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Original Article

Renal expression of trefoil factor 3 mRNA in association with tubulointerstitial fibrosis in IgA nephropathy

KEIKO TANAKA,[1] HITOSHI SUGIYAMA,[2] TOSHIO YAMANARI,[1] KOKI MISE,[1] HIROSHI MORINAGA,[1] MASASHI KITAGAWA,[1] AKIFUMI ONISHI,[2] AYU OGAWA-AKIYAMA,[1] KATSUYUKI TANABE,[1] JUN EGUCHI,[1] YASUKAZU OHMOTO,[3] KENICHI SHIKATA[4] and JUN WADA[1]

Departments of [1]Nephrology, Rheumatology, Endocrinology and Metabolism, and [2]Human Resource Development of Dialysis Therapy for Kidney Disease, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, [4]Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, and [3]Otsuka Pharmaceutical Co., Ltd, Tokushima, Japan

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fibrosis, IGA nephropathy, renal biopsy, TFF3, tubular epithelium.

Correspondence
Professor Hitoshi Sugiyama, Department of Human Resource Development of Dialysis Therapy for Kidney Disease, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan.
Email: hitoshis@okayama-u.ac.jp

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SUMMARY AT A GLANCE

Trefoil factor 3 is a peptide that, generally speaking, has protective functions in epithelial biology. This study reports that TFF3 is increased in IgA nephropathy and correlates with injury. Whether TFF3 is functionally a counter-regulatory, protective factor or whether its overexpression denotes a pathogenic role remains an outstanding question.

ABSTRACT:

Aim: **Trefoil factor 3 (TFF3) is a small peptide that is involved in mucosal protection. TFF3 is widely expressed in multiple tissues including kidney tissue. Previous studies have reported that the levels of urinary TFF3 are significantly increased in patients with chronic kidney disease. The aim of this study is to detect the TFF3 mRNA in kidney and elucidate the relationship between renal TFF3 mRNA and tubulointerstitial fibrosis in IgA nephropathy (IgAN).**

Methods: **We investigated the renal mRNA expression of TFF3 by real-time PCR analysis in biopsy specimens from patients with IgAN, other glomerulonephritis (OGN) and minor glomerular abnormalities (MGA). We also determined the renal localization of TFF3 and the levels of urinary TFF3 by immunostaining and ELISA, respectively.**

Results: **The renal TFF3 mRNA expression was significantly associated with the urinary TFF3 secretion and the tubulointerstitial fibrosis score in the IgAN group alone. Immunostaining of the renal specimen of IgAN patients revealed that TFF3 is located in the renal tubular epithelial cells. The locations were almost the same as those that showed uromodulin positivity; specifically, the thick ascending limb (TAL) of the loop of Henle and the early portion of the distal tubule. The urinary TFF3 levels were positively correlated with the levels of urinary biomarkers of tubulointerstitial injury in such patients.**

Conclusion: **Renal TFF3 mRNA is associated with renal tubulointerstitial fibrosis in IgAN patients. The TFF3 located in the renal tubular epithelial cells may play a role in the progression of tubulointerstitial fibrosis in IgAN patients.**

Trefoil factor 3 (TFF3) is a small peptide that has essential roles in restitution, such as cell migration to heal superficial lesions, and in regeneration, including differentiation and proliferation in the repair of the epithelia.[1–3] It has been suggested that TFF3 is expressed in all mucus-secreting tissues, including the renal tubules.[4] The TFF3 expression increases in various organs as the consequence of tissue damage such as inflammation or fibrosis.[5] With regard to the kidney, it has been shown that serum and urinary TFF3 significantly increase in patients with chronic kidney disease (CKD).[6–8] However, it is unclear whether the renal TFF3 expression is localized in the tubular epithelial cells, and whether it increases in the association with the tubulointerstitial injury in patients with CKD. No previous study has analyzed the mRNA expression in patients with CKD such as glomerulonephritis.

IgA nephropathy (IgAN) is globally the most common chronic glomerulonephritis.[9,10] It has been reported to cause

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end-stage renal disease (ESRD) in 30–40% of the patients within 20 years.[11] The renal prognosis of IgAN is more closely associated with the severity of tubulointerstitial injury and fibrosis than that of glomerular lesions.[12,13] However, the detailed mechanism of the tubulointerstitial injury that develops in association with IgAN has not been elucidated. No established biomarker of the tubulointerstitial damage of IgAN has ever existed.

