Role of uromodulin and complement activation in the progression of kidney disease

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Abstract. Uromodulin (UMOD) is a glycoprotein that is selectively expressed on the epithelial cells of the thick ascending limb of Henle’s loop and the early distal renal tubule. The present study aimed to investigate whether UMOD was associated with complement activation in patients with renal diseases. In addition, its biological function was examined in vitro. The expression levels of UMOD and complement components, including C1q, C3, C4 and C3a, and membrane attack complex (MAC) in the plasma of patients with IgA nephropathy (IgAN; n=58) and lupus nephritis (LN; n=36) were detected using ELISA, which was used to determine the association between UMOD expression and complement components. In addition, a simulated hypoxia-reoxygenation (H/R) model was used to stimulate UMOD expression in mouse inner medullary collecting duct cells. Additionally, the association between UMOD expression and complement components C1q and C3d at the cellular level was identified using western blotting and immunofluorescence, respectively. It was revealed that the plasma UMOD concentration was significantly decreased in patients with IgAN and LN compared with healthy controls, and the levels of C3a and MAC were significantly increased in the plasma of patients with IgAN and LN. Furthermore, the plasma levels of C1q, C3 and C4 in patients with LN, but not in patients with IgAN, were significantly decreased compared with healthy controls. The plasma levels of UMOD were negatively correlated with the plasma C3a and MAC concentrations. However, the plasma levels of UMOD were significantly and positively correlated with the plasma C1q concentration, but not with that of C3 and C4. It was identified that UMOD expression started to increase after 1 h of simulated H/R, and continued to increase at 6 and 12 h. In addition, cells with lower UMOD expression had higher C3d expression in vitro. Collectively, the present results suggested that UMOD was associated with severe complement activation and may be involved in complement-mediated immune protection by inhibiting complement activation in renal disease.

Introduction

Chronic kidney disease is ubiquitous and associated with significant morbidity and mortality, and is caused by hypertension, infection, diabetes and the excess of complement activation (1). Complement activation accelerates progressive kidney diseases by stimulating the synthesis and release of pro-inflammatory cytokines, including TNFα and interleukins, and reactive oxygen species, as well as increasing the synthesis of matrix proteins (2). Uromodulin (UMOD) is the most abundant protein in normal human urine and is selectively expressed by epithelial cells of the thick ascending limb of Henle’s loop and the early distal renal tubule (3,4). Previous studies have revealed that UMOD serves an important role in patients with acute and chronic kidney diseases (5,6). The levels of UMOD in urine and blood are closely associated with the estimated glomerular filtration rate (eGFR) in patients with chronic kidney disease (7-9). Our previous studies have suggested that the levels of UMOD were decreased in urine and were associated with interstitial fibrosis, tubular atrophy and low eGFR in patients with IgA nephropathy (IgAN) (10-12). Furthermore, UMOD-knockout mice exhibit more serious injury compared with wild-type mice after ischemia-reperfusion injury (13,14); however, the potential underlying mechanism requires to be elucidated.

Complement is an important component of the innate immune system that consists of ≥30 types of proteins that widely exist in the circulation, tissues and cell membrane surface (15-17). Abnormal complement activation exists in a variety of kidney diseases, including lupus nephritis (LN) (18), IgAN (19) and membranoproliferative glomerulonephritis (20). Previous studies have reported that numerous types of kidney cells present a variety of complement components and complement receptors, such as C2, C3, C4, factor B, factor H, CR1, CR3 and C3aR (21,22). Moreover, Schiano et al (23) revealed that mice with complement factor H gene-knockout exhibited renal tubulointerstitial damage due to excessive complement activation. However, the exact mechanism remains unknown.
The present study aimed to investigate the potential clinical significance of UMOD, and its association with complement activation and histopathological characteristics in patients with kidney disease.

