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Expression of serum soluble Klotho protein in patients with renal damage induced by anti-neutrophil cytoplasmic antibody-associated small-vessel vasculitis and influence on prognosis

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Abstract

***Background**: Anti-neutrophil cytoplasmic antibody-associated small-vessel vasculitis (AASV) is an autoimmune disease with unclear pathogenesis, which causes damage to multiple organs and systems, renal failure or even death. We aimed to explore the expression of serum soluble Klotho protein in patients with AASV-induced renal damage and influence on prognosis.* ***Methods**: A total of 330 AASV patients treated from June 2012 to June 2014 were divided into renal damage and non-renal damage groups. Clinical symptoms and laboratory examination results were compared. They were divided into Klotho <935.05 pg/mL and ≥935.05 pg/mL groups, and renal damage and pathological indices were compared. Survival curves were plotted using Kaplan-Meier method, and 5-year and renal survival rates were compared.* ***Results**: Compared with the non-renal damage group, the mean arterial pressure, urine protein and blood creatinine levels significantly rose, while the red blood cell count, hemoglobin, serum albumin, and Klotho protein levels declined in the renal damage group (P<0.05). The optimal cut-off value of Klotho protein in assessing renal damage was 935.05 pg/mL. Compared with Klotho ≥935.05 pg/mL group, the levels of blood creatinine and urine protein significantly increased, and the proportion of normal glomeruli decreased, while that of fibrous crescents rose in Klotho <935.05 pg/mL group (P<0.05). The 5-year renal survival rate was significantly lower in Klotho <935.05 pg/mL group than that in Klotho ≥935.05 pg/mL group (P<0.05).* ***Conclusions**: Klotho protein is lowly expressed in patients with renal damage induced by AASV as a potential marker for early diagnosis and prognostic evaluation.*

***Keywords**: Klotho protein, anti-neutrophil cytoplasmic antibody, small-vessel vasculitis, renal damage, prognosis*

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Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated small-vessel vasculitis (AASV) is an autoimmune disease with unclear pathogenesis, characterized by necrosis of small and medium vessels, which mainly involves capillaries, venules, arterioles and small arteries. It can

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cause damage to multiple organs and systems, the renal involvement rate can be as high as 80.0-87.1%, and renal failure or even death can be caused in severe cases (1,2). AASV frequently occurs in middle-aged and elderly people, and the peak incidence is mainly at the age of 50-70 years old (3).

Klotho protein is a calcium-phosphorus regulatory protein and its direct effect on the kidney is to promote urine phosphorus excretion and reduce urine calcium loss (4). Serum soluble Klotho protein has a variety of important biological functions in the human body, such as regulating calcium-phosphorus metabolism, and participating in the regulation of apoptosis and cell senescence, and it plays a protective role in the heart, brain, and kidney (5). Additionally, Klotho can enhance calcium reabsorption in distal renal tubules, promote the production of phosphaturia in proximal renal tubules, and inhibit phosphorus uptake by vascular smooth muscle cells (6). Klotho protein is highly expressed in the kidney, and the abnormal deletion of its expression can lead to obstructive nephropathy, renal ischemia-reperfusion injury, and chronic renal disease. It has potential value in the application as a diagnostic factor and drug target (7).

According to the severity, glomerular lesions can be divided into focal type, crescent type, mixed type, and sclerotic type. Such classification plays an important role in predicting the renal prognosis of patients (8). Among patients without developing into end-stage renal disease, the 5-year renal survival rate can reach 93% in focal type, while it is 76%, 61% and 50%, respectively, in crescent, mixed, and sclerotic types (9).

At present, there are few reports on the relationship between Klotho protein and renal damage in AASV patients. In the present study, therefore, the expression of Klotho protein in patients with renal damage induced by AASV and its value for prognostic evaluation were explored, aiming to provide references for clinical diagnosis and treatment.