We hypothesized that renal TFF3 mRNA is expressed in human renal biopsy specimens and that it is associated with the tubulointerstitial injury in IgAN patients. We subjected kidney biopsy specimens of patients with IgAN, other glomerulonephritis (OGN), and minor glomerular abnormalities (MGA) to an mRNA analysis. The results of our study suggested that TFF3 was located in the renal tubular epithelial cells, and that the renal TFF3 mRNA expression increased in correlation with the intensity of tubulointerstitial fibrosis and urinary TFF3 levels, which was significantly associated with the urinary markers of tubulointerstitial injury in IgAN patients. The data suggested that TFF3 plays a role in the progression of the tubulointerstitial injury in patients with IgAN.

METHODS

Patients and data collection

This study included a total of 25 patients (IgA nephropathy (IgAN group), $n = 12$; other glomerulonephritis (OGN group), $n = 10$; and patients with minor glomerular abnormalities as controls (MGA group), $n = 3$) who underwent renal biopsy in our division. A sufficient amount of tissue was obtained to allow for the analysis to detect mRNA TFF3. The causes of glomerulonephritis other than IgAN were the following: lupus nephritis (LN) ($n = 3$), microscopic polyangiitis (MPA) ($n = 5$) and granulomatous angiitis (GPA) (n = 2). We did not include the patients with nephrotic syndrome, such as minimal change nephrotic syndrome, focal segmental glomerulosclerosis or membranous nephropathy in the OGN group, because we needed to compare the findings with those of patients with a similar disease state to IgAN. We used the normal parts of surgical specimens obtained after medically necessary intervention as a result of renal cell carcinoma (RCC) as a control for the mRNA expression and the immunohistochemical analysis. The clinical data were obtained from the patients' medical records. All of the subjects gave their written informed consent. The study was approved by the Ethical Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (KEN 1607–010, 2016).

RNA extraction and the quantitative PCR

The samples for the mRNA analysis consisted of cortex area of approximately 3 mm that was obtained from close to the medulla. Total RNA was isolated from human kidney tissue using NucleoSpin RNA XS (Takara, Japan), according to the manufacturer's instructions. Total RNA (500 ng) from each sample was reverse transcribed using PrimeScript RT Master Mix (Takara, Japan) at 37°C for 15 min, 85°C for 5 s, and 4°C for 5 min. For the quantification of the mRNA levels of TFF3, a real-time quantitative PCR (qPCR) was performed, using Power SYBR Green PCR Master Mix (ThermoFisher Scientific, MA, USA). The PCR amplification was performed using an Applied Biosystems StepOnePlus Real-time PCR System. The sequences of the primer pairs were as follows: TFF3 (Sense 5′ → 3′; CCAAGCAAACAATCCAGAGCA, Antisense 5′ → 3′; GCTCAGGACTCGCTTCATGG, product size 79 bp) and GAPDH (Sense 5′ → 3′; CTGGGCTACACTGAGCACC, Antisense 5′ → 3′; AAGTGGTCGTTGAGGGCAATG, product size 101 bp). The PCR amplification program consisted of 15 s of initial denaturation at 95°C followed by 40 PCR cycles at 95°C for 15 s, and then 60°C for 60 s. The Ct (threshold cycle) values of the target gene amplifications were normalized to those of the GAPDH control. Specificity was verified by a melt curve analysis. The comparative Ct method was used to calculate the relative quantification of gene expression. The normal part of the surgical specimen dissected from the RCC was used as a control of the TFF3 mRNA expression. PCR products were analyzed by 3% agarose gel electrophoresis and stained with ethidium bromide.

The evaluation of the inflammation and fibrosis scores

We evaluated the tubulointerstitial cell infiltration and fibrosis of biopsy specimens with Hematoxylin and Eosin- and Masson's trichrome-stained slides, respectively. The degrees of inflammation and fibrosis were semi-quantitatively graded as described previously,[14] with some modification as follows: mild, <10%; moderate, 10–30%; severe, >30% of injury (inflammation or fibrosis). We statistically compared the following two groups: mild injury and moderate to severe injury.

