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Original Article

Association of urinary NGAL with renal tubulointerstitial lesions and its clinical significance in LN patients

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Abstract: To investigate the relationship between the levels of urinary neutrophil gelatinase-associated lipocalin (NAGL) and activity of tubulointerstitial lesions in patients with lupus nephritis (LN), and to find a new biomarker reflecting renal tubulointerstitial lesions activity in LN. Kidney tissues were collected from 60 patients, including 39 cases of active group and 21 cases of inactive group, who were diagnosed with LN for the first time. As control group, normal kidney tissues were collected from 10 patients, who had accepted nephrectomy because of injury or benign tumor. An immunohistochemistry was performed to detect the NGAL levels in kidney tissues. The NGAL levels in blood and urine samples were investigated using ELISA. The levels of urinary NAGL in patients of LN active group were significantly higher than that in LN inactive group and control group, and were positively correlated with 24-hour urinary protein excretion (24 h UP), SLE disease activity index (SLEDAI), renal activity index of SLE patients (R-SLEDAI), glomerular activity index (GAI), tubulointerstitial activity index (TLAI), and activity Index (AI). The levels of NGAL in kidney of LN patients were higher than that in control group, and were positively correlated with serum creatinine (SCr), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), GAI, TLAT and AI. NAGL levels in urine and kidney tissues of LN patients exhibited a significant up-regulating trend, in accordance with the severity of renal tubulointerstitial lesions. Urinary NAGL levels can be treated as a sensitive indicator of LN activity, tubulointerstitial lesions activity and severity.

Keywords: Lupus nephritis (LN), neutrophil gelatinase-associated lipocalin (NGAL), tubulointerstitial lesions

Introduction

Lupus nephritis (LN) is one of the most common and severe complication of systemic lupus erythematosus (SLE) with an incidence up to 60% [1], and is one of the main factors affecting SLE prognosis [2]. It has already been reported that 10% to 26% of LN patients can develop into renal disease of end stage [3]. So, early monitoring and effective treatment of LN is very important to the prevention of kidney failure.

Previous studies have shown that renal tubulointerstitial lesions of LN patients, including tubulointerstitial inflammation, epithelial cell necrosis and interstitial scarring, are not secondary to glomerular injury, but the independent risk factor of LN [4, 5]. Renal tubulointerstitial lesions can predict progression and prognosis of kidney disease even better than tubular lesions [6]. Currently, renal biopsy is still the gold standard of LN diagnosis and evaluation of renal tubulointerstitial lesions severity, and can directly reflect the severity of acute or chronic lesions of LN patients. However, the invasion and risk limit the repeated implementation of renal biopsy [7]. Besides, serum creatinine, 24-hour creatinine clearance rate, 24-hour urinary protein quantity, anti-ds-DNA antibody and titer cannot reflect kidney pathological changes of LN patients exactly [8]. Therefore, searching for clinical marker reflecting renal tubulointerstitial lesions and their activities in LN patients in early stage is of great significance in treatment and prognosis of LN.

Neutrophil gelatinase-associated lipocalin (NGAL) is a small molecular protein of 25 KD, which belongs to apolipoprotein family [9]. Urinary NGAL can be used as an early diagnostic marker of acute kidney injury, diabetic nephropathy and contrast-induced nephropathy [10-12]. Moreover, urinary NGAL level can be used as clinical standard reflecting LN disease activity [13]. However, the relationship between urinary

NGAL level and tubulointerstitial lesions in LN is rarely reported. This paper aims to detect NGAL levels in blood, urine and kidney tissues of LN patents, to analyze the association between NGAL levels and clinical/pathological indicators, LN disease activity and tubulointerstitial lesions severity degrees, and to further explore the relationship and clinical significance of urinary NGAL and tubulointerstitial lesions.