Materials and methods

Clinical data

A total of 330 patients with AASV treated in our hospital from June 2012 to June 2014 were selected, including 185 males and 145 females aged (55.92±10.84) years old. The inclusion and exclusion criteria were as follows:

Inclusion criteria: 1) Patients meeting the diagnostic criteria for AASV (10); 2) those clinically diagnosed with AASV; 3) those who signed the informed consent with the approval of the Ethics Committee of our hospital. Exclusion criteria: 1) Patients accompanied by mental illness; 2) those complicated with other autoimmune diseases; 3) those with other primary or secondary kidney diseases, such as chronic glomerulonephritis, hepatitis B-related nephritis or hypertensive renal damage; 4) those with incomplete medical data.

Observation indices

The following data were collected from all patients enrolled. 1) General conditions: age, gender and mean arterial pressure; 2) initial clinical manifestations: fever, fatigue, and emaciation; 3) laboratory examinations: blood routine (red blood cell count, white blood cell count, hemoglobin, and platelet count), blood biochemistry (serum albumin, blood creatinine, triglyceride, and total cholesterol), immunological and inflammatory indices (C-reactive protein, ANCA, and complement C3); renal manifestations (glomerular filtration rate, urine protein (24-hour urine collection), and gross hematuria); 4) renal pathology: renal biopsy; 5) disease activity was assessed with the Birmingham Vasculitis Activity Score (BVAS).

Sample collection

Samples were collected after diagnosis and before treatment. In the early morning, 4 mL of fasting venous blood was drawn from all enrolled cases and controls who were healthy subjects receiving physical examinations in our

hospital. After standing for 20 min, the blood was centrifuged at 2,000 rpm for 20 min, and the supernatant was collected and stored in a refrigerator at -80°C. Besides, 10 mL of midstream morning urine was collected and centrifuged at 1,500 rpm for 10 min, and the supernatant was collected and stored in the refrigerator at -80°C.
Hemoglobin level was measured by XE-2100D automatic hematology analyzer (Sysmex Corporation, Japan). The levels of serum creatinine, triglyceride, serum albumin, total cholesterol, C-reactive protein, complement C3, and ANCA, as well as the counts of platelets, white blood cells, and red blood cells were detected by 9760 series automatic biochemical analyzer.
The level of serum Klotho protein was detected by double-antibody sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (eBioscience, USA). Purified human Klotho protein antibody was coated onto a microtiter plate to prepare a solid phase antibody. Klotho protein was added into the well of coated monoclonal antibody, and then conjugated with horseradish peroxidase (HRP)-labeled Klotho protein antibody. The resulting antibody-antigen-enzyme-labeled antibody complex was washed and color-developed by adding substrate tetramethylbenzidine (TMB). TMB became blue under the catalysis of HRP, and finally yellow in the presence of acid. The color intensity was positively correlated with the Klotho protein level. The optical density at 450 nm was measured with a microplate reader, and the Klotho protein level in sample was calculated from the standard curve.

Diagnostic criteria

1) Criteria for renal damage were set as follows: urine red blood cells ≥5/hpf, with or without urine protein quantification >150 mg/d, and blood creatinine >106 μmol/L.
2) Pathological classification of ANCA-associated glomerulonephritis was performed as follows: If the patient was suspected of renal damage, the short-term and long-term renal prognosis was predicted according to the pathological classification of ANCA-associated glomerulonephritis in 2010 (11). There are four types: focal type (normal glomeruli ≥50%), crescent type (crescent formation in ≥50% of glomeruli), mixed type (normal glomeruli <50%, crescent formation in <50% of glomeruli, and glomerular sclerosis <50%), and sclerotic type (glomerular sclerosis ≥50%).

Follow-up

All AASV patients with renal damage were followed up through telephone, outpatient visits, and hospitalization for 5 years (the follow-up was terminated if the patient died). During the observation period, renal death event was recorded if the disease progressed to end-stage renal disease, or long-term renal replacement therapy was needed, or the patient died. The 5-year survival rate and renal survival rate of patients in each group were recorded.