Materials and methods

Patients and samples. The present study was approved by the Ethical Committee of Qi Lu Hospital of Shandong University (Jinan, China). Written informed consent was obtained from all patients and healthy controls involved in the study. The study population included 58 patients with IgAN composed of 38 men and 20 women with a median age of 33 years (range, 27-48 years) and 36 patients with LN composed of 7 men and 29 women with a median age of 34 years (range, 28-46 years) between January 2014 and December 2018 at Qi Lu Hospital of Shandong University. Patients were included in the study according to the following requirements: i) Age of ≥18 years; and ii) biopsy-confirmed primary IgAN or LN. Additionally, 30 healthy volunteers (median age, 41 years; range, 25-61 years) were enrolled as controls, including 15 males and 15 females.

UMOD, C3a, membrane attack complex (MAC), C1q, C3 and C4 measurements. The plasma UMOD levels were measured using a commercial human uromodulin ELISA kit (cat. no. RD1916320R; BioVendor R&D) according to the manufacturer’s protocol as described previously (25). Based on median expression levels of plasma UMOD in the IgAN (217 ng/ml) and LN (169 ng/ml) groups, patients were classified into low and high expression subgroups. The plasma concentrations of C3a and MAC were determined using ELISA (cat. no. A031 and A020, respectively; Quidel Corporation) according to the manufacturer’s instructions. The plasma concentrations of C1q, C3 and C4 were measured using immunoturbidimetric assays (Shanghai BeiJi Biochemical reagent Co., Ltd.) according to the manufacturer’s instructions.

Renal biopsy. All renal perforation specimens were collected and fixed in 4% paraformaldehyde at room temperature for 24 h. Following fixation, the samples were embedded in paraffin and cut into 4-μm-thick sections, which were then dewaxed in xylene (80-90°C), rehydrated using a descending ethanol series (100, 85 and 75% ethanol) and washed in water. Following antigen retrieval, the spontaneous fluorescence was eliminated to β-actin and quantified using ImageJ software (V1.8.0; National Institutes of Health).

Immunofluorescence assays. The cells in each group were seeded on a coverslip and cultured as aforementioned. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, treated with 0.1% Triton X-100 for 15 min (at room temperature) and blocked with 5% fetal bovine serum for 15 min (at room temperature) and blocked with 5% fetal bovine serum.
serum (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The cells were incubated with anti-C3d primary antibody (1:50; cat. no. AF2655-SP; R&D Systems, Inc.) overnight at 4˚C. After washing, cells were incubated with secondary antibodies (1:200; cat. no. 34312ES60; Shanghai Yeasen Biotechnology Co., Ltd.) at room temperature for 1 h and DAPI was added dropwise to completely cover the cells for nuclei staining. The coverslip with cells was inversely placed and mounted on a slide with anti-fluorescence quenching agent (Beijing Solarbio Science & Technology Co., Ltd.). Sections were evaluated under fluorescence microscopy (magnification, x200).

**Statistical analysis.** Data were analyzed using SPSS 23.0 software (IBM Corp.). Data are presented as the mean ± SD of at least three experiments, median and interquartile range for continuous variables or numbers (proportions) for categorical variables. Kruskal-Wallis test was used for comparisons of UMOD and complement components levels in plasma of patients, and Dunn-Bonferroni test was used as the post-hoc test. The correlation between plasma UMOD levels and complement components levels in plasma samples using ELISA. It was revealed that the plasma UMOD concentration was significantly decreased in patients with IgAN or LN compared with in healthy controls (P<0.0001; Fig. 1A). The plasma levels of C3a and MAC in the three groups are shown in Fig. 1B and C. Compared with in the control group, C3a and MAC levels were significantly higher in the IgAN and LN groups (P<0.05; Fig. 1B and C). Furthermore, the levels of C1q, C3 and C4 in plasma were significantly decreased in the LN group compared with in the control groups (P<0.05 and P<0.0001, respectively; Fig. 1D-F). However, there was no significant difference in the plasma levels of C1q, C3 and C4 between the IgAN and healthy control groups (P>0.05; Fig. 1D-F).

