relatively lower 28 d survival rate, compared with sepsis patients with low levels ($p = 0.026$; Figure 4).

Discussion

A biomarker is a pivotal pole in early diagnosis of sepsis. As of 2010, 170 biomarkers had been evaluated for application in sepsis[19]. Circulating miRNAs have shown potential as biomarkers for the detection of various diseases, including sepsis and sepsis-associated AKI. The values of circulating miRNAs lie not only in their minimally invasive nature, but also in the fact that they can be measured repeatedly and there are several established methods for their extraction and measurement. Besides, urine samples contain fewer proteins compared to blood-based samples, therefore reducing the interference of proteins during RNA extraction and further analysis. In the field of sepsis, a multitude of deregulated miRNAs have been documented from the viewpoint of diagnostic properties[20-23]. In 2017 Ge et al[24] reported that the serum levels of miR-4321 and miR-4270 were increased in the patients with sepsis-induced AKI compared with non-AKI sepsis patients. For quantitative analysis of miRNA levels, RT-qPCR analysis is regarded as a gold standard[25]. Normalization of circulating miRNAs is a critical issue. RNU6B (U6), widely used for data normalization of cell/tissue-based miRNAs, is not suitable for normalization of circulating miRNAs in inflammatory diseases[26]. Some studies have chosen endogenous miR-16 as an internal control, such as Tsai et al[27]. In our report, spiked-in Caenorhabditis elegans miRNAs were selected as an internal control owing to its absence in human samples[28].

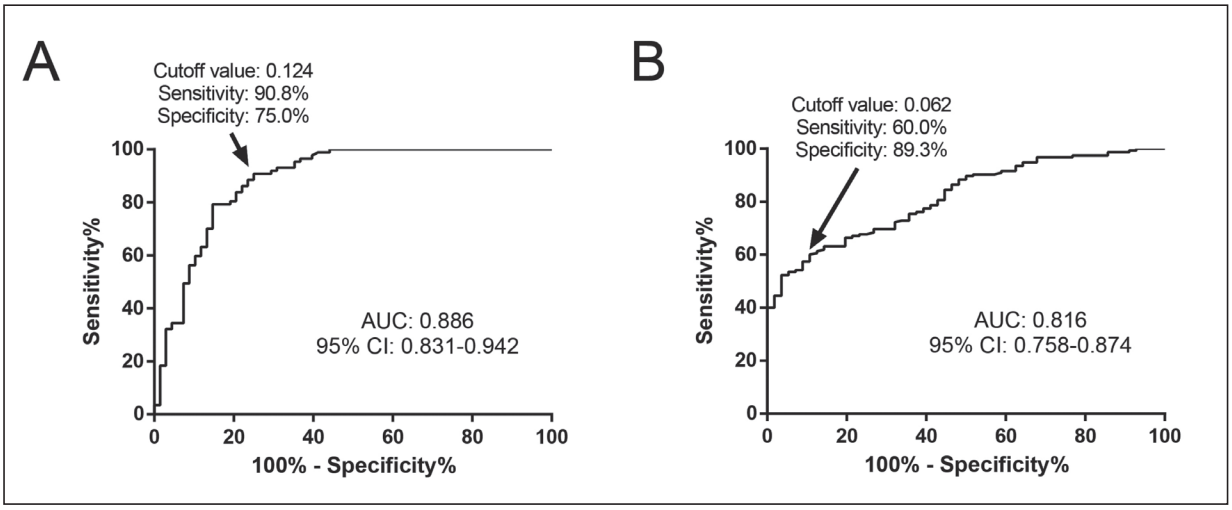

Figure 2. The receiver operating characteristic curve (ROC) analysis was performed to determine the diagnostic performance of urinary miR-26b. ***(A)*** AKI sepsis *vs.* non-AKI sepsis. ***(B)*** Sepsis *vs.* SIRS.