Materials and methods

Subjects

Totally 60 patients diagnosed with LN in the hospital were collected from December 2011 to January 2014. All the patents must meet the following conditions: (1) Diagnostic criteria formulated by American Rheumatism Association on 1997. LN was diagnosed by renal biopsy. (2) Older than 18 years. (3) The prednisone dose within one month used by active LN patients should be less than 30 mg per day. Immunosuppressants should not be used. (4) Patients with primary or secondary kidney diseases, such as glomerulonephritis, diabetic nephropathy and hypertensive nephropathy, were excluded. (5) Female patients should not be pregnant. (6) Patients with infections were excluded. Additionally, 10 patients who had accepted nephrectomy because of renal trauma or benign tumor were collected as control group. All enrolled subjects have signed informed consent. The study was approved by the ethics review board of Affiliated Hospital of Qingdao University.

According to the scoring criteria of SLE disease activity index (SLEDAI) published on 2000 [14], the 60 patients mentioned above were divided into SLE active group (≥9 points) of 39 cases and SLE inactive group of 21 cases (<9 points). Renal activity index of SLE patients (R-SLEDAI) was determined based on the performance of hematuria, proteinuria, pyuria and tubular urine of patients, with each of 4 points (The minimum score was 0 point, and the maximum score was 16 points).

Sample collection

The kidney tissues were collected from the 60 LN patients, and the kidney tissues of 10 control patients were collected as well. Kidney tissues which were more than 5 cm away from tumor were collected in patients with benign tumor. All kidney tissues were confirmed by microscopy. Clinical samples of patients, including age, serum creatinine (SCr), blood urea nitrogen (BUN), serum albumin (ALB), complement C3 and C4, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and 24-hour urinary protein excretion (24 h UP) were collected before and after renal biopsy. The detections of these samples were completed with the help of test center of the hospital. In addition, blood and urine samples of all the patients were collected before surgery, centrifuged at 2000 r/min at 4°C, and then stored at -80°C for further use.

Reagents and instruments

Human lipocalin2 (NGAL) ELISA Kit was purchased from R&D Systems Inc (QC115, Minneapolis, MN, USA). Rabbit anti-human NGAL polyclonal antibody was purchased from BOSTER Co., Ltd (BA3407, Wuhan, China). Microscope was purchased from Hitachi Japan, Ltd (SU1510, Tokyo, Japan). HMIAS2 2000 high-definition color pathological graphic analysis system was purchased from Qingping Image Technology Co. Ltd (Wuhan, China).

HE staining and determination of LN pathological scores

Kidney tissues were placed in neutral 10% formalin to be fixed, and then were produced into slides with a thickness of 3 μm. HE staining was conducted. The slides were observed using microscope. LN pathology scores were determined according to the Hill lupus nephritis renal pathology scores criteria published in 2000 [15]. The judging indicators included glomerular activity index (GAI), tubulointerstitial activity index (TLAI), renal pathology Activity Index (AI, AI = GAI + TLAI) and chronic index (CI). Among these indicators, TLAI included renal tubular karyopyknosis, nuclear activation, cell necrosis, cell flat, macrophage infiltration, epithelial cell loss and interstitial inflammation, each was 0 to 3 points. The proportion of lesions which was less than 10% was 0.5 points, the proportion of lesions which was more than 10% but less than 25% was 1 point, the proportion of lesions which was more than 25% but less than 50% was 2 points, and the proportion of lesions which was more than 50% was 3 points. Active LN patients were divided into 4 groups with different severity degrees according to the

Table 1. Clinical indicators of all the patients

Items	Control group	LN group	
		Active group	Inactive group
Cases (n)	10	39	21
Age (year)	30.26±7.24	30.32±12.67	30.92±14.73
24 h UP (g/24 h)	0.09±0.35	3.46±1.20[a,d]	1.35±0.68[b]
C3 (g/L)	1.04±0.23	0.47±0.20[a,d]	0.71±0.06[b]
C4 (g/L)	0.37±0.11	0.10±0.005[a,c]	0.24±0.13[b]
SCr (μmol/L)	86.23±9.82	110.42±52.30[a,d]	78.22±24.78
BUN (mmol/L)	5.50±1.45	13.15±9.42[a,d]	5.80±3.16
CRP (mg/L)	1.7±1.09	47.73±35.34[a,c]	20.97±18.70[b]
ESR (mm/h)	10.20±4.47	42.52±36.28[a,c]	27.19±20.72[b]
ALB (mmol/L)	38.47±2.68	25.32±5.46[a]	29.19±5.82[b]

Note: a, P<0.01 compared with control group. b P<0.05 compared with control group. c, P<0.01 compared with LN inactive group. d, P<0.05 compared with LN inactive group.