**Levels of plasma UMOD and complement components in healthy controls, patients with IgAN and patients with LN.** To investigate the potential association between UMOD and complement components, the levels of plasma UMOD, C3a, MAC, C1q, C3 and C4 in patients with IgAN or LN and healthy controls were analyzed in plasma samples using ELISA. It was revealed that the plasma UMOD concentration was significantly decreased in patients with IgAN or LN compared with in healthy controls (P<0.0001; Fig. 1A). The plasma levels of C3a and MAC in the three groups are shown in Fig. 1B and C. Compared with in the control group, C3a and MAC levels were significantly higher in the IgAN and LN groups (P<0.05; Fig. 1B and C). Furthermore, the levels of C1q, C3 and C4 in plasma were significantly decreased in the LN group compared with in the control groups (P<0.05 and P<0.0001, respectively; Fig. 1D-F). However, there was no significant difference in the plasma levels of C1q, C3 and C4 between the IgAN and healthy control groups (P>0.05; Fig. 1D-F).

**Correlation between UMOD levels and complement components.** To further assess whether the levels of plasma UMOD were correlated with complement components, the Spearman correlation coefficients of all patients were calculated. The results indicated that the plasma levels of UMOD were significantly negatively correlated with the plasma concentration of C3a and MAC (r=-0.410 and P<0.001; r=-0.301 and P=0.003, respectively; Fig. 2A and B).

**Results**

**Clinical characteristics of patients.** Demographic and clinicopathological characteristics of the patients are presented in Table I. The median age of the patients with IgAN (33 years) and patients with LN (34 years) was not markedly different compared with that of healthy controls (41 years). Sex distribution was markedly different among the three groups. The immunofluorescence staining results of IgA, IgG, Clq, C3, IgM and Fibrinogen are presented in Table II.; IgA expression (++) occurred more frequently in IgAN than in LN. IgG (++) and Clq (++) were expressed in LN, but not in IgAN. The histopathological features of patients with IgAN and patients with LN are listed in Tables III and IV.
Subsequently, the correlation of plasma levels of C1q, C3 and C4 with plasma UMOD was investigated in all patients. It was demonstrated that the plasma levels of UMOD were significantly positively correlated with the plasma concentration of C1q (r=0.283; P=0.006; Fig. 2C), However, plasma UMOD levels were not significantly correlated with C3 (r=0.147; P=0.157; Fig. 2D) and C4 (r=0.195; P=0.06; Fig. 2E). In addition, the simulated H/R model was used to assess these correlation coefficients in mIMCD3 cells. Compared with the control group, the protein expression levels of complement C1qA decreased at 1 h after H/R and began to increase at 6 h after H/R, but no significant change was observed at 12 h after

