Determination of urine cofilin-1 level in acute kidney injury using a high-throughput localized surface plasmon-coupled fluorescence biosensor

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Abstract. The actin-depolymerizing factor (ADF)/cofilin protein family has been reported to be associated with ischemia-induced renal disorders. We examine whether cofilin-1 is associated with acute kidney injury (AKI) using human urine samples. We exploited a 96-well based high-throughput biosensor that uses gold nanoparticles and a sandwich immunoassay to detect the urine cofilin-1 level of AKI patients. The mean urine cofilin-1 level of the AKI patients ($n = 37$ from 47 cases analyzed) was twofold higher than that of healthy adults ($n = 21$ from 29 cases analyzed). The receiver operating characteristic (ROC) curve showed that cofilin-1 was acceptable for discriminating AKI patients from healthy adults. However, an increase of the sample size is required to conclude the importance of urine cofilin-1 on AKI diagnosis, and the high-throughput ultrasensitive biosensor used in this study would greatly accelerate the measurement of urine cofilin-1 in an increased sample size. © *2014 Society of Photo-Optical Instrumentation Engineers (SPIE)* [DOI: 10.1117/1.JBO.19.1.011004]

Keywords: acute kidney injury; urine cofilin-1; oxidative stress; gold nanoparticles–based; high-throughput biosensor.

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1 Introduction

Acute kidney injury (AKI) is a severe syndrome that usually leads to high mortality in the intensive care unit (ICU). This clinical problem increases the utilization of health resources and the risk of chronic kidney disease.[1–3] Approximately 80% of patients with AKI are suffering from acute tubular damage.[4] In addition, AKI is a common and severe complication associated with circulatory shock.[5] Clinically, ischemia/reperfusion injury of the kidney can be induced by shock and resuscitation.[6] However, AKI is a complex pathophysiological syndrome that cannot be simply explained by ischemia–reperfusion during shock.[7] The classification of AKI is dependent on risk, injury, failure, loss and end-stage (RIFLE) and the acute kidney injury network criteria.[3,8] Whether AKI can be assessed by measuring specific molecules in body fluid remains to be studied.

Serum creatinine is routinely used for the preliminary identification of AKI in a clinical situation. However, several lines of evidence indicate that the creatinine concentration cannot be considered a decisive factor for AKI because of several difficulties; these include a lack of specificity with respect to

AKI, low sensitivity to kidney malfunction, time consuming biochemical detection, and the presence of other interfering factors.[9–14] Up to the present, a number of serum and urine biomarkers have been reported to be useful for the early detection of AKI. Urinoanalysis of AKI-related biomarkers is particularly interesting because it is noninvasive, ideal for early detection, and more specifically targets kidney disorders than serum biomarkers. The urine biomarkers for the diagnosis of AKI can be classified into three categories. These are low-molecular-weight proteins such as kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), cytokines/chemokines such as interleukine-18 (IL-18), and structural and functional proteins such as F-actin.[15,16] These biomarkers have their own pros and cons when detecting AKI, and a combination of the detected biomarkers is likely to be useful for the prediction of AKI.

Gold nanoparticles (GNPs) possess particular optical properties, namely they act as localized surface plasmons (LSPs) and exhibit selective wavelength absorption with extremely large molar extinction coefficients as well as significant enhancement of the localized electromagnetic field within 50 to 60 nm of the GNP surface.[17–19] The LSP-coupled fluorescence (LSPCF) technique, when associated with a sandwich immunoassay, has been used to study antigen–antibody interactions at very low concentrations.[20–25] Such an ultrasensitive high-throughput LSPCF biosensor (HT-LSPCFB) is essential for detecting

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and assessing the level of urine actin-depolymerizing factor (ADF)/cofilin in ICU patients.

In this study, we investigated the levels of urine cofilin-1, a major nonmuscle isoform of ADF/cofilin family of proteins, in AKI patients and healthy adults using a HT-LSPCFB. Moreover, the receiver operating characteristic (ROC) assay based on the value of the area under curve (AUC) was used to assess the association between urine cofilin-1 and AKI.