Immunohistochemistry

Serial 4-μm-thick formalin-fixed, paraffin-embedded sections from renal biopsy specimens were used for immunohistochemistry. Deparaffinized sections were treated with heat-induced antigen retrieval with citric acid buffer solution (pH 6.0) for 20 min at 98°C. Endogenous peroxidase was blocked with 3% hydrogen peroxidase, and nonspecific antibody binding was blocked with 10% goat serum. Rabbit anti-human TFF3 antibody[15] (dilution; 1:100) or Uromodulin (UMOD) antibody (11911–1-AP, Proteintech, Rosemont, IL, USA) (dilution; 1:50) was used as the primary antibody. Rabbit antibody IgG control (ab172730, Abcam, Cambridge, UK) was used at the same concentration as the primary antibody. After incubation with the primary antibody at 4°C overnight, the sections were incubated with biotinylated goat anti-rabbit as a secondary antibody, followed by

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treatment with horseradish peroxidase-conjugated avidin (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). 3,3′-Diaminobenzene was used as a chromogen. The renal tubular staining of TFF3 was semi-quantitatively evaluated as follows: (−), none; (±), trace staining; (+), mild staining; and (++), moderate staining.

ELISA for urine TFF3 level

The urinary TFF3 concentrations were determined by a specific ELISA, as described previously.[15] The urinary levels of creatinine, albumin, α1-microglobulin (α1MG), β2-microglobulin (β2MG), and N-acetyl-β-D-glucosaminidase (NAG) were