Statistical analysis

SPSS 19.0 software was used for data analysis. Measurement data in line with normal distribution were expressed as mean ± standard deviation, and independent-samples *t* test was performed for comparison between two groups, while analysis of variance for comparison among groups. Measurement data not in line with normal distribution were expressed as median and inter-quartile range, and rank sum test was performed for intergroup comparison. Numerical data were expressed as rate, and χ^2 test or Fisher's exact probability test was employed for intergroup comparison. Two-tailed $P<0.05$ suggested a statistically significant difference.

Results

Clinical symptoms and laboratory examination results

Indirect immunofluorescence assay showed that all the enrolled cases were ANCA-positive, of

which 310 were positive for p-ANCA and all positive for MPO in antigen-specific ELISA. The remaining 20 patients were positive for c-ANCA and all positive for PR3. Among the 330 patients with AASV enrolled, 280 cases were accompanied by renal damage. The clinical symptoms and laboratory examination results were compared between renal damage group and non-renal damage group. Compared with non-renal damage group, BVAS, mean arterial pressure, urine protein and blood creatinine levels significantly rose (P<0.05), while the red blood cell count, hemoglobin, serum albumin and Klotho protein levels significantly declined in the renal damage group (P<0.05) (**Table 1**).

ROC curve analysis results of predictive value of serum Klotho protein level for renal damage in AASV patients

Klotho protein was subjected to receiver operating characteristic (ROC) curve analysis to find the optimal cut-off value for predicting renal damage in AASV patients. It was found that the optimal cut-off value of Klotho protein in assessing renal damage was 935.05 pg/mL, and the area under the curve, sensitivity and specificity were 0.83, 86.27%, and 80.13% (P<0.001), respectively, indicating a higher diagnostic value (**Figure 1**).

Table 1. Clinical symptoms and laboratory examination results.

Clinical characteristic	Renal damage group (n=280)	Non-renal damage group (n=50)	χ^2	P
Age	56.27±10.12	55.74±9.34	0.345	0.730
Gender [male/female(n)]	155/125	30/20	0.371	0.542
Mean arterial pressure (mmHg)	103.56±11.21	98.43±10.30	3.001	0.003
Fever [n (%)]	66 (23.57%)	14 (28.00%)	0.453	0.501
Fatigue [n (%)]	136 (48.57%)	21 (42.00%)	0.735	0.391
Emaciation [n (%)]	53 (18.93%)	9(18.00%)	0.024	0.877
Hemoglobin (g/L)	87.28±11.91	126.14±18.70	19.249	0.000
Red blood cell (10^{12}/L)	2.91±0.46	4.43±0.67	19.919	0.000
White blood cell (10^9/L)	8.45±3.21	8.26±2.24	0.401	0.689
Platelet (10^9/L)	257.82±50.13	248.56±48.17	1.210	0.227
Serum albumin (g/L)	29.24±2.70	35.75±4.50	13.960	0.000
Blood creatinine (μmol/L)	409.21±105.8	312.13±98.6	6.036	0.000
Triglyceride (mmol/L)	1.83±0.57	1.73±0.36	1.198	0.232
Total cholesterol (mmol/L)	4.28±0.90	4.30±1.01	0.142	0.887
Urine protein (mg/d)	419.63±185.34	152.16±48.23	10.132	0.000
C-reactive protein (mg/L)	3.63±2.84	4.41±2.92	1.781	0.076
ANCA (RU/mL)	135.31±36.82	127.23±34.65	1.442	0.150
Complement C3 (mg/d)	105.21±22.45	99.23±17.69	1.786	0.075
Klotho protein (pg/mL)	623.2±158.3	986.4±203.1	14.271	0.000
Interstitial lung disease	54 (19.29%)	10 (20.00%)	0.014	0.906
Hypoxemia	51 (18.21%)	9 (18.00%)	0.001	0.971
Respiratory failure	32 (11.43%)	6 (12.00%)	0.014	0.907
Diffuse dysfunction	78 (27.86%)	16 (32.00%)	0.357	0.550
Pulmonary infection	143 (51.07%)	26 (52.00%)	0.015	0.904
BVAS	13.9±2.8	11.7±2.4	5.222	<0.001
p-ANCA positive/c-ANCA positive	245/35	45/5	0.249	0.619

