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Original Article

Study the role of neutrophil gelatinase-associated lipocalin on rat renal anemia

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Abstract: Studies investigating neutrophil gelatinase-associated lipocalin (NGAL) as a prominent marker of renal injury are limited. This study analyzed the association between NGAL and anemia indices in rats with renal anemia. Serum ferritin, IL-1, IL-6, TNF-α, and plasma NGAL levels were significantly higher in the observation group than in the regular group. Serum EPO and TAST levels were lower in rats with renal anemia than in healthy rats. In the observation group, renal tissue NGAL mRNA expression and NGAL protein increased, whereas renal tissue EPOR mRNA expression and EPOR protein decreased, compared to the control group. In rat renal anemia models, decreased bone marrow NGAL protein and EPOR mRNA expression were observed, whereas no differences were noted in NGAL mRNA expression and EPOR protein levels, compared to those in the control group. Multivariate logistic regression analysis indicated that plasma NGAL concentrations were inversely correlated with hemoglobin, and positively correlated with IL-6 and renal tissue NGAL protein (R^2 = 0.734, F = 23.955; P < 0.001), after adjusting for iron parameters. The receiver operating characteristic curve based diagnostic accuracy of NGAL (area under the curve [AUC], 0.811; 95% CI, 0.663-0.959) and IL-6 (AUC, 0.800; 95% CI, 0.656-0.944) for identifying anemia were higher (P < 0.01) than that for IL-1 (AUC, 0.775; 95% CI, 0.627-923) and TNF-α (AUC, 0.767; 95% CI, 0.617-0.916). In conclusion, enhanced NGAL levels may contribute to the development of anemia in mild inflammatory states associated with chronic kidney disease.

Keywords: Neutrophil gelatinase-associated apolipoprotein (NGAL), chronic kidney disease, anemia pathway, animal experiment

Introduction

Neutrophil gelatinase-associated lipocalin (NGAL) belongs to the lipocalin family. Glomerular filtration rate is a major factor regulating the stability of this molecule, and NGAL is found increased in damaged nephrons. Increased NGAL is associated with the progression of acute kidney damage and slow kidney disease [1]. And the urinary NGAL can predict acute rejection following deceased donor kidney transplantation [2]. Previous studies suggest that NGAL may be an iron regulatory protein that binds to bacterial iron carriers and inhibits bacterial growth via antagonism [3]. Moreover, NGAL may be affected by the reactive oxygen species (ROS) system via the Tf transfer pathway, thus contributing to secondary anemia [4].

At present, NGAL is largely studied in relation to renal function damage, due to which the role of NGAL in anemia is less studied. The objective of our group was to investigate whether NGAL elevation is a potential risk factor for renal anemia patients. Based on previous clinical observations of non-dialysis renal anemia patients, the authors have confirmed that NGAL mediated iron metabolism pathways may be associated with chronic kidney disease [5]. As disease and anemia display an important relationship, serum NGAL may be a risk factor for renal anemia progression. Subsequent studies by Kim et al., reported results that were similar to ours, where it was found that plasma NGAL concentrations were associated with iron status in CKD patients with pre-dialysis anemia [6].

Inflammatory reactions often coincide with renal dysfunction. Interference with iron utilization in the inflammatory state is considered to be a common cause of anemia [7]. Concurrently, some *in vitro* experiments have indicated that

NGAL may also regulate the hematopoietic system *in vivo*. NGAL inhibits the induction of apoptosis in the medulla and blocks the differentiation of erythroid progenitor cells, thus inhibiting erythropoiesis [8, 9]. Therefore, we surmised that NGAL may inhibit erythropoiesis and interfere with iron utilization, leading to renal anemia.

To further clarify the mechanism underlying the role of NGAL in renal anemia, the current study established a rat renal anemia model to investigate whether NGAL is a potential risk factor for renal anemia, with particular reference to iron status and inhibition of erythropoiesis.