2 Materials and Methods

2.1 Patients

Urine was collected from 47 ICU patients diagnosed as suffering from AKI, which was determined using modified RIFLE kidney disease criteria[26] and serum creatinine level (above 1.4 mg/dL). For comparison, 29 healthy adults were also recruited at National Taiwan University Hospital, Hsin-Chu branch. The admitted AKI patients were emergency treated with intravenous fluid and/or inotropic agents for 24 h or longer, depending on their clinical state. The urine samples were collected no less than 4 h after ICU admission. The sample donors signed a consent form and completed a structured questionnaire, either personally or via their legal representatives. This study has been approved by the institutional review board of National Taiwan University Hospital, Hsin-Chu branch.

2.2 Materials

The GNP conjugate streptavidin (Au-SA, GNP diameter 25 nm) was purchased from Aurion Inc. (Wageningen, the Netherlands). Anti-cofilin-1 antibody (Abnova Inc., Taipei, Taiwan) was used both as a capture antibody (c-Ab) and as a detection antibody (d-Ab). The fluorescent labeling kit (Lightning-Link™ Atto633) and biotin labeling kit were purchased from Innova Biosciences (Cambridge, United Kingdom). In addition, the 96-well immunoplate was purchased from Corning Costar (Corning, New York). Bovine serum albumin (BSA) and phosphate-buffered saline (PBS) tablets were purchased from Sigma Inc. (St. Louis, Missouri).

2.3 Optical Setup of HT-LSPCFB

For the experimental setup of the HT-LSPCFB (Fig. 1), a He-Ne laser (17 mW, 632.8 nm, Newport) was used as the excitation light source. The intensity of the laser beam was modulated at a frequency of 1 kHz using a chopper. A beam splitter was applied to split the laser beam into a signal beam and a reference beam. The intensity-modulated signal beam was directed incident to each well of a 96-well immunoplate to measure the antigen–antibody interaction. The immunocomplexes were coated on the bottom of the immunoplate wells. Two identical long-pass filters (cutoff wavelength = 640 nm) were introduced into this instrument to detect the intensity-modulated fluorescence signal via a photomultiplier tube. Two lock-in amplifiers were used to simultaneously measure the magnitude of the laser intensity from the reference channel and the fluorescence signal from the signal channel for cofilin-1 detection.

2.4 Protein Detection Using the HT-LSPCFB

Briefly, the 96-well immunoplates were first coated with 360 ng of c-Ab per well at 4°C overnight. After interacting with 1% BSA–PBS solution at 37°C for 1 h, 100 μL of urine samples were added and incubated at room temperature for 2 h. The immunoplates were then washed six times using the PBS solution. Finally, the LSPCF probe solution was added to each well, followed by incubation of the, c-ab/cofilin-1/LSPCF probe immunocomplex for 2 h. After washing six times with the PBS solution, the immunoplates were measured using the HT-LSPCFB.

2.5 Statistical Analysis

The independent samples t-test was used to compare urine cofilin-1 between the healthy control and the AKI groups assuming unequal variances. The ROC curve and AUC that were used for discriminating the level of urine cofilin-1 from patients relative to healthy adults were based on the criteria providing optimal discrimination as described previously.[27] The binomial exact was used to analyze the 95% confidence interval (95% CI) for the AUC. The p value tests the null hypothesis that the AUC = 0.5. If $p < 0.05$, this means that the AUC is significantly different from 0.5 and, therefore, there is evidence that the laboratory test does have an ability to distinguish between the two groups. All of the tests and statistical evaluations were performed using MEDCALC software (Ver. 12.1.4.0, Mariakerke, Belgium).
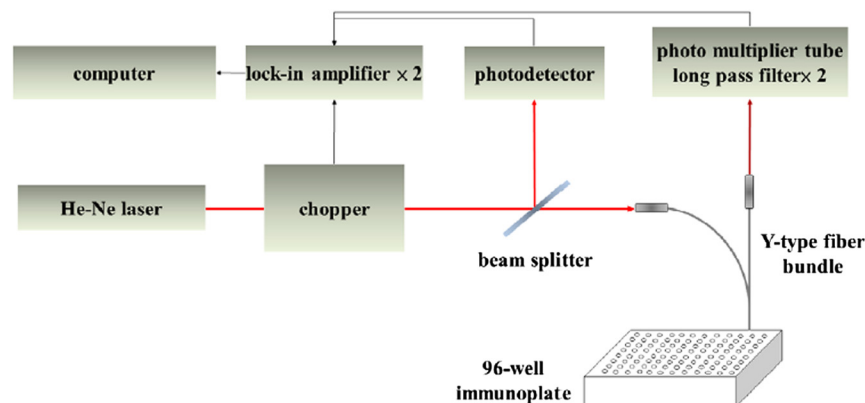

Fig. 1 Optical setup of HT-LSPCFB.