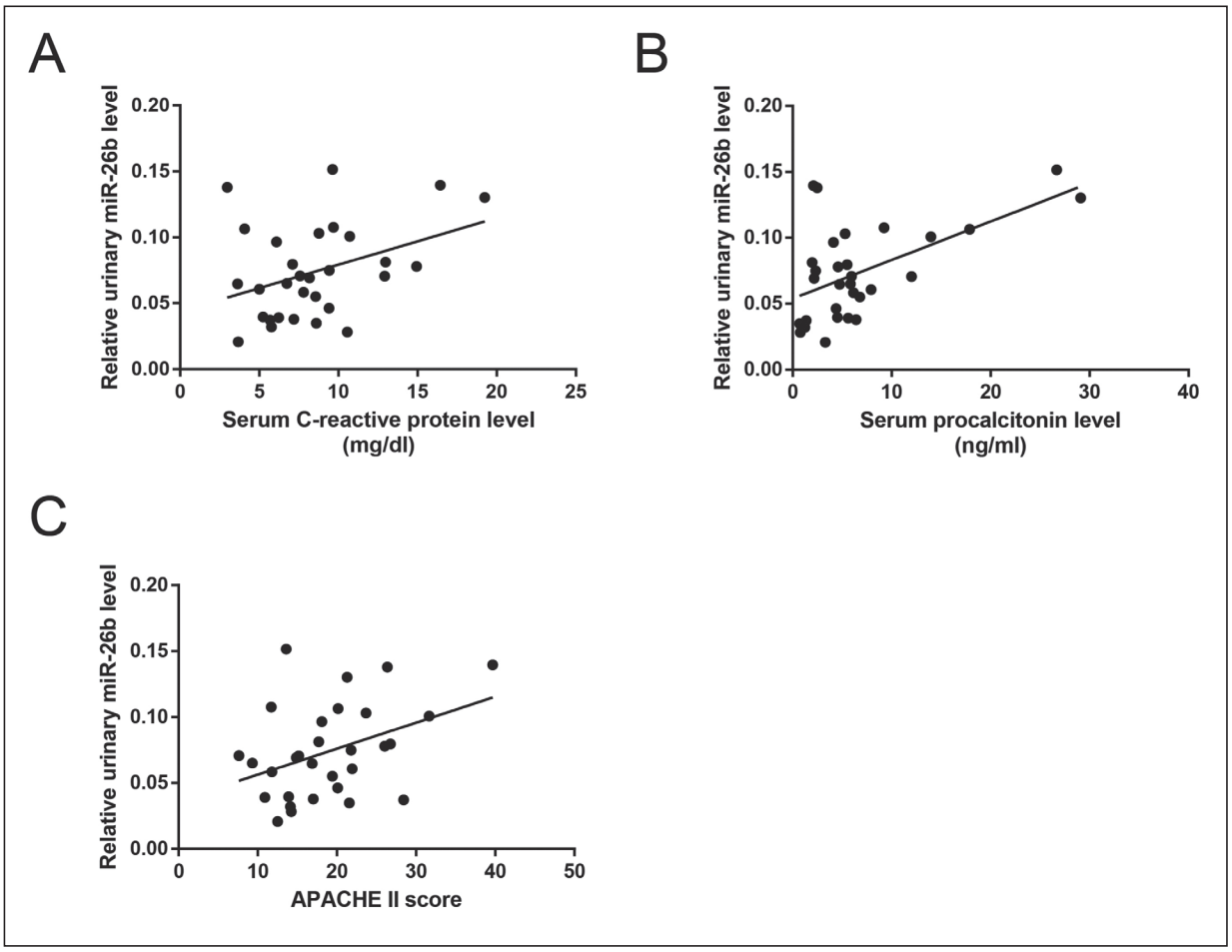

Figure 3. The increased urinary miR-26b levels were positively associated with ***(A)*** higher serum C-reactive protein levels, ***(B)*** higher serum procalcitonin levels and ***(C)*** higher APACHE II score on ICU admission.

Recently, scholars reported[29,30] that circulating miR-26b levels were deregulated in various inflammation-associated diseases, including psoriasis and ulcerative colitis-associated colorectal carcinoma, and could function as a useful biomarker. An elevation of miR-26b in the urine was associated with kidney injury induced by cisplatin treatment in rats[31]. Accordingly, it is reasonable to speculate that the fluctuation of urinary miR-26b levels could reflect kidney damage caused by sepsis. In the present study, human urine samples were used to identify urinary miR-26b as a candidate biomarker for the detection of sepsis-associated AKI. In patients with diagnosed sepsis-associated AKI, urinary miR-26b levels were significantly increased in comparison to non-AKI sepsis patients. We also found a closely positive correlation between urinary miR-26b levels

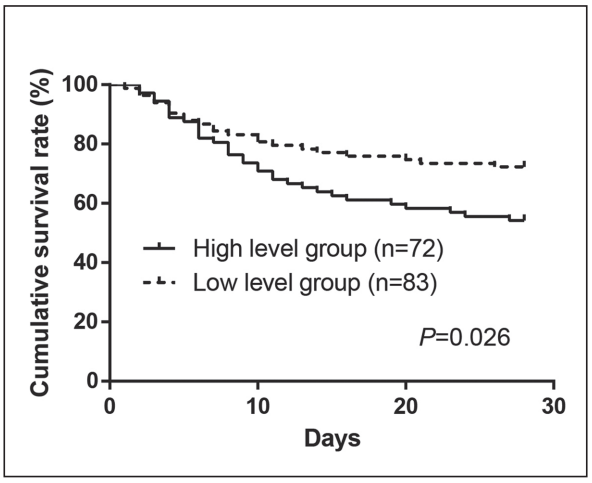

Figure 4. Kaplan-Meier survival curves showed that patients with high urinary miR-26b levels on ICU admission had an increased mortality. *p*-value was assessed by log-rank test.

and APACHE II score, indicating that urinary miR-26b levels were also a good parameter for reflecting the severity of sepsis.

It is undeniable that there are some limitations in this paper. Firstly, the participant samples were collected from only three hospitals and the number of participants was relatively small. Thus, further validation in a larger number of participants from more hospitals is required to confirm the efficacy of this marker. Secondly, no analysis of urinary miR-26b levels during follow-up was conducted. In addition, we did not fully elucidate the underlying mechanism of the association between urinary miR-26b and sepsis-associated AKI. Thus, the sequential measurement should be performed to unravel the potential role of miR-26b in sepsis-associated AKI.

Conclusions

We for the first time, demonstrated that urinary miR-26b were significantly upregulated in the patients with sepsis-associated AKI, and its levels were closely associated with disease severity and prognostic outcomes. Our data, therefore, strongly highlighted the potential utility of urinary miR-26b as a biomarker in sepsis-associated AKI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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