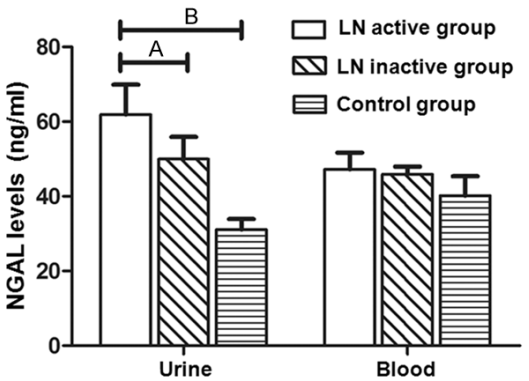

Figure 1. NGAL levels in urine and blood of patients in each group. ELISA was performed to detect NGAL levels in urine and blood of patients. B. P<0.05 compared with control group. A. P<0.05 compared with LN inactive group.

Tubulointerstitial lesions score criteria, including the mild group (0 to 4 points), the moderate group (5 to 8 points), the severe group (9-12 points) and the extremely severe group (more than 12 points).

ELISA

The NGAL levels in blood and urine of LN patients were detected by ELISA in accordance with the instructions of detection kit. Briefly, micro-plate wells were coated with a monoclonal antibody specific for NGAL. Standards and samples were pipetted into wells and any NGAL present was bound by the immobilized antibody. After incubation, the unbound conjugate was washed off and substrate solution was added to each well. The intensity of color developed was proportional to the concentration of NGAL in the patient urine sample.

Immunohistochemical staining

The Streptomyces avidin-peroxidase Quick method (SP) was according to the instructions. The primary anti-NGAL polyclonal antibody was used with a dilution of 1:200. The results were analyzed using HMIAS2 2000 high-definition color pathological graphic analysis system and presented by positive target expression index (EI). The calculated formula was: positive target average optical density value × positive area/(positive area + negative area) %. The average of EI from 10 fields expressed in % was taken as the EI of one slide.

Statistical analysis

Statistical analysis was conducted using SPSS 19.0 software. Data were presented as means ± standard deviation. Comparison among groups were performed using one-way AVNOA test when the variance were homogenous, and using nonparametric rank sum test and LSD-t test when variance were not homogenous. The correlation analysis of normally distributed data was detected using Pearson, and that of skewed data were analyzed using Spearman. P<0.05 was considered as significant difference.

Results

Clinical data of patients

In order to detect the changes of related factors of LN patients in vivo, the test center of hospital was commissioned to conduct related detections. Compared with control group, levels of several factors of LN active patients, including 24 h UP, SCr, BUN, CRP, and ESR were significantly higher (P<0.05). However, levels of complement C3, C4 and ALB were significantly lower (P<0.05). In comparison with LN inactive patients, levels of 24 h UP, SCr, BUN, CRP and ESR in LN active patients were increased significantly (P<0.05) whereas levels of complement C3 and C4 were decreased significantly (P<0.05), and there was no significant difference between ALB levels of the two groups