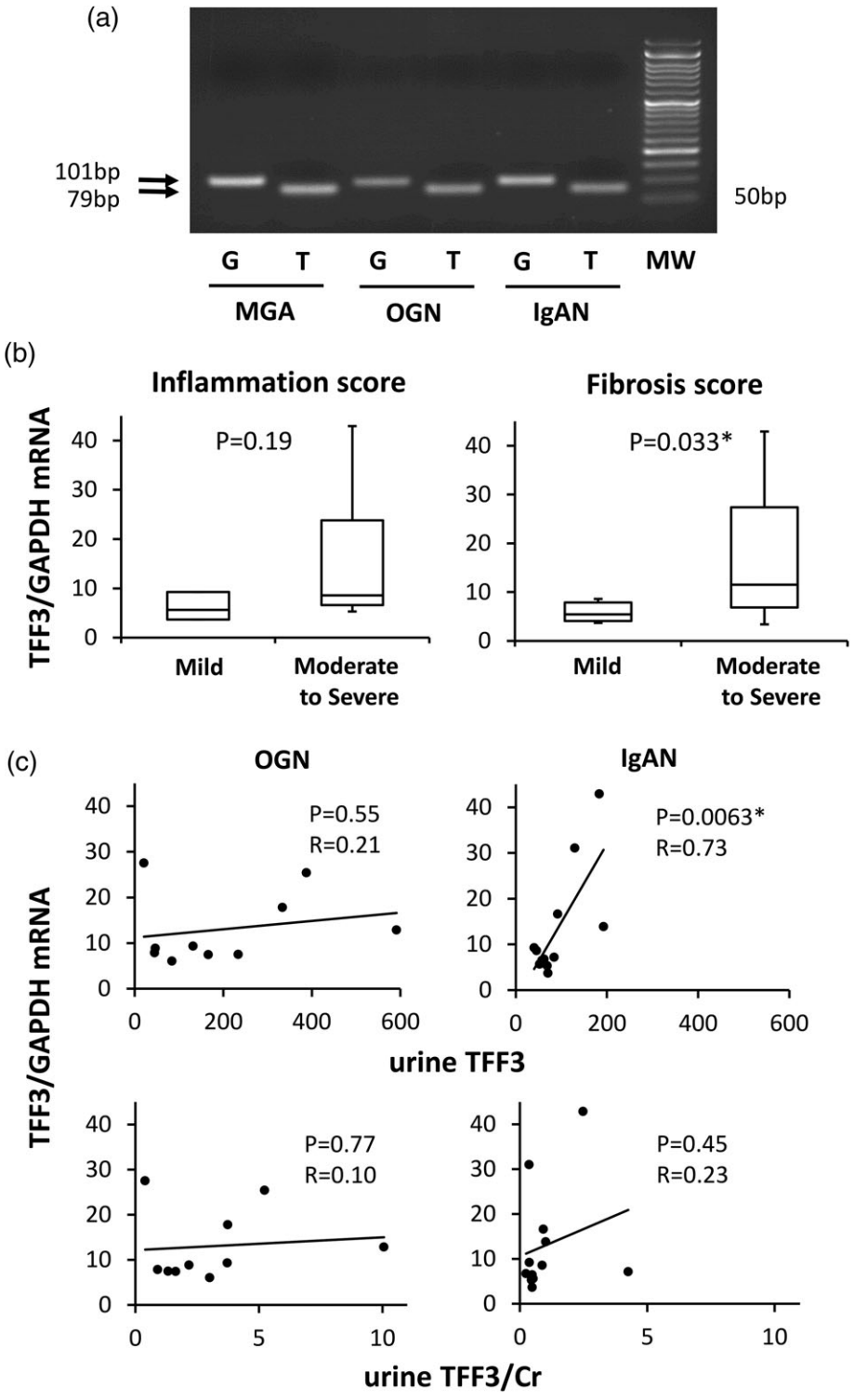

Fig. 1 The agarose gel electrophoresis of PCR products from biopsy specimens (a), the renal trefoil factor 3 (TFF3) mRNA levels (as determined by a quantitative PCR) according to the degree of tubulointerstitial inflammation and fibrosis in IgAN patients (P = 0.19; P = 0.033*) (b) and the relationship between the renal TFF3 mRNA level and the urinary TFF3 level (P = 0.55, R = 0.21; P = 0.0063*, R = 0.73; P = 0.77; R = 0.10; P = 0.45; R = 0.23) (c).(a) PCR products from biopsy specimens in patients with minor glomerular abnormalities (MGA), other glomerulonephritis (OGN) and IgA nephropathy (IgAN), and 50 bp molecular weight (MW) markers are shown. The PCR products of TFF3 cDNA (T) are 79 bp, and those of GAPDH cDNA (G) are 101 bp, both of which were of the expected size. (b) The renal TFF3 mRNA levels in patients with mild degree (n = 3) and moderate to severe degrees (n = 9) of inflammation score (left panel) and with mild degree (n = 4) and moderate to severe degrees (n = 8) of fibrosis score (right panel) in patients with IgAN (n = 12). The renal TFF3 mRNA level significantly increased in patients with moderate to severe of fibrosis. (c) The renal TFF3 mRNA level was significantly correlated with the urinary TFF3 concentration in IgAN patients (top right panel).

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Table 1 The background characteristics of the subjects

	MGA group (n = 3)	OGN group (n = 10)	IgAN group (n = 12)
The number of glomeruli	30.3 ± 19.5	19.0 ± 8.48	13.5 ± 6.45
Age (years)	26.3 ± 8.6[‡]*	61.4 ± 12.9[†]*	48.2 ± 18.4
Male gender, *n* (%)	2 (66.6%)	3 (30.0%)	3 (25.0%)
eGFR (mL/min/1.73 $m^{2)}$	91.4 ± 36.0	56.6 ± 27.8	64.0 ± 27.2
Urinary total protein (g/gCr)	0.049 (0.016–0.25)[§]*	0.98 (0.17–1.88)	0.85 (0.60–1.61)[†]*
Urinary albumin (mg/gCr)	2.17 (0.47–18.3)[§]*	53.5 (5.76–141.0)	58.8 (41.3–120.8)[†]*
Urinary α1MG (mg/gCr)	0.57 (0.48–0.99)[‡,§]*	11.7 (7.42–26.3)[†,§]*	3.95 (2.20–13.0)[†,‡]*
Urinary β2MG (μg/gCr)	56.6 (52.4–87.6)[‡]*	995 (223–4801)[†,§]*	107 (28.5–232)[‡]*
Urinary NAG (U/gCr)	1.64 (0.74–3.96)[§]*	6.44 (3.39–14.4)	6.76 (3.99–14.3)[†]*
Serum TFF3 (ng/mL)	12.5 (5.21–15.5)	12.1 (10.1–21.8)	9.76 (6.96–17.1)
Urinary TFF3 (ng/mL)	56 (6.5–222)	149 (46.1–347)	69.6 (53.4–120)
Urinary TFF3 (μg/gCr)	46.6 (7.80–99.1)	259 (122–411)[§]*	50.0 (39.3—9.2)[‡]*

*P < 0.05. [†]*versus* MGA. [‡]*versus* OGN. [§]*versus* IgAN. α1MG, alpha 1 microglobulin; β2MG, beta 2 microglobulin; eGFR, estimated glomerular filtration rate; IgAN, immunoglobulin A nephropathy; MGA, minor glomerular abnormalities; NAG, N-acetyl-β-D-glucosaminidase; OGN, glomerulonephritis; TFF3, trefoil factor 3.

determined using routine laboratory methods (SRL, Inc., Okayama, Japan).