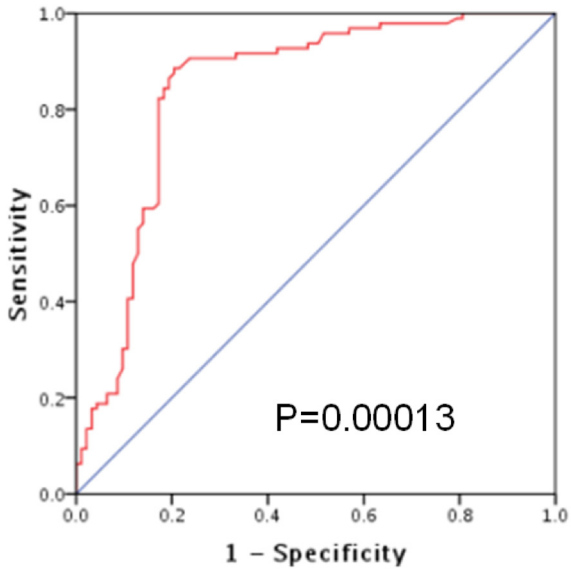

Fig. 1. ROC curve analysis results of predictive value of serum Klotho protein level for renal damage in AASV patients.

Renal damage-related indices in different Klotho protein expression groups

Based on the optimal cut-off value of Klotho protein, patients were divided into Klotho <935.05 pg/mL group and Klotho ≥935.05 pg/mL group, and the changes in renal damage-related indices were compared between the two groups. Compared with those in Klotho ≥935.05 pg/mL group, the levels of blood creatinine and urine protein were significantly increased (P<0.05), the glomerular filtration rate was slightly lower, and the proportion of gross hematuria was slightly higher in Klotho <935.05 pg/mL group (P>0.05) (**Table 2**).

Renal pathological indices in different Klotho protein expression groups

According to the severity, glomerular lesions can be divided into focal type (≥50% of glomeruli were normal), crescent type (≥50% of glomeruli underwent crescent formation), mixed type (<50% of glomeruli were normal, <50% of glomeruli underwent crescent formation and <50% of glomeruli underwent sclerosis), and sclerotic type (≥50% of glomeruli underwent sclerosis). The proportion of each type of lesions was 55.75%, 27.43%, 12.39%, and 4.42%, respectively, in Klotho ≥935.05 pg/mL group, dominated by the focal type. The proportion of each type of lesions was 30.54%, 44.91%, 16.17%, and 8.38%, respectively, in Klotho <935.05 pg/mL group, dominated by the crescent type. Compared with those in Klotho ≥935.05 pg/mL group, the proportion of normal glomeruli was significantly decreased, while that of fibrous crescents was significantly higher in Klotho <935.05 pg/mL group (P<0.05), suggesting that the degree of renal damage was lower in Klotho ≥935.05 pg/mL group than that in Klotho <935.05 pg/mL group (**Table 3**).

Survival rates of patients with different Klotho expression levels

All patients were followed up for 5 years. Thirty-two cases were lost to follow-up, and the remaining 248 cases were followed up until the endpoint, including 152 cases in Klotho <935.05 pg/mL group and 96 cases in Klotho ≥935.05 pg/mL group. The survival curves in the two groups were plotted using the Kaplan-Meier method. In

Table 2. Renal damage-related indices in different Klotho protein expression groups.

Index	<935.05pg/mL (n=167)	≥935.05pg/mL (n=113)	χ^2	P
Blood creatinine (μmol/L)	423.16±161.06	328.74±120.05	5.312	0.000
eGFR [mL•min^{-1}•(1.73m^2)$^{-1}$]	56.21±43.07	66.22±49.83	1.790	0.075
Urine protein (mg/d)	441.35±198.31	309.63±185.34	5.597	0.000
Gross hematuria [n (%)]	16 (9.58)	5 (4.42)	2.583	0.108

eGFR: Estimated glomerular filtration rate.