3 Results

3.1 General Characterization and Classification of the AKI Patients

The admission of patients to the ICU was based on several criteria, including sepsis, peritonitis, pneumonia, acute coronary failure, cardiopulmonary resuscitation, respiratory insufficiency, and upper gastrointestinal bleeding. They were clinically analyzed for AKI based on the biochemical analysis of their serum creatinine level. The ICU patients, classified as AKI, were also characterized with respect to age, sex, mean arterial pressure, intravenous fluid volume (mL)/1 day, application of inotropic agents, and survival rate. Table 1 summarizes the characteristics of the 47 AKI patients and 29 healthy adults.

3.2 Detection of Cofilin-1 Levels in Urine Samples Using HT-LSPCFB

To examine if cofilin-1 was excreted into the urine of AKI patients, 100 μL of urine sample from each donor was loaded

Table 1 The characteristics of the AKI and health urine donors.[a]

Characteristics	AKI group	Healthy adults
Cases (n)	47	29
Basic data		
Age (year)	72.7 ± 14.6	36.0 ± 10.0
Male (n)	30	20
Female (n)	17	9
Mean arterial pressure (mmHg)	75.9 ± 29.3	N.D.
Intravenous fluid volume (mL)/1 day	2115.9 ± 1511.4	0
Applied for inotropic agents (%)[b]	40.4	0
Survivors (%)[c]	63.3	100
Cause of ICU admission		
Sepsis	16	
Peritonitis	1	
Pneumonia	11	
Acute coronary syndrome	8	
Cardiopulmonary resuscitation	1	
Respiratory failure	2	
Upper gastrointestinal (UGI) bleeding	1	
Others[d]	7	

[a]For the AKI group, the mean creatinine level was 3.759 ± 2.29 mg/Dl.
[b]The percentage was calculated from the cases using (or not using) inotropic agents divided by total number of patients.
[c]The percentage was calculated from the survived cases divided by the total number of patients.
[d]Any patient that displayed AKI due to uncertain causes.

Table 2 Comparison of urine cofilin-1 (CFL-1) levels detected in healthy adults and AKI patients.

	Cases (n)		
	Total	Urine CFL-1 detectable	Ratio (%)[a]
AKI	47	37	78
Healthy adults	29	21	72

[a]The number of urine CFL-1 detectable cases divided by the total cases.

into a 96-well immunoplate setup for the HT-LSPCFB. The results were measured relative to a normalized cofilin-1 level that was defined as the measured fluorescence intensity of each well normalized against the positive controls on the same plate. BSA in PBS solution (1%) was used as the negative control. Only measured values of samples higher than that of the negative control were included for further statistical analysis. The results showed that urine cofilin-1 was detected in 37 of 47 urine samples from AKI patients and in 21 of 29 urine samples from healthy adults (Table 2). Thus, the percentages of detectable urine cofilin-1 in AKI patients and healthy controls were 78% and 72%, respectively.