Materials and methods

Materials

Forty-five male SD rats (weight 180-220 g) were provided by the Experimental Animal Center of Xiamen University (license number: SYXK 2013-0006). The following materials were obtained: RNA extraction kit (Promega, US), reverse transcription kit (Promega), SYBR® Green Realtime PCR Master Mix kit (TOYOBO, Japan), NGAL ELISA kit (Abcam, UK), EPOR ELISA kit (Nanjing Jiancheng Biotechnology Co., Ltd. China), Ferritin ELISA kit (Nanjing Jiancheng Biotechnology Co., Ltd), rabbit anti-rat NGAL, EPOR polyclonal antibody (Proteintech, US) and protein extraction kit (Promega). All other reagents and antibodies were obtained from Beyotime (China).

Animal studies

Forty-five healthy SPF rats were fed with standard rat food for 1 week and randomly divided into 2 groups as follows: a control group consisting of 20 rats and an observation group consisting of 25 rats. Rats in the observation group were gavaged with adenine (1.5%, 300 mg/kg/d) slightly modified [10] for 6 weeks until they died, while the control group was orally administered with an equal volume of distilled water. Six rats died during the modeling period. All animal experiments were carried out in accordance with Institutional Animal Care and Use Committee of Xiamen University and that all efforts were made to minimize animal suffering.

Samples preparation

Renal samples and sacral bone marrow were recovered 6 weeks after molding. Next, sacral bone marrow and renal tissues were divided into 2 parts. Half of the sacral bone marrow and the right renal tissues were utilized for protein and RNA extraction, and were frozen in liquid nitrogen and stored at 80°C. The other half of the sacral bone marrow and left renal tissues were intended for histological studies, and were fixed in formaldehyde for 24 h prior to paraffin embedding.

Enzyme-linked immunosorbent assay (ELISA)

Circulating NGAL, Fer, and EPO levels were determined using ELISA kits according to the manufacturer's instructions. 1. Prepare all reagents, samples and standards as instructed. 2. Add 100 µl standard or sample to each well. Incubate for 2.5 h at room temperature or overnight at 4°C. 3. Add 100 µl prepared biotin antibody to each well. Incubate for 1 h at room temperature. 4. Add 100 µl prepared Streptavidin solution. Incubate for 45 min at room temperature. 5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate for 30 min at room temperature. 6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

Measurement of other serum parameters

Complete total iron binding capacity (TIBC), serum iron (SI), and serum creatinine (Scr) were measured using an automated analyzer (BECKMAN, US), while hemoglobin was measured via a CELL-DYN 3700 hematology analyzer (Abbott, US). TAST = SI/TIBC*%.

Real-time quantitative reverse transcription PCR

Total RNA from renal tissue or bone marrow was extracted using TRIzoland reverse transcribed to cDNA using a Reverse Transcription Kit. These cDNAs were fluorescently amplified with 7500 Software using the SYBR® Green Real-time PCR Master Mix Kit, following the manufacturer's instructions. Cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, and 60°C for 1 min. GAPDH was used as the internal control. Relative mRNA expression levels were obtained using a relative, quantitative, standardized gene expression method (2-ΔΔCt), primers were shown in the **Table 1**.

Western blot

Kidney and bone marrow tissues were washed with cold PBS, and lysed in RIPA lysate buffer

Table 1. The primers were used in the manuscript

Name	Forward primers	Reverse primers
NGAL	5'-CCGACACTGACTACGACCAG-3'	5'-CATTGGTCGGTGGGAACAGA-3'
EPOR	5'-TAGCGACTTGGACCCTCTCA-3'	5'-GCAACAGCCATAGCTGGAAGT-3'
GAPDH	5'-TCCTGCACCACCAACTGCTTAG-3'	5'-AGTGGCAGTGATGGCATGGACT-3'