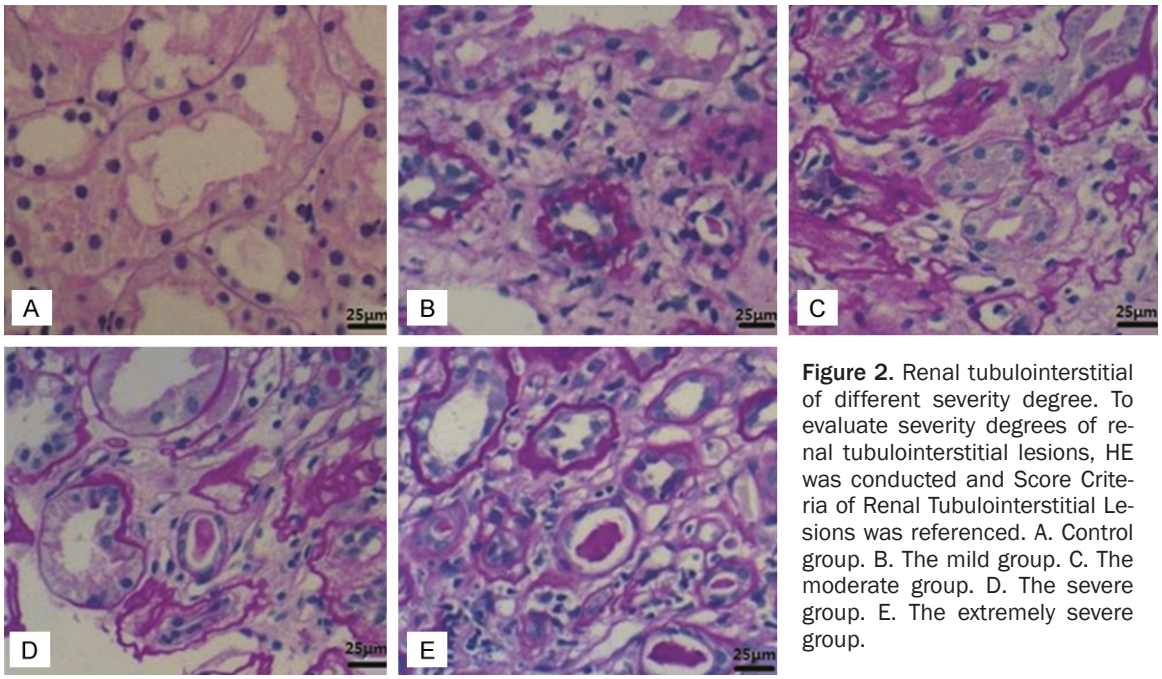

Figure 2. Renal tubulointerstitial of different severity degree. To evaluate severity degrees of renal tubulointerstitial lesions, HE was conducted and Score Criteria of Renal Tubulointerstitial Lesions was referenced. A. Control group. B. The mild group. C. The moderate group. D. The severe group. E. The extremely severe group.