3.3 Comparisons of Urine Cofilin-1 Levels between Healthy Adults and AKI Patients

We next compared the arithmetic means of the urine cofilin-1 between AKI patients and healthy adults. The results showed that the mean urine cofilin-1 level was significantly higher in AKI patients than in healthy adults (0.4985 versus 0.2302, $p = 0.0003$; Fig. 2). The median detected that the normalized cofilin-1 level of the AKI patients was 0.4021, while that of the healthy controls was 0.2095. There was a significant difference in mean age between AKI patients and healthy controls (73 years versus 36 years) and therefore, we also analyzed the urine cofilin-1 levels after selecting the AKI patients who were under 50 years old in the AKI group. The arithmetic
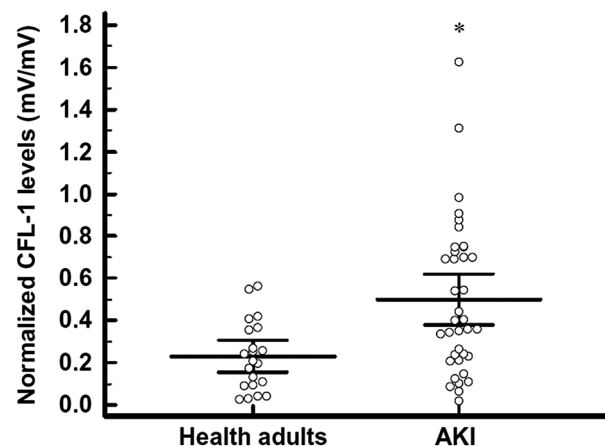
Fig. 2 Comparisons of the urine cofilin-1 levels between healthy adults and AKI patients. Each datum represented the normalized fluorescent signal, which is defined as the measured fluorescence intensity of each well normalized against the positive controls in the same plate. The error bars represented the 95% confidence intervals for arithmetic means. *$p < 0.05$.

mean of the younger AKI patients was 0.6755 ($n = 6$), and the value remained significantly higher than that of the healthy adults. Although the mean of urine cofilin-1 level of patients below 50 years old was higher than that of those over 50 years old (0.6755 versus 0.4445), there was no significant difference between these two groups ($p = 0.1601$ using t-test). Hence, the current results suggest that the level of urine cofilin-1 is correlated with the presence of AKI and might be important and useful for the diagnosis of AKI in patients.

3.4 Assessment of the Association Between Urine Cofilin-1 Levels and AKI

To understand whether urine cofilin-1 can be used optimally as a means of distinguishing between healthy controls and AKI patients, the ROC curve was used to calculate the maximum likelihood of fitness of the binominal model.[28] The ROC curve uses a binary classification system, namely the true positive rate (sensitivity) versus the false positive rate (100%-specificity%), as its discrimination threshold varies. Each datum fitted to the ROC curve corresponds to a unique pair of values for sensitivity and specificity. Our results showed that the empirical and fitted AUC for urine cofilin-1 were 0.727 and 0.734, respectively ($p < 0.0005$, Fig. 3). At a criterion value of 0.563, the sensitivity and specificity were 38% and 100%, respectively. Alternatively, at 0.267, the sensitivity was 65% and the specificity was 71.4%. When the criterion value was set as 0.0963, the sensitivity was 92% and the specificity was 28.6%. Although the AUC value revealed that the cofilin-1 level seemed acceptable for use as a means of discriminating AKI patients from healthy individuals, the sensitivity and specificity were slightly lower than other reported AKI biomarkers such as plasma, NGAL and cystatin C, and urine NGAL, IL-18, and KIM-1.[10] A combinational analysis of urine cofilin-1 and other biomarkers would be required to improve the diagnosis of AKI and the putative outcomes.
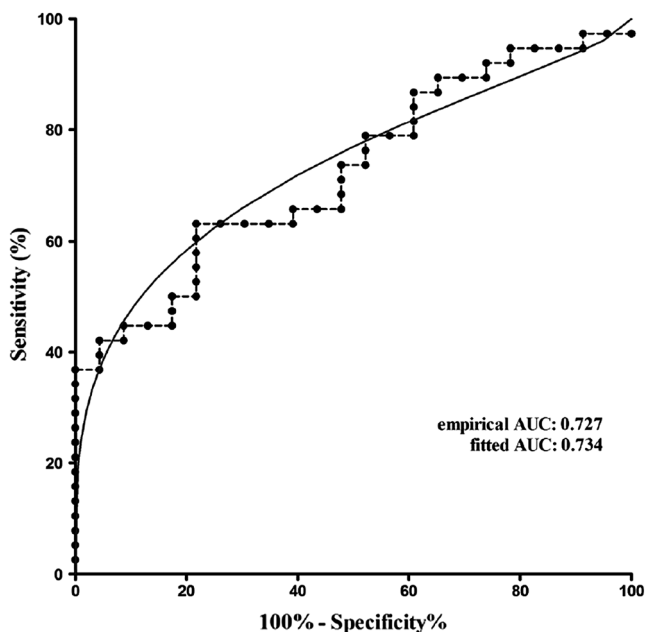

Fig. 3 The ROC assay discriminating urine cofilin-1 of AKI patients from that of healthy adults. All of the data were obtained from the values of the normalized fluorescence signals over negative controls. The empirical and fitted AUCs are shown.