Table 2. Plasma NGAL levels and hematologic parameters in the study

	Observation group (n = 19)	Control group (n = 20)	*P* value
NGAL			
Plasma NGAL level (pg/mL)	10083.70 ± 411.15	7016.13 ± 904.98	< 0.001
Renal function parameters			
Serum creatinine (μmol/L)	161.95 ± 23.97	62.81 ± 4.56	0.003
Blood urea nitrogen (mmol/L)	18.39 ± 3.74	9.03 ± 0.74	0.002
Hematologic parameters			
Hemoglobin (g/L)	114.67 ± 6.99	150.57 ± 4.89	0.003
Erythrocytes (× 10^{12}/L)	5.41 ± 0.33	7.79 ± 0.44	< 0.001
Iron parameters			
Serum iron (μmmol/L)	24.98 ± 2.78	31.23 ± 6.36	0.048
Transferrin saturation (%)	28.84 ± 3.73	35.41 ± 6.36	0.043
Serum ferritin (ng/mL)	63.14 ± 11.97	45.67 ± 3.75	0.003
Inflammation indices			
TNF-α (pg/ml)	30.25 ± 8.52	20.18 ± 7.23	0.034
IL-1 (pg/ml)	41.17 ± 11.09	26.34 ± 6.81	0.022
IL-6 (pg/ml)	56.32 ± 13.96	39.81 ± 10.86	0.006
EPO			
Plasma EPO level (ng/mL)	0.59 ± 0.37	1.90 ± 0.75	< 0.001

Data are expressed as mean ± SD or frequency (%). NGAL, neutrophil gelatinase associated lipocalin; TNF-α, tumor necrosis factor-α; EPO, erythropoietin.

(1% Triton X-100, 1% deoxycholate, 0.1% SDS) following which the supernatant was extracted. Protein concentration was measured using a BCA Protein Assay Kit. An appropriate amount of concentrated SDS-PAGE sample loading buffer was added to collected protein samples and heated at 100°C for 3-5 min to denature the protein completely. Protein samples were then run through 10-12% SDS-PAGE gels and transferred to nitrocellulose membranes, which were subsequently blocked with 5% non-fat dry milk and TBST for 1 h. Membranes were incubated with corresponding primary antibodies (concentration 1:1000) overnight at 4°C. After washing, they were incubated with secondary antibodies for 1 h at room temperature. Membrane signals were detected via a chemiluminescence detection system (ECL).

Statistical analysis

All values are presented as mean ± standard deviation. Differences between the 2 groups were analyzed using student's t-test for normally distributed data, whereas the Mann-Whitney U test was used for non-normal data. Multivariate logistic regression analysis was used to assess the association between anemia and NGAL concentration. A provisional cutoff point for NGAL concentration was determined using a receiver operating characteristic (ROC) curve, which showed maximal sensitivity and specificity for renal anemia. Statistical significance was set at $P < 0.05$.

Results

Plasma NGAL levels and hematological parameters

Renal anemic rats exhibited higher NGAL levels than regular rats did (10083.70 ± 411.15 pg/mL versus 7016.13 ± 904.98 pg/mL; $P < 0.001$) (**Table 2**). Mean values of TNF-α, IL-1, and IL-6 in the observation group (30.25 ± 8.52, 41.17 ± 11.09 and 56.32 ± 13.96 pg/ml) were significantly higher than those in the control group (20.18 ± 7.23, 26.34 ± 6.81, and 39.81 ± 10.86 pg/ml; $P = 0.034$, $P = 0.022$, and $P = 0.006$, respectively) (**Table 2**). Iron deficiency was diagnosed based on TAST values < 20%. The observation group exhibited lower levels of serum iron and transferrin saturation (TAST) compared to those of the control group (24.98 ± 2.78 μmmol/L and 28.84 ± 3.73% versus 31.23 ± 6.36 μmmol/L and 35.41 ± 6.36%; $P = 0.048$ and $P = 0.043$). Although TAST in the observation group was > 20%, this result indicated that iron utilization was inhibited. Rats with renal anemia exhibited significantly decreased EPO levels compared to those of healthy rats (0.59 ± 0.37 versus 1.90 ± 0.75 ng/mL, $P < 0.001$) (**Table 3**).