Table 3. Renal pathological indices in different Klotho protein expression groups.

Clinical characteristic	<935.05pg/mL (n=167), %	≥935.05pg/mL (n=113), %	χ^2	P
Focal/crescent/mixed/sclerotic type	51/75/27/14	63/31/14/5	18.174	0.000
Number of normal glomeruli	40.02±23.91	54.87±29.43	4.640	0.000
Glomerulosclerosis	20.45±18.07	19.38±24.79	0.418	0.677
Fibrous crescent	22.53±20.81	11.32±10.44	5.291	0.000
Cellular crescent	17.36±13.42	15.87±11.65	0.960	0.338
Mixed crescent	12.14±11.04	10.27±10.60	1.413	0.159
Fibrinoid necrosis	109(65.27)	71(62.83)	0.174	0.676

Klotho <935.05 pg/mL group, 34 patients died, and the 5-year survival rate was 77.63%. In Klotho ≥935.05 pg/mL group, 16 patients died, and the 5-year survival rate was 83.33%. It can be seen that the 5-year survival rate was lower in Klotho <935.05 pg/mL group than that in Klotho ≥935.05 pg/mL group (P>0.05) (**Figure 2**).

Renal survival rates of patients with different Klotho expression levels

In Klotho <935.05 pg/mL group and Klotho ≥935.05 pg/mL group, 7 cases and 2 cases developed end-stage renal disease before death, 26 cases and 9 cases required dialysis at the time of treatment, and 52 cases and 21 cases had renal death, respectively. The renal survival rate of patients in Klotho <935.05 pg/mL group (65.78%) was significantly lower than that in Klotho ≥935.05 pg/mL group (78.13%) (P<0.05) (**Figure 3**).

Discussion

The kidney is the most common organ involved in AASV, and nearly 90% of patients with AASV suffer from renal damage. Of the 330 patients with AASV enrolled in this study, 84.8% of them had renal damage, being consistent with previous studies (12). The initial manifestations of renal damage in AASV patients are diverse. In this study, compared with the non-renal damage group, the levels of urine protein and blood creat-

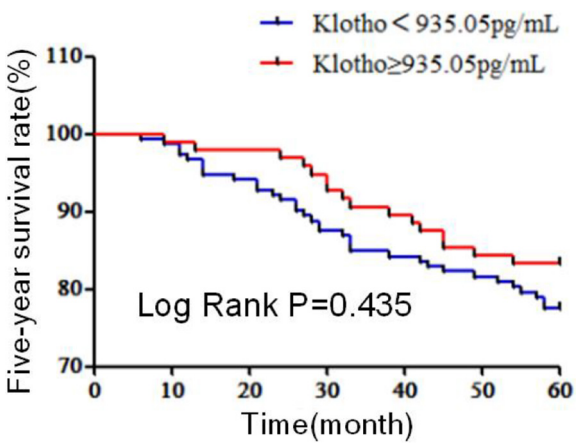

Fig. 2. 5-year survival rates of patients with different Klotho expression levels.

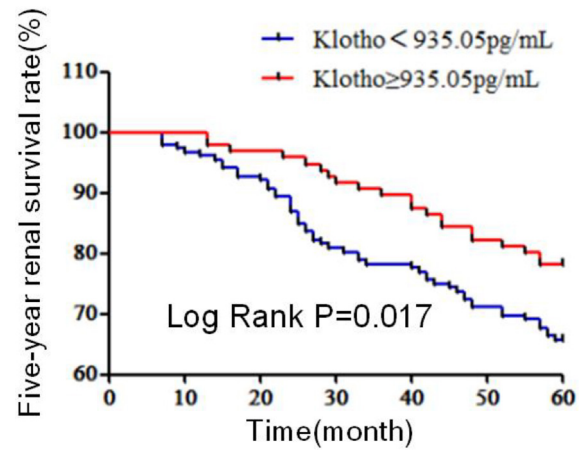

Fig. 3. 5-year renal survival rates of patients with different Klotho expression levels.