| Characteristics                      | Total                  | IgAN                   | LN                     | Healthy volunteers |
|--------------------------------------|------------------------|------------------------|------------------------|--------------------|
| Males, n (%)                         | 45 (47.9)              | 38 (65.5)              | 7 (19.4)               | 15 (50)            |
| Females, n (%)                       | 49 (52.1)              | 20 (34.5)              | 29 (80.6)              | 15 (50)            |
| Age, years (range)                   | 33 (28-46)             | 33 (27-48)             | 34 (28-46)             | 41 (25-61)         |
| Height, cm (range) cm                | 167.5 (150-192)        | 170 (162-175)          | 163 (160-167)          | 164.6 (150-180)    |
| Weight, kg                           | 69 (60-75)             | 71 (64-78)             | 65 (59-70)             | 64 (46.5-92)       |
| SBP (range), mmHg                    | 137 (128-154)          | 137 (128-153)          | 137 (129-153)          | 134.5 (107-184)    |
| DBP ± SD, mmHg                       | 87.89±14.79            | 87 (77-95)             | 90 (78-98)             | 85.56 (69-114)     |
| RBC ± SD, x10¹²/l                   | 4.21±0.81              | 4.49±0.66              | 3.78±0.84              | 4.49±0.66          |
| HGB ± SD, g/l                        | 121±25.8               | 132±22.8               | 105±21.8               | 118±28             |
| ESR (range), mm/h                    | 19 (11-32)             | 17 (10-23)             | 26 (13-48)             | 34 (18-55)         |
| Total protein (range), g/l           | 58.7 (47.9-66.3)       | 61.2 (52.47-66.8)      | 50.2 (37.28-64.57)     | 59.6 (48.7-76.8)   |
| Albumin (range), g/l                 | 33.25 (25.2-39.93)     | 35.55 (30.7-42.03)     | 27.95 (18.83-33.25)    | 32.62 (15.7-41.4)  |
| ALT (range), U/l                     | 15 (11-19)             | 15 (11-18)             | 15 (11-22)             | 19.7 (5.35)        |
| AST (range), U/l                     | 18 (15-22)             | 18 (16-22)             | 19 (15-26)             | 20.2 (11-26)       |
| Total cholesterol (range), mmol/l    | 5.64 (4.47-6.63)       | 5.48 (4.63-6.59)       | 5.73 (3.77-7.19)       | 5.16 (3.13-9.19)   |
| HDL cholesterol ± SD, mmol/l         | 1.32±0.34              | 1.32±0.30              | 1.31±0.40              | 1.32±0.39          |
| LDL cholesterol (range), mmol/l      | 3.35 (2.41-4.13)       | 3.30 (2.60-4.09)       | 3.56 (2.20-4.23)       | 3.15 (2.09-4.88)   |
| Triglycerides (range), mmol/l        | 2.02 (1.33-2.83)       | 1.93 (1.22-2.72)       | 2.17 (1.45-3.16)       | 1.87 (0.97-3.17)   |
| Creatinine (range), mg/dl            | 95 (73.75-134.25)      | 107 (79-139.5)         | 91 (68.25-131.25)      | 126.4 (77-287)     |
| BUN (range), μmol/l                  | 6.57 (5.1-11.23)       | 5.78 (4.79-7.83)       | 9.38 (6-16.15)         | 7.81 (5.1-13.21)   |
| Cystatin C (range), ng/l             | 1.43 (1.03-1.86)       | 1.16 (0.94-1.67)       | 1.67 (1.16-2.43)       | 1.58 (0.74-2.42)   |
| Uric acid (range), μmol/l            | 393 (311-523)          | 373 (294-470)          | 430 (344-572)          | 359.3 (239-420)    |
| eGFR (range), ml/min/1.73 m²         | 92 (72-110)            | 97 (82-110)            | 86 (66-107)            | 90 (64-112)        |
| GLU ± SD, mmol/l                     | 4.56±0.69              | 4.56±0.58              | 4.56±0.84              | 4.44±0.75          |
| UACR (range), mg/g                   | 2.65 (1.28-3.82)       | 2.16 (1.08-3.43)       | 2.95 (1.73-7.45)       | 2.49 (0.99-4.49)   |
| C3a (range), ng/l                    | 157.6 (92.4-240.4)     | 144.8 (79.2-233.9)     | 175 (112.5-310.6)      | 147 (60.9-277.1)   |
| MAC (range), ng/l                    | 190.8 (135.5-364.9)    | 150.7 (117.4-222.2)    | 354.4 (179.8-491)      | 281 (118.76-435.7) |
| C1q ± SD, mg/l                       | 157±41.4               | 165±42.1               | 143±36.5               | 169±43.4          |
| C3 (range), g/l                      | 0.98 (0.5-1.18)        | 1.14 (0.96-1.29)       | 0.4 (0.31-0.69)        | 0.9 (0.26-1.57)    |
| C4 (range), g/l                      | 0.22 (0.07-0.30)       | 0.26 (0.14-0.32)       | 0.14 (0.07-0.23)       | 0.25 (0.067-0.533) |
| UMOD (range) ng/l                    | 149.88 (96.53-245.04)  | 147.12 (96.53-230.44)  | 156.79 (95.42-250.25)  | 189.91 (80.27-231.23) |
| K ± SD, mmol/l                       | 4.19±0.56              | 4.16±0.50              | 4.23±0.71              | 8.58±0.24          |
| Na (range), mmol/l                   | 141 (139-143)          | 142 (141-143)          | 140 (138-142)          | 138.3 (123-143)    |
| Cl ± SD, mmol/l                      | 106±3.6                | 106±2.6                | 106±4.9                | 102±1.9            |