Table 3. Univariate and multivariate linear regression analysis between plasma NGAL levels and indices of anemia

	Univariate		Multivariate*	
	Standard β	*P* value	Standard β	*P* value
Hematologic parameters				
Hemoglobin (g/dL)	-0.745	< 0.001	-0.540	0.001
Erythrocytes (× 10^{12}/L)	-0.699	< 0.001	-0.729	0.167
Inflammation indices				
TNF-α (pg/ml)	0.604	< 0.001	0.105	0.450
IL-1 (pg/ml)	0.718	< 0.001	0.096	0.522
IL-6 (pg/ml)	0.443	0.005	0.441	0.001
NGAL				
Renal tissue NGAL protein	0.741	< 0.001	0.328	0.042
Bone marrow NGAL protein	-0.745	< 0.001	-0.039	0.874
EPO				
Serum erythropoietin (ng/ml)	-0.762	< 0.001	-0.303	0.051
Renal tissue EPOR protein	-0.508	0.001	-0.074	0.644
Bone marrow EPOR protein	-0.175	0.286	0.128	0.258

NGAL, neutrophil gelatinase-associated lipocalin; TNF-α, tumor necrosis factor-α; EPO, erythropoietin; EPOR, erythropoietin receptor. *adjusted for iron parameters.

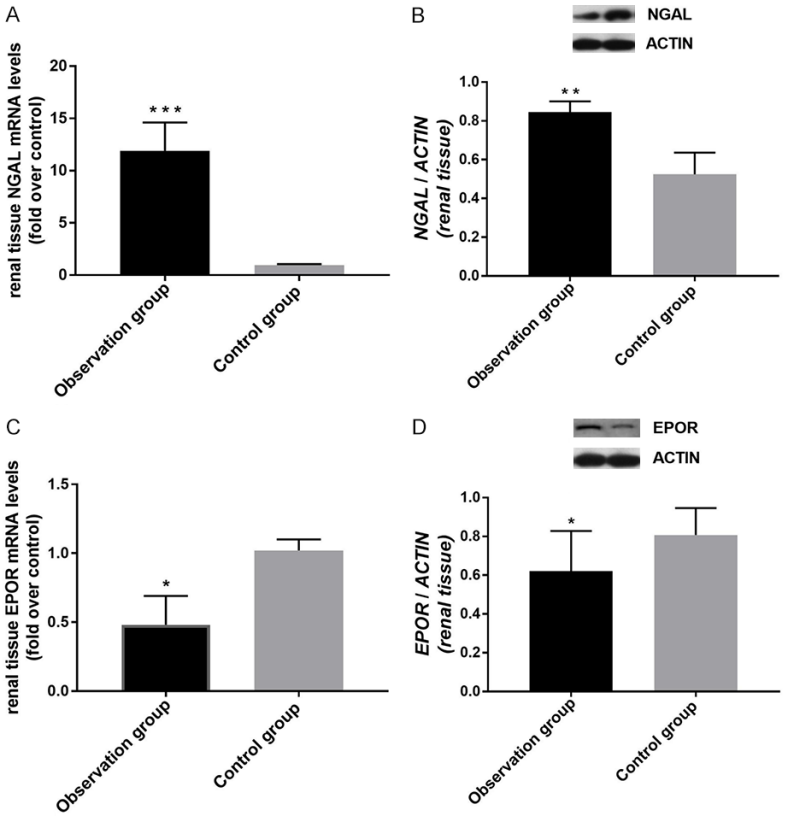

Figure 1. Renal tissue NGAL, EPOR mRNA expression and associated protein levels in renal anemia rats. A. NGAL mRNA levels were determined via quantitative RT-PCR. Relative fold change values were normalized against GAPDH as the endogenous control. B. NGAL protein levels and ACTIN as endogenous control were evaluated via immunoblotting. C. EPOR mRNA levels. D. EPOR protein levels. Results are expressed as mean + S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001 versus control group).

Renal tissue NGAL and EPOR levels in renal anemia

To analyze the effect of renal tissue NGAL on renal anemia, we analyzed NGAL, and EPOR mRNA and protein levels in the 2 groups. NGAL mRNA expression was significantly higher in the observation group (**Figure 1A**). Changes in NGAL mRNA were confirmed by analyzing the NGAL protein content using western blot (**Figure 1B**). We observed a significant decrease in EPOR mRNA expression and protein level in the observation group (**Figure 1C** and **1D**).