Statistical analysis

The results are expressed as the mean ± SD or the median and interquartile range (IQR) for continuous data, and as integers, frequencies and percentages for categorical data. *P* values of <0.05 were considered to indicate statistical significance. Differences between groups were analyzed using Student's *t*-test and the Mann–Whitney U-test for continuous data, and the Fisher's exact test for categorical data. The statistical analyses were performed using the JMP software program (version 11, SAS Institute Inc.; Cary, NC, USA).

RESULTS

The background characteristics of the patients in the 3 groups are shown in Table 1. The urinary markers of tubular injury, such as α1MG, β2MG, and NAG were higher in the OGN group than in the other groups. The median serum and urinary TFF3 levels were also higher in the OGN group than in the other groups.

The results of agarose gel electrophoresis of PCR products are shown (Fig. 1a). The expected sizes of the PCR products of TFF3 and GAPDH were 97 and 101 bp, respectively, and were detectable in all groups. The mean TFF3 mRNA levels were 10.5 ± 3.96 (MGA group), 13.0 ± 7.85 (OGN group) and 13.1 ± 11.9 (IgAN group). In the IgAN group, the TFF3 mRNA expression level (as determined by the qPCR) was significantly associated with the interstitial fibrosis score, rather than the inflammation score (Fig. 1b). In the OGN group, the TFF3 mRNA expression level was neither associated with the inflammation score nor the interstitial fibrosis score. The TFF3 mRNA expression level was significantly correlated with the urinary TFF3 level in the IgAN group (Fig. 1c). In the MGA and OGN group, the relationship between the TFF3 mRNA expression and the urinary level of TFF3 was not significant.

Immunohistochemical staining revealed that the tubular epithelial cells positive for TFF3 in renal tissue were from the normal portion of the kidney of a patient with RCC, and from patients with MGA, OGN and IgAN (Fig. 2). No clearly positive reaction for TFF3 was observed in the renal glomeruli (Fig. S1, Supporting information). We evaluated the semi-quantitative intensity and localization of immunostaining for TFF3 antibody in the cortex and medulla of renal biopsy tissues (Table S1). The intensities of staining of the RCC and MGA specimens were only at trace levels, while those of the OGN and IgAN specimens a mild to moderate intensity. More tubules showed positive staining for TFF3 antibody in the medulla than in the cortex. On the serial sections that were subjected to immunostaining with TFF3 and UMOD antibodies, the tubular lumens that were positive for TFF3 were found to overlap with those that were positive for UMOD (Fig. 2).

The urinary TFF3 levels were significantly associated with higher urinary markers of tubular injury, including urinary α1MG and β2MG in both the OGN and IgAN group (Fig. 3). The same relationship between the urinary TFF3 levels and urinary markers of tubular injury was not found in the MGA group.

DISCUSSION

In the present study, we demonstrated, for the first time, that TFF3 mRNA was expressed in renal biopsy specimens from human patients with kidney disease, and showed that the significant TFF3 level was positively correlated with the degree of tubulointerstitial fibrosis of renal tissue in patients with IgAN. Immunohistochemical staining indicated that TFF3 protein was localized in the renal tubular epithelial cells of such patients. In patients with IgAN, the urinary TFF3 levels were significantly associated with the urinary markers of tubulointerstitial injury. Taken together, we