SBP, systolic blood pressure; DBP, diastolic blood pressure; RBC, Red blood cell; HGB, Hemoglobin; ESR, erythrocyte sedimentation rate; ALT, alanine transaminase; AST, Aspartate aminotransferase; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; BUN, blood urea nitrogen; GLU, Glucose; UACR, urine albumin/creatinine ratio; IgAN, IgA nephropathy; LN, lupus nephritis; MAC, membrane attack complex; UMOD, uromodulin; eGFR, estimated glomerular filtration rate. Age is presented as the median value for all the variables.
H/R compared with 6 h after H/R (Fig. 3A). On the other hand, the protein expression levels of UMOD started to increase at 1 h after H/R treatment, and continued to increase at 6 and 12 h after H/R (Fig. 3A and B). In addition, the expression levels of the complement activation product C3d on the surface of the cell membrane was measured via immunofluorescence staining, revealing that C3d expression was increased at 1 h and began to decrease at 6 h after H/R (Fig. 3C). No significant change was observed at 12 h compared with 6 h post H/R (data not shown).

Association between plasma UMOD levels and clinicopathological variables. To determine whether plasma UMOD expression was associated with clinicopathological parameters, kidney function parameters and histopathological stage were analyzed in all patients. As shown in Fig. 4A-D, plasma UMOD concentration was closely correlated with the following kidney function biomarkers: eGFR (r=0.255; P=0.013; Fig. 4A), creatinine (r=−0.307; P=0.003; Fig. 4B), BUN (r=−0.359; P<0.001; Fig. 4C) and cystatin C (r=−0.294; P=0.004; Fig. 4D). Based on the median expression levels of plasma UMOD in the IgAN groups, patients were classified into two subgroups: Low and high expression group. In patients with IgAN, the plasma levels of UMOD were significantly associated with tubular atrophy and interstitial fibrosis (P=0.0219; Table III). In addition, it was revealed that in patients with LN plasma UMOD expression was significantly associated with AI (P=0.0354; Table IV). No other clinicopathological feature was identified to be associated with plasma UMOD expression.

Discussion

In present study, it was demonstrated that UMOD was negatively and significantly correlated with complement activation product C3a and MAC in patients with IgAN and LN. Moreover, UMOD was positively correlated with complement components C1q in the IgAN group. This correlation was further assessed using a simulated H/R model in vitro.
Previous studies have reported that UMOD expression is closely associated with kidney diseases (3-9). It has been demonstrated that UMOD gene mutations result in familial juvenile hyperuricemic nephropathy, medullary cystic kidney disease type 2 and glomerulocystic kidney disease (8). Furthermore, UMOD-deficient mice are more susceptible...
to nephrolithiasis (9). Serum UMOD is a promising potential biomarker in the progression of renal disease (29). Mechanistically, the role of UMOD is associated with renal ion channel activity (9,30), urinary tract infections (31) and urinary cast formation (32). UMOD−/− mice exhibit a decrease in renal outer medullary potassium channel (ROMK) immunoreactivity in the plasma membrane-enriched fractions and an increase of ROMK accumulation in intracellular vesicular compartments compared with wild-type mice (33,34). Moreover, in a previous study, >100 µg/ml UMOD predominantly prevented the adherence of Escherichia coli to transitional cells (35,36). Additionally, UMOD is involved in the gel and potential cast formation in acute tubular necrosis partially via oligosaccharide residues (37). Consistent with previous studies (10-12), the present study identified the potential association between UMOD and kidney diseases, and revealed that plasma UMOD levels were decreased in patients with IgAN and LN. This may be associated with interstitial fibrosis, renal tubular atrophy and decreased EGFR, which is consistent with previous reports on the decrease of urinary UMOD levels in patients with IgAN (10-12).