Bone marrow tissue NGAL and EPOR levels in renal anemia

To analyze the effect of increased systemic NGAL levels on bone marrow homeostasis, we analyzed NGAL and EPOR mRNA and protein levels in 2 groups of bone marrow tissues. No differences were observed in NGAL mRNA levels (**Figure 2A**). In contrast, we observed a significant decrease in NGAL protein levels in the observation group compared with that in the control group (**Figure 2B**). In this assay, the observation group showed a significantly lower EPOR mRNA level compared with that of the control group (**Figure 2C**), but no differences were observed in EPOR protein levels (**Figure 2D**).

Univariate and multivariate regression analysis

Baseline variables considered clinically relevant or showing a univariate rela-

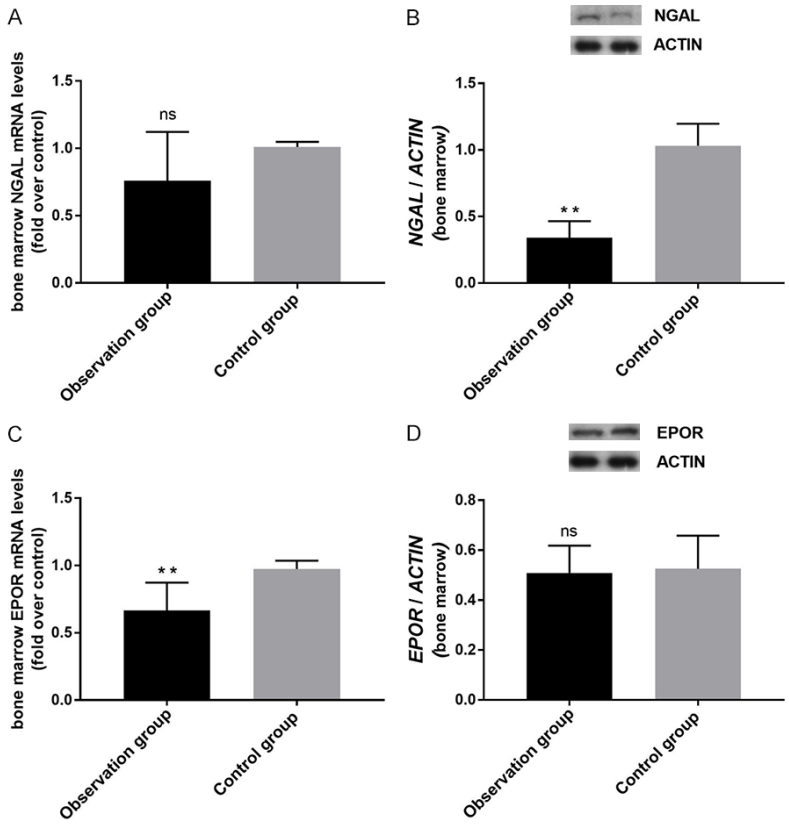

Figure 2. Bone marrow tissue NGAL, EPOR mRNA expression and protein levels in renal anemia rats. A. NGAL mRNA levels were determined via quantitative RT-PCR. B. NGAL protein levels were evaluated via immunoblotting. C. EPOR mRNA levels. D. EPOR protein levels. Results depict mean ± S.E.M. (**P < 0.01; ^{ns}P > 0.05 versus control group).

tionship with the outcome were entered into a multiple linear regression model. Plasma NGAL concentrations were inversely correlated with hemoglobin, and positively correlated with IL-6 and renal tissue NGAL protein (R^2 = 0.734, F = 23.955; P < 0.001), after adjusting for iron parameters (**Table 3**).