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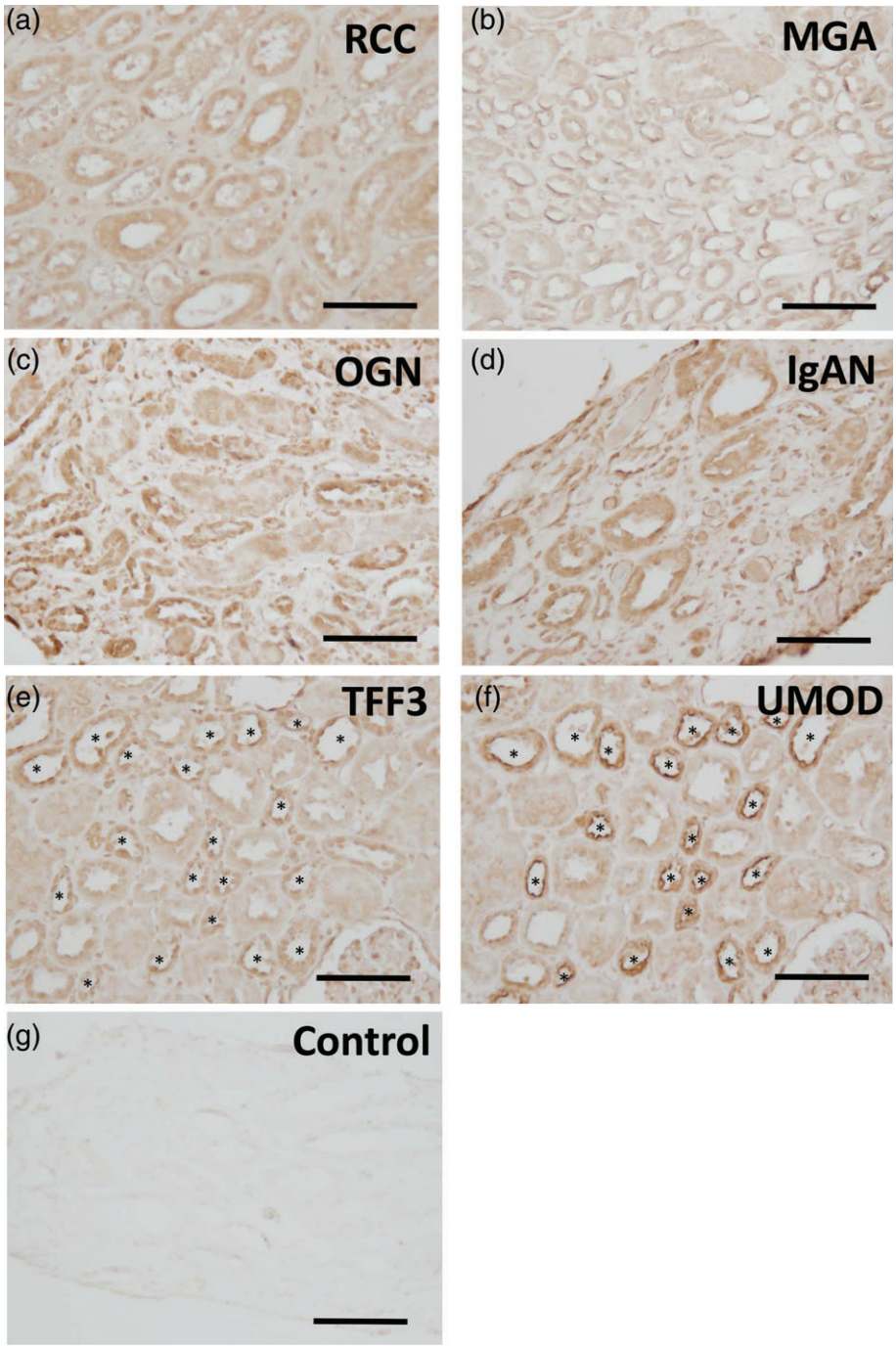

Fig. 2 Immunohistochemical staining of TFF3 antibodies in renal tissue specimens (tubules) (a–d), the serial sections of immunostaining with TFF3 and uromodulin (UMOD) antibody (IgAN) (e,f) and the non-immune rabbit IgG control (IgAN) (g).Representative micrographs of the normal part of the surgical specimen from a patient with renal cell carcinoma (RCC) (61-year-old, male) (a), and renal biopsy specimens from patients with minor glomerular abnormalities (MGA) (34 year-old, male) (b) other glomerulonephritis (OGN) (microscopic polyangiitis, 69year-old, female) (c), and IgA nephropathy (IgAN) (43-year-old, female) (d) are shown. Serial sections of a renal biopsy specimen from a patient with IgAN were subjected to immunostaining with TFF3 (e) and UMOD (f) antibodies. The same sections of renal tubules (asterisks) were positive for both TFF3 and UMOD. The rabbit antibody IgG control (staining for a patient with IgAN) is shown (g). Scale bars indicate 100 μm.

hypothesized that TFF3 was expressed in the tubular epithelial cells, and that the TFF3 expression and secretion into the urine increased along with the progression of the tubulointerstitial fibrosis in IgAN patients.