3.5 Induction of Cofilin-1 Expression in a HK-2 Proximal Tubular Cell Line Exposed to Oxidative Stress

Oxidative stress has been reported to play an important role in the development of AKI.[29] In addition, excretion of cofilin-1 seems to be associated with damage to renal proximal tubular cells (PTCs) based on animal experiments.[30,31] To examine if there is an increase in urine cofilin-1 when PTCs are exposed to the oxidative stress, we used H_2O_2 and arsenic trioxide (ATO) to treat a human kidney-2 (HK-2) PTC line and then detected the expression of cofilin-1. The result showed that there was an apparent upregulation of total cofilin-1 in HK2 cells after treatment [Fig. 4(a)]. However, the change in serine-3 phosphorylated cofilin-1 was relatively weak. The blots were quantified using densitometry to confirm the increase in cofilin-1 level after HK2 cells had been exposed to the oxidative stress [Fig. 4(b)]. These findings suggest that, at least in part, oxidative stress may influence the expression of cofilin-1 in human PTCs and thus the excretion of cofilin-1 into urine.

4 Discussion

In this study, we showed that urine cofilin-1 was increased in patients with AKI syndrome. However, several limitations need to be considered with respect to this conclusion. First, the sample sizes for both ICU patients and healthy adults were not large enough, and the number of individuals as well as the age distribution between the two groups was significantly different.
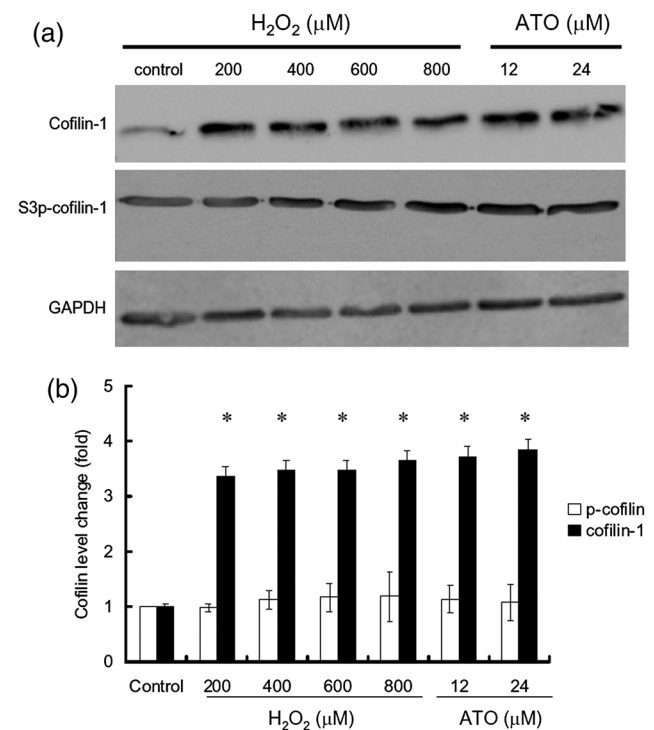

Fig. 4 Induction of total cofilin-1 in proximal tubular cell (PTC) line by oxidative stress. (a) Use of Western blot analysis to detect the level of total and ser-3 phosphorylated cofilin-1 in HK-2 cells exposed to different concentrations of H_2O_2 and ATO for 48 h. GAPDH was used as the loading control. (b) Quantification of the blot results using the densitometry. Each datum represented the mean of three independent experiments ± SD. *$p < 0.05$ compared to the control.

Given that the cases were collected from a single hospital, a cross-institutional investigation involving several hospitals is needed to confirm the results of this preliminary clinical study. Second, the mean ages of the AKI group and the healthy group were significantly different. To clarify the effect of this confounding factor, we calculated the mean cofilin-1 level from AKI patients younger than 50 years old. The urine cofilin-1 level remains high in this AKI subgroup compared to the healthy controls (0.6755 versus 0.2302). This implies that age may be not a major factor influencing the amount of urine cofilin-1 in ICU patients. However, more cases and a better age distribution for the two groups, particularly the control group, are required to further confirm these preliminary results.