ROC curve analysis

The diagnostic values of NGAL, IL-1, IL-6, and TNF-α required to identify renal anemia were investigated using ROC curve analysis (**Figure 3**). Diagnostic accuracy of NGAL (area under the curve [AUC], 0.811; 95% CI, 0.663-0.959) and IL-6 (AUC, 0.800; 95% CI, 0.656-0.944) in identifying anemia was higher (P < 0.05) than that for IL-1 (AUC, 0.775; 95% CI, 0.627-923) and TNF-α (AUC, 0.767; 95% CI, 0.617-0.916).

Discussion

Extensively heterogeneous results have been reported regarding the association between plasma NGAL levels and anemia indices under various clinical conditions. In the current study, serum Scr of rats in the observation group increased, while Hb level decreased, thus establishing the model of renal anemia. However, 6 rats died during the modeling process, accounting for a large amount of adenine in the modeling method. At the same time, we observed inverse association between serum Hb concentration and plasma and renal tissue NGAL levels, indicating that increased NGAL levels may promote the development of anemia in chronic kidney disease.

The current study investigated the association between NGAL levels and renal anemia indices. NGAL has recently emerged as an important factor in iron homeostasis and erythrocyte growth regulation that may contribute to anemia when chronically elevated [11]. This is supported by important results observed in our study. In observation group, the plasma NGAL was associated with iron indices (TSAT, SI and ferritin). This findings indicate the role of the plasma NGAL in iron metabolism in renal anemia.

Although the NGAL/iron/oxidative stress triad appears to play a leading role in anemia, other studies have indicated that not all anaerobic models are NGAL. Elevation is accompanied by a decrease in iron levels, suggesting that NGAL upregulation is not strictly regulated by increased iron demand during anemia [12]. Reportedly, NGAL mediates apoptosis directly and blocks erythrocyte maturation [13], which is activated by IL-1, but is inhibited by IL-3. Under physiological conditions, this may represent antagonism of stimulation by EPO and other endogenous promoters, which is a harmful mechanism regulating erythropoiesis. The current study found that serum EPO, renal tissue EPOR, and bone marrow EPOR mRNA levels in

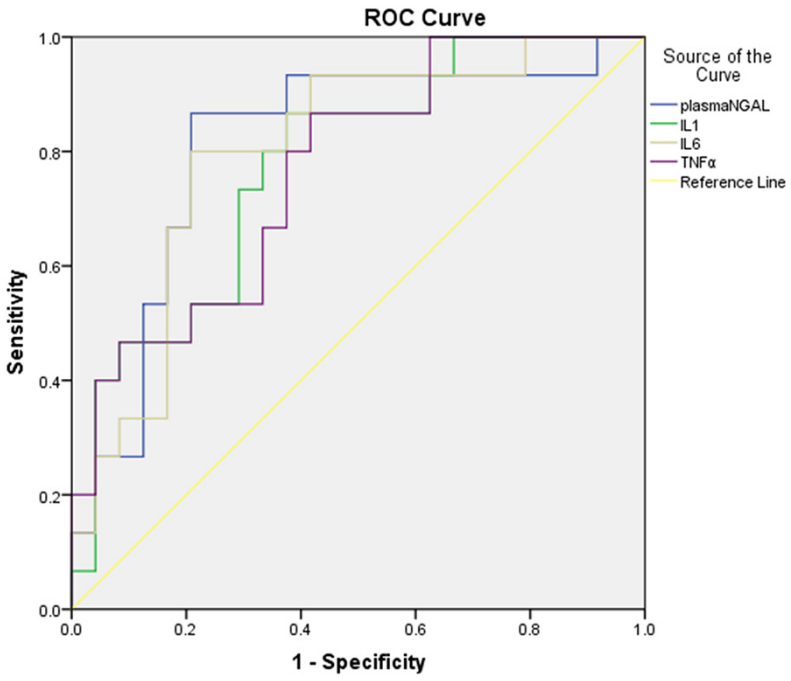

Figure 3. ROC curves showing diagnostic abilities of NGAL, IL-1, IL-6, and TNF-α to identify renal anemia. Area under the curve was calculated for NGAL (AUC, 0.811; 95% CI, 0.663-0.959; sensitivity 86.7% and specificity 79.2% at the optimal cutoff of 8145.72 ng/mL), IL-1 (AUC, 0.775; 95% CI, 0.627-923; sensitivity 86.7% and specificity 62.5% at the optimal cutoff of 34.79 pg/mL), IL-6 (AUC, 0.800; 95% CI, 0.656-0.944; sensitivity 80.00% and specificity 79.20% at the optimal cutoff of 48.23 pg/mL) and TNF-α (AUC, 0.767; 95% CI, 0.617-0.916; sensitivity 80.00% and specificity 62.5% at the optimal cutoff of 25.73 pg/mL).