IgAN is globally the most common glomerulonephritis. Previous long-term observational studies have shown that 30–40% of these patients progress to ESRD within 20 years.[11] Despite the better understanding of the pathogenic mechanisms of glomerular injury, such as being triggered by the aberrant glycosylation of IgA1,[16,17] the pathogenic mechanism of the tubulointerstitial injury of IgAN has not been fully elucidated. At present, the extent of tubulointerstitial injury can be clinically assessed by a renal biopsy. In this study, we showed the significance of TFF3 in IgAN patients, which could be used as a valuable biomarker of tubulointerstitial injury in patients with IgAN.

The precise distribution of TFF3 in human kidney tissue remains unknown. We have shown that TFF3 immunostaining was positive in renal epithelial cells, which is in agreement with other previous reports.[7] No former reports

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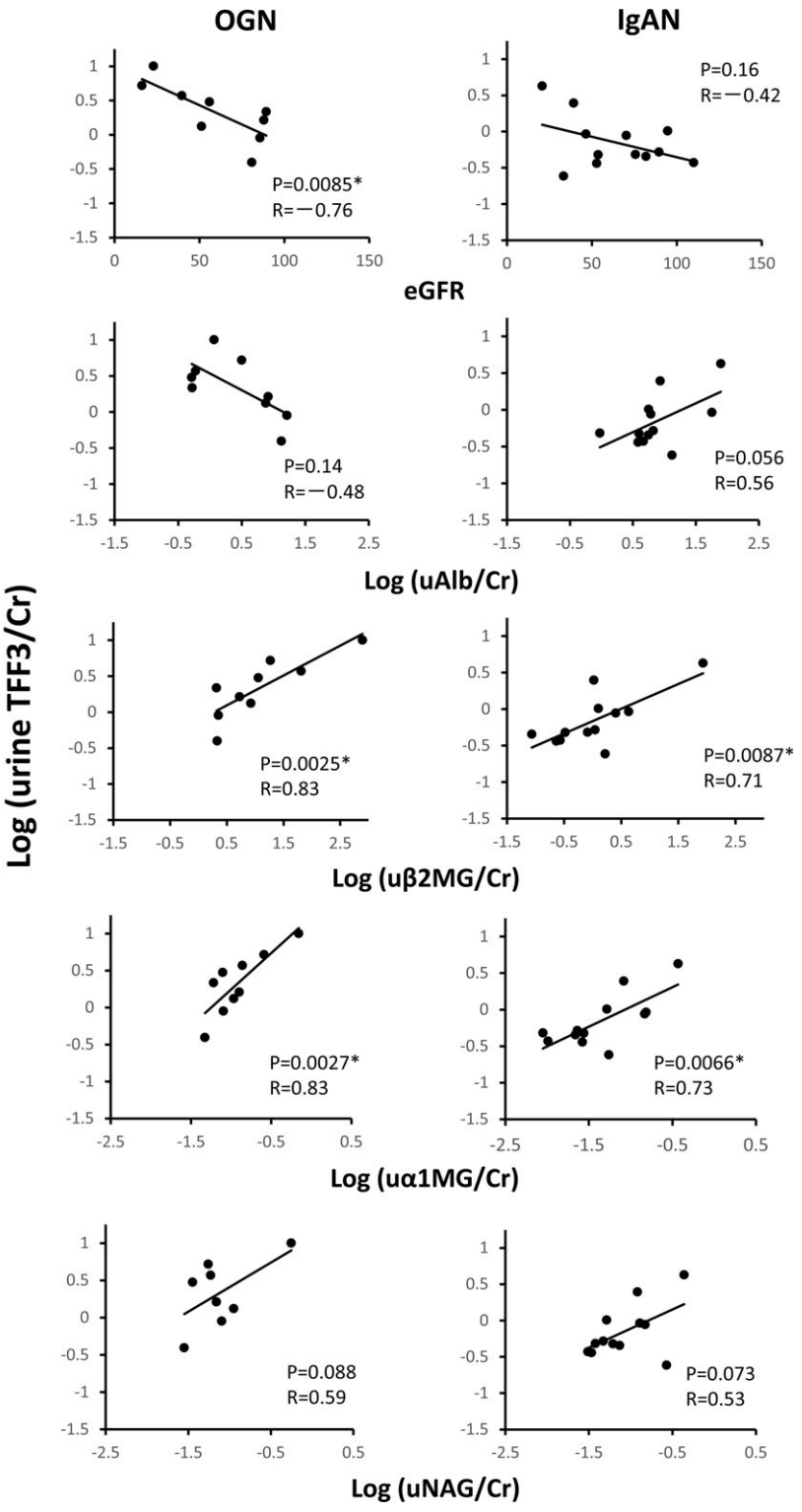