Under normal conditions, human urine contains <0.1g/L of total protein. Thus, an extremely sensitive method for measuring urine protein is essential for the early detection of certain syndromes. In this study, we have accessed a modified LSPCFB that uses a reusable 96-well plate for high-throughput screening, and have been able to detect small amounts of urine cofilin-1. The limit of detection (LOD) is 20 to 36 pg/mL depending on the background value. Using purified recombinant human cofilin-1 as the antigen, cofilin-1 can be detected at levels as low as 10 pg/mL, though the LOD is 36 pg/mL.[32] A similar metal-based fluorescent technique without high-throughput has been used to detect several other serum proteins that are related to breast cancer, prostate cancer, and infectious diseases.[20,22–25] The application of this sensor to urine samples is particularly favorable when carrying out *in vitro* diagnosis because of the ease of specimen collection, the noninvasive nature of the sample, and the easy of study of the sample. Additionally, the detection of other urine proteins using this technique is likely to be of interest to researchers when investigating other renal-related diseases in the future.

The current findings supported the hypothesis that the level of cofilin-1 urine is associated with AKI. Morgan Gilman has reported that a model of renal ischemia that uses ATP depletion leads to a rapid activation of cofilin biosynthesis in proximal tubule cells and repletion of ATP for 24 h further increases cofilin activation, leading to disruption of actin stress fibers and cell–cell contact.[33] These findings suggest that the response of proximal tubule cells to AKI may be a result of cofilin activation within these cells. Release of actin and ADF/cofilin into the renal tubule lumen via casts and vesicles has also been reported in the urine of animals as well as during human AKI.[34] Compared to their studies, our preliminary clinical study has focused on the detection of cofilin level rather than measuring cofilin activity in AKI patients. Detection of cofilin activity would be another approach to assessing AKI in patients. One possibility that should be considered is the development of a biosensor, that can specifically detect cofilin activity in the urine of AKI patients, particularly the phosphorylated form of cofilin.

A recent report has shown that obesity is an independent risk factor for AKI and this association is in part mediated by oxidative stress.[35] Changes in actin cytoskeletal architecture have also been visualized during reperfusion after induced oxidative stress.[36] Cofilin-1 is important for actin organization and is associated with ischemia in the renal system. Therefore, we further investigated whether this effect is associated with ischemia-/reperfusion-induced oxidative stress in PTCs. The current findings show that both H_2O_2 and ATO are able to induce the expression of total cofilin-1 in human HK-2 proximal tubular cells although the change in phosphorylated cofilin-1 was relatively weak. This observation suggests that cofilin-1 activity is not repressed when the total cofilin-1 level is increased by the oxidative stress. Little is known about whether oxidative stress is able to influence the expression of cofilin-1. A recent report has shown that phosphorylated cofilin-1 translocates to nuclei of podocytes during nephritic glomerular diseases, but the biological role of this phenomenon is unknown.[37] Whether phosphorylated cofilin-1 also relocates to the nuclei of HK2 PTCs after oxidative stress would be interesting to explore.

We have previously shown that cofilin-1 level is acceptable for assessing patients hospitalized in the ICU.[32] These patients were stratified for AKI, and the results revealed that cofilin-1 was just unacceptable as an approach to discriminating AKI patients from healthy individuals (AUC = 0.681). Here, therefore, we recruited more urine samples from healthy controls and excluded non-AKI cases among the ICU patients and interestingly found that the urine cofilin-1 level now seems to be a useful approach to assessing AKI. It would be of interest to increase the sample size and investigate whether urine cofilin-1 is suitable for the diagnosis of AKI in the future.

5 Conclusions

In summary, we have demonstrated for the first time based on this preliminary clinical study that the level of urine cofilin-1 is elevated in AKI patients compared to healthy adults. The ROC analysis revealed that urine cofilin-1 may be acceptable for distinguishing AKI patients from healthy individuals. Additionally, a pilot study using H_2O_2 treatment of human HK-2 PTCs suggests that the increased cofilin-1 found in urine is possibly associated with oxidative stress that upregulates cofilin-1 in PTCs. Although the current data showed that increase of urine cofilin-1 level is associated with clinically determined AKI, larger studies, a better aged matched control group, and an interhospital investigation are required to confirm this conclusion in the future.

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