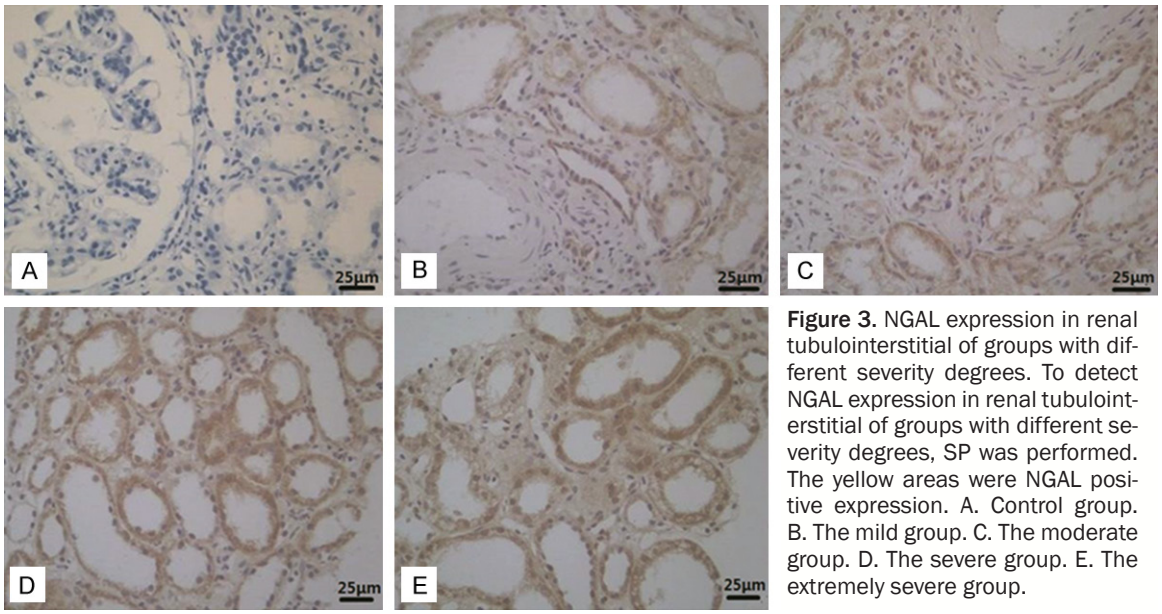

Figure 3. NGAL expression in renal tubulointerstitial of groups with different severity degrees. To detect NGAL expression in renal tubulointerstitial of groups with different severity degrees, SP was performed. The yellow areas were NGAL positive expression. A. Control group. B. The mild group. C. The moderate group. D. The severe group. E. The extremely severe group.

(**Table 1**). These results suggest different immune indicators between LN active patients and LN inactive patients.

NGAL levels in blood and urine in each group

To determine changes of NGAL levels in blood and urine of patients in different groups, ELISA was performed. NGAL levels in blood of LN patients, neither active group nor inactive group, were not significantly different from control group ($P>0.05$). However, urinary NGAL levels of LN patients were significantly higher than control group ($P<0.05$), moreover, urinary NGAL levels of active LN patients were significantly higher than that of inactive LN patients ($P<0.05$) (**Figure 1**). These results suggest the higher urinary NGAL levels in active LN patients compared to that in inactive LN patients.

NGAL levels in urine and kidney tissues of active LN patients with different severity degrees of tubulointerstitial lesions

To explore the relationship between severity degrees of tubulointerstital lesions and NGAL

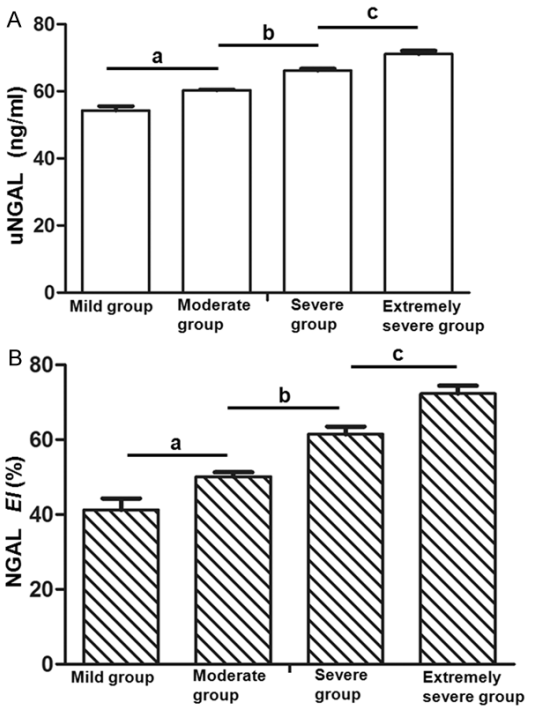

Figure 4. Analysis of NGAL levels in LN patients. A. Urinary NGAL levels in LN patients with different severity degrees of renal tubulointerstitial lesions. ELISA was used to detect urinary NGAL levels in different groups. a, P<0.05 compared with the mild group. b, P<0.05 compared with the moderate group. c, P<0.05 compared with the severe group. B. Renal tubulointerstitial NGAL expressions in LN patients with different severity degrees of renal tubulointerstitial lesions. NGAL expressions were presented as EI, which was calculated with the formula: positive target average optical density value × positive area/(positive area + negative area) %. a, P<0.05 compared with the mild group. b, P<0.05 compared with the moderate group. c, P<0.05 compared with the severe group.

levels in urine and kidney tissues in active LN patients, the severity degrees of tubulointerstitial lesions were determined firstly using score criteria of tubulointerstital lesions and HE staining of kidney slices. **Figure 2A** represented normal renal interstitial tissue. Active LN patients were divided into four groups with different severity degrees of tubulointerstitial lesions, including the mild group (**Figure 2B**), the moderate group (**Figure 2C**), the severe group (**Figure 2D**) and the extremely severe group (**Figure 2E**). As presented, the more severe the disease, the more severe renal interstitial injury and inflammatory cell infiltration and proliferation. After that, NGAL levels in urine and kidney tissues of active LN patients of the four groups were detected using ELISA and SP, respectively.