Fig. 3 The relationship between the urinary TFF3 concentration and the eGFR, albuminuria and other urinary markers of tubular injury, including beta 2-microglobulin (β2MG), alpha 1-microglobulin (α1MG), N-acetyl-β-d-glucosaminidase (NAG) in patients with OGN (left panels) and IgAN (right panels) (P = 0.085*, R = −0.76; P = 0.16, R = −0.42; P = 0.14, R = −0.48; P = 0.056, R = 0.56; P = 0.0025*, R = 0.83; P = 0.0087*, R = 0.71; P = 0.0027*, R = 0.83; P = 0.0066*; R = 0.73). The urinary TFF3 concentration was significantly correlated with urinary markers of tubular injury such as β2MG and α1MG in patients with OGN and IgAN.

have examined the TFF3 mRNA expression in the renal tissue of kidney disease patients. In a rodent model of acute kidney injury (AKI), the kidney tubules of the outer stripe of the outer medulla (abundant with straight proximal tubules) were a major site for the expression of TFF3 mRNA.[4] As for the normal surgical specimens that were dissected from patients with RCC, the TFF3 expression was detectable in all portions of the urinary tract with peaks in the renal medulla and the urethra.[18] Immunostaining of serial sections with TFF3 and UMOD antibodies revealed that the renal TFF3 protein was located in almost the same area as UMOD, which is specifically expressed in the thick

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ascending limb (TAL) of the loop of Henle and the early portion of the distal tubule.[19,20] Our mRNA expression analysis demonstrated that TFF3 was synthesized in the renal tissue and that the level was associated with the urinary TFF3 concentration, which was also associated with the urinary markers of interstitial injury. The biopsy specimens used for the mRNA analysis were mainly obtained from the cortex, in close proximity to the medulla, including the outer stripe of the outer medulla. The increased expression of TFF3 may be a reaction of the tubular epithelial cells to stimulation from the damage that occurs in the course of the progression of interstitial fibrosis.

Some previous reports have shown that TFF3 may be a biomarker associated with the kidney function of CKD patients.[7,8,21] The urinary TFF3 excretion was low in a rodent model of AKI, where the renal epithelial cells were observed to have decreased.[4] Urinary TFF3 excretion is also low in the early stages of CKD, and constantly increase during the progression of CKD to end-stage renal failure.[22] These factors imply that the continuous and certain stimulation of renal epithelial cells is necessary for the increase of TFF3 expression.

The molecular mechanisms of TFF3 action are still unclear. Although no definite receptor or binding molecule has been identified, it has been hypothesized that TFF3 may act in a receptor-mediated manner.[23] The HK-2 cultured human cell line (proximal tubular epithelial cells) can synthesize and excrete more TFF3 after exposure to damage such as nutrient starvation, hypoxia and immunoglobulin λ light chain.[24,25] Hypoxia induces the transcription of HIF-1 and TFF3 mRNA *in vitro,*[26] and it is known that TFF3 is regulated by HIF-1α.[25] The relationship between TFF3 and the molecular mechanisms of fibrosis has not been studied thus far. The expression and release of TFF3 may be a response to chronic hypoxia due to ischemia, which is related to tubulointerstitial fibrosis in the final common pathway to end-stage renal disease.[27]

With regard to inflammatory bowel disease, previous *in vitro* analyses revealed that TNF-α negatively regulates TFF3 gene transcription via NF-κB, resulting in delayed cytoprotection, restitution and mucosal renewal, which are the same with IL-6, IL-1β (pro-inflammatory cytokines).[28] On the other hand, IL-4 and IL-13 (anti-inflammatory cytokines) have been shown to potently increase TFF3 gene transcription in colon cancer cell lines, in a STAT6-dependent