the observation group were decreased. This decrease was negatively correlated with NGAL, while EPOR protein levels did not change significantly. Therefore, these findings, indicated that deficiency in erythropoietins and iron may be the leading cause of renal anemia. We speculate that NGAL may mediate iron metabolism disorders as well as abnormal red blood cell maturation.

Inflammatory factors may aggravate renal anemia [14]. Studies suggest that IL-6 and TNF-α may reduce erythroid proliferative activity of the bone marrow, and cause a disturbance in iron metabolism and decrease the erythropoietin (EPO) response effect, leading to anemia [15]. To test whether the above findings are related to the NGAL-mediated inflammatory response, we further improved detection of serum IL-1, IL-6, and TNF-α. The results suggested that serum IL-1, IL-6, and TNF-α in the model group were higher than those in the regular group, which is related to an increase in serum and kidney NGAL. Based on these findings, it may be speculated that renal anemia is presumably due to NGAL induced inhibition of erythrocyte production in conjunction with disturbed iron utilization resulting from systemic inflammatory response.

According to previous reports [8], when primary anemia occurs, the body has a dual response to NGAL, which is a protective antioxidant factor, and an increase in its synthesis represents an important defense mechanism in response to oxidative stress. Studies have shown [16] that administration of recombinant NGAL immediately after the induction of anemia significantly inhibits its hematopoietic recovery, where when more red blood cells are needed, immature red blood cells may activate IL-3 to inhibit NGAL synthesis. Effects of the induction of cell survival systems (e.g., BCL-XL, etc.) ultimately result in inhibition feedback systems which maintain red blood cell growth and differentiation. In our study, the results showed that the NGAL level in the bone marrow of CKD rats in the observation group was decreased. Therefore, we speculated that, during renal anemia, the body increases the synthesis of NGAL by reducing the production of medullary NGAL.

Choi et al., [17] demonstrated that enhanced NGAL production may contribute to the development of anemia in patients with systemic inflammation. Shrestha et al., [18] reported that plasma NGAL concentrations were closely linked to hemoglobin associated inflammation in patients with chronic systolic heart failure. In our study, multivariate regression analysis revealed that plasma NGAL concentrations were inversely correlated with hemoglobin, and positively correlated with IL-6 and renal tissue NGAL protein, after adjustment for iron parameters in renal anemia. These findings suggest that ele-

vated NGAL concentrations, activated by IL-6, were presumably caused by the inhibition of erythrocyte production in conjunction with disturbed iron utilization in chronic renal failure with systemic inflammation.

ROC curve analysis was used to evaluate the diagnostic value of NGAL, IL-1, IL-6, and TNF-α in chronic kidney disease anemia. The AUC of serum NGAL was similar to that of IL-6 but significantly more extensive than IL-1 and TNF-α. These results indicated that the diagnostic specificity of NGAL was superior to that of IL-1 and TNF-α, and thus NGAL may be used to identify renal anemia, similar to IL-6. A provisional cutoff point was determined (8145.72 pg/mL) using a receiver operating characteristic (ROC) curve, based on an optimal cutoff value corresponding to maximal sensitivity and specificity for renal anemia.

The current study displayed certain limitations. Primarily, renal anemia was not studied following NGAL knockout, thus limiting the opportunity for detecting a causal relationship between NGAL and anemia. Despite these limitations, our data may provide additional diagnostic criteria for anemia associated with chronic kidney disease.

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Disclosure of conflict of interest

None.

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