Table 2. Correlation analysis between clinical/pathological indicators of NGAL levels in urine and kidney of LN active patients

Items	Urinary NGAL levels		NGAL levels in kidney	
	r	P	*r*	P
SCr	0.322	0.149	0.606	0.028*
BUN	0.146	0.473	0.322	0.149
ESR	0.215	0.397	0.742	0.012*
CRP	0.105	0.673	0.619	0.021*
C3	-0.252	0.292	-0.297	0.251
C4	-0.142	0.562	-0.218	0.331
eGFR	-0.318	0.182	-0.245	0.121
SLEDAI	0.473	0.046*	0.325	0.147
R-SLEDAI	0.522	0.035*	0.450	0.051
ALB	0.079	0.742	0.213	0.409
24 h UP	0.460	0.049*	0.262	0.281
GAI	0.467	0.043*	0.611	0.036*
TLAI	0.626	0.020*	0. 467	0.043*
AI	0.611	0.036*	0.473	0.046*
CI	0.298	0.216	0.324	0.163

Note: *P<0.05. SCr, serum creatinine; BUN, blood urea nitrogen; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; SLEDAI, systemic lupus erythematosus disease activity index; R-SLEDAI, renal activity index of SLE patients; ALB, serum albumin; 24 h UP, 24-hour urinary protein excretion; GAI, glomerular activity index; TLAI, tubulointerstitial activity index; AI, activity index; CI, chronic index.

In normal renal tissues presented in **Figure 3A**, no NGAL expression was found. However, NGAL levels in renal tissues were increased gradually while the severity degrees increased gradually (**Figures 3B**, **3E**, **4B**) (P<0.05). Meanwhile, levels of urinary NGAL were increased gradually along with the increase of severity degrees of renal tubulointerstitial lesions as well, especially in the extremely severe group (**Figure 4A**) (P<0.05). The results indicate that the more sever renal tubulointerstitial lesions, the higher NGAL levels in renal tissues and urine.

Correlation analysis of NGAL levels in urine and kidney of active LN patients and clinical/pathological indicators

To clarify the correlation of NGAL levels in urine and kidney of active LN patients and clinical/pathological indicators, correlation analysis using Pearson and Spearman were conducted. It was shown that urinary NGAL levels of active LN patients were positively correlated with 24 h

UP, SLEDAI, R-SLEDAI, GAI, TLAI and AI ($P< 0.05$), and negatively correlated with SCr, BUN, CRP, ESR, ALB, complement C3 and C4, eGFR and CI ($P>0.05$). There were positive correlations between NGAL levels in kidney of active LN patients and SCr, BUN, ESR CRP, GAI, TLAI and AI ($P<0.05$), however, there was no correlation between NGAL levels in kidney of active LN patients and ALB, 24 h UP, complement C3 and C4, eGFR, R-SLEDAI, SLEDAI and CI ($P>0.05$) (**Table 2**).

Discussion

LN is one of the main factors influencing the prognosis and mortality in SLE patients. It has been reported that a variety of kidney damages may occur in up to 50% of SLE patients, including asymptomatic proteinuria and/or hematuria, nephritic syndrome, nephrotic syndrome, even rapidly progressive glomerulonephritis [16]. As the independent participant of LN onset, tubulointerstitial lesions not only develop following glomerular injury, but are closely related with manifestations and prognosis of patients [17]. Therefore, the understanding of tubulointerstitial lesions may play important roles in condition evaluations, treatment programs and prognosis assessment of LN patients. To a certain degree, SLEDAI and R-SLEDAI can reflect disease progression and help to guide treatment and recurrence in LN patients [18, 19]. However, SLADAI and R-SLEDAI can not reflect the pathological changes in kidney.

Thus, renal biopsy is still the "golden standard" evaluating tubulointerstitial lesions and their severity, but the invasion and risk limit its clinical use. It is of great significance to find a clinical marker which can reflect tubulointerstitial lesions sensitively.

Urinary NGAL levels can be the sensitive biomarkers of early acute kidney injury [20, 21], and the effective markers which can reflect LN incidence, monitor LN activity and predict children LN recurrence as well [22, 23]. However, the relationship between urinary NGAL levels and tubulointerstitial lesions in LN patients is rarely reported. In research of Alharazy SM et al. [24] on 100 LN patients including 47 active ones and 53 inactive ones, urinary NGAL levels are significantly related with SLEDAI, and can be taken as sensitive indicator predicting progression of recurrent LN. But the relation between urinary NGAL levels and tubulorinterstitial lesions in LN was not explored in this research [24]. In this paper, it was found that the urinary NGAL levels of active LN patients were significantly higher than that of control patients and inactive LN patients. Additionally, urinary NGAL levels of active LN patients were positive correlated with several clinical/pathological indicators, such as 24 h UP, SLEDAI, R-SLEDAI, GAI, TLAI and AI, meaning urinary NGAL levels can reflect disease activity of LN, which is consistent with results reported by Suzuki M [25]. Moreover, urinary NGAL levels of active LN patients increased gradually not only along with increased NGAL levels in kidney, but along with increased degrees of tubulonterstitial lesions in this paper. All the results indicate that urinary NGAL levels can be a noninvasive, effective and sensitive indicator monitoring degrees of tubulointerstitial lesions severity in LN patients, and may help to adjust the treatment in time.

Until now, the reason for the increased urinary NGAL levels in tubulointerstitial lesions is still unclear. As reported previously, NGAL can regulate the morphogenesis of renal tubular epithelial cells [26], and is involved in kidney formation via regulating cell growth as an iron transport protein. Levels of NGAL are usually low in kidney, lung and stomach physically [27]. However, NGAL can protect renal tubular cells from injury and promote cell proliferation in ischemic renal injury due to its resistance to apoptosis [28]. In renal ischemia, levels of NGAL increase, and the expression areas are consistent with the locations of renal tubular epithelial cells [29]. The results indicate that the generation of renal tubular epithelial cells may be induced by the up-regulation of NGAL. Meanwhile, Bolignano et al. indicated that impaired renal tubular cells may protect kidney tissue through receptor mediated oxidative stress and apoptosis which are induced by increased NGAL levels [30]. In combination with results of this paper, we speculate that the main cause of increased urinary NGAL levels in tubulointerstitial lesions of active LN patients is the increased NGAL levels secreted by renal tubulointerstitial cells. NGAL may involve in renal tubular regeneration after the impairment of renal tubular epithelial cells and delay the progression of renal tubulointerstitial lesions in LN, which is consistent with research of Morik et al. [31].

This study explored the relationship between urinary NGAL levels and activity of renal tubulointerstitial lesions in LN patients for the first time. However, the present study has some limitations. (1) This study is a retrospective analysis. The relationship between urinary NGAL levels and renal tubulointerstitial lesions in LN patients was not explored dynamically, and the prognosis of selected subjects was not monitored. (2) This study is a single-center study. The blood and urine samples of subjects may be affected by different drugs, infections or potential diseases. (3) There were not enough subjects in this study. This may lead to biased results.

In conclusion, urinary NGAL levels may reflect the activity and severity of renal tubulointerstitial lesions in LN patients. Thus, urinary NGAL levels can be applied in monitoring LN activity, evaluating LN progression and guiding the rational diagnosis and treatment of LN. However, the exact mechanism should be further explored with a larger sample size.

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Disclosure of conflict of interest

None.

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