Complement is an important component of the immune system, and complement components are produced by kidney parenchymal tissues, as well as the liver. Moreover, the complement system is involved in various renal disease, such as LN (38), IgAN (39) and diabetic nephropathy (40).

Table IV. Pathological characteristics of 36 patients with lupus nephritis with high (n=18) and low (n=18) uromodulin expression.

| Characteristics                          | N   | High   | Low   | P-value |
|-----------------------------------------|-----|--------|-------|---------|
| Histological classification             |     |        |       |         |
| II+III                                  | 7   | 6 (85.7) | 1 (14.3) | 0.0877 |
| IV+V                                    | 29  | 12 (41.4) | 17 (58.6) |         |
| Activity index                          |     |        |       |         |
| <10                                     | 23  | 15 (65.2) | 8 (34.8) | 0.0354 |
| ≥10                                     | 13  | 3 (23.1)  | 10 (76.9) |         |
| Chronicity index                        |     |        |       |         |
| <3                                      | 24  | 14 (58.3) | 10 (41.6) | 0.2890 |
| ≥3                                      | 12  | 4 (33.3)  | 8 (66.7)  |         |

Figure 4. Correlation between plasma uromodulin and kidney function parameters. Correlation between plasma uromodulin and (A) eGFR, (B) serum creatinine, (C) blood urea nitrogen and (D) serum cystatin C. eGFR, estimated glomerular filtration rate.
Individuals with homozygous hereditary deficiency of the classical pathway components, such as C1q, C1r, C1s, C4 and C2, are more susceptible to systemic lupus erythematosus (41). In addition, complement activation promotes the pathogenesis of IgAN via alternative and lectin pathways, and likely occurs systemically on IgA-containing circulating immune complexes, as well as locally in glomeruli (42). A clinical study has reported that the C1q, mannose-binding lectin, Bb, C4d, C3a, C5a and sC5b-9 levels in the plasma of patients with diabetic nephropathy are significantly higher than in the plasma of patients with diabetes without renal disease (43). Mechanically, activation of the lectin and alternative pathways of the three possible complement pathways is associated with renal damage (21). In accordance with the aforementioned findings, the present results indicated that C3a and MAC levels were increased in the plasma of patients with IgAN and LN.

UMOD is considered to be a component of the innate immune system (42,43), and it has been revealed that UMOD binds with immune proteins, such as IgG28, complement factors, such as C1q (34,35), cytokines and TNF (36). In addition, our previous study has reported that UMOD significantly enhances the function of complement H factor cleavage of C3b and inhibits complement activation (44). Collectively, the aforementioned findings suggest that UMOD is closely associated with complement activation. However, the association between UMOD and complement components remains unknown. In the present study, it was identified that UMOD participated in the progression of kidney disease, which was partially dependent on complement activation.

Although the clinical significance of UMOD and complement activation in renal disease has been clarified, there are several limitations to the present study that warrant discussion. Firstly, the number of patients enrolled in the current study was small. Secondly, the underlying mechanism is still unclear. The study confirmed that UMOD was involved in the progression of renal disease, which partly depended on the activation of complement. Whether UMOD promoted the activation of complement by binding to complement factor C1q protein and then promote the progress of IgAN and LN in renal disease, needed further investigated in future studies. In addition, functional studies should be performed to identify the biological mechanisms of UMOD in renal disease.

In conclusion, the present results suggested that low UMOD plasma levels were associated with severe complement activation and may be involved in complement-mediated immune protection by inhibiting complement activation in renal disease.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

XY designed the study and revised the manuscript. FS and ML performed the experiments. FP and LY analyzed the data. FP provided technical support. LY drafted the manuscript. XY and FP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Qi Lu Hospital of Shandong University (Jinan, China; approval no. KYLL-2017-004), and written informed consent was obtained from all patients and healthy controls.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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