inine as well as mean systolic pressure were significantly higher, while the red blood cell count, hemoglobin, and serum albumin levels were lower in the renal damage group. The elevation in blood pressure may be related to the decrease in glomerular filtration rate in renal damage and the increase in circulating blood volume due to water-sodium retention, the decrease in red blood cell count and hemoglobin may indicate that anemia is aggravated in renal damage, and the decrease in serum albumin may be related to the loss of urine protein in renal damage.

Ullah *et al.* confirmed that Klotho protein deficiency caused by kidney disease accelerated cell senescence through oxidative stress, thereby reducing the anti-injury and self-healing ability of the kidney (13). In addition, Klotho$^{-/-}$ mice have stem cell loss in multiple organs and kidney progenitor cell senescence, leading to delayed tissue repair after renal damage (14,15). Promoting the expression of Klotho through gene manipulation can accelerate vascular formation in Klotho$^{-/-}$ mice and promote the limb recovery after ischemic injury, demonstrating that Klotho protein replacement therapy can promote renal recovery (16). Moreover, Klotho protein plays an important role in regulating renal function and improving acute kidney injury. As an early index for acute kidney injury, it also possesses etiological effects (17). In this study, the expression level of Klotho protein in the renal damage group was far lower than that in the non-renal damage group, suggesting that there is a lack of Klotho protein expression in renal damage in AASV patients.

To further explore the predictive value of serum Klotho protein expression for renal damage in patients with AASV, ROC curve analysis was conducted in this study. It was confirmed that the optimal cut-off value of Klotho in assessing the occurrence of renal damage in patients with AASV was 935.05 pg/mL, based on which the patients were divided into Klotho <935.05 pg/mL group and Klotho≥935.05 pg/mL group. The differences in renal damage and renal pathological changes were compared between the two groups. The clinical manifestations of renal damage in patients with AASV are proteinuria, hematuria, and renal insufficiency (18). In renal biopsy, necrotizing and crescentic glomerulonephritis can be observed under a light microscope (19). In this study, it was found that compared with those in Klotho ≥935.05 pg/mL group, the levels of blood creatinine and urine protein were significantly increased in the Klotho <935.05 pg/mL group. The proportion of focal lesions was higher in the Klotho ≥935.05 pg/mL group, while the proportion of crescentic lesions was higher in the Klotho <935.05 pg/mL group. Moreover, compared with those in the Klotho ≥935.05 pg/mL group, the proportion of normal glomeruli was decreased, while that of fibrous crescents rose in the Klotho <935.05 pg/mL group. It can be seen that the decline in expression level of Klotho protein worsens the renal damage in patients with AASV to a certain extent. Furthermore, it was found that the 5-year overall survival rate was slightly lower in the Klotho <935.05 pg/mL group than that in the Klotho ≥935.05 pg/mL group, but there was no significant difference. The 5-year renal survival rate of patients in the Klotho <935.05 pg/mL group was lower, suggesting that the expression level of Klotho protein can affect the prognosis of patients to a certain extent.

In conclusion, there is a lack of expression of Klotho protein in AASV patients with renal damage, and the occurrence of renal damage can be well predicted with 935.05 pg/mL as the cut-off value. The decline in expression level of Klotho protein worsens the renal damage and significantly harms the renal prognosis of AASV patients to a certain extent. In this study, the role of Klotho protein in renal damage induced by AASV was innovatively explored, providing a certain reference for clinical diagnosis. Nevertheless, this study still has limitations. This is

a single-center study with a small sample size. Further multi-center studies with larger sample sizes are ongoing in our group.

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Authors' contribution

FM - Study design, Data collection; JL - Data analysis, Writing.

Conflict of interest

The authors declare no conflict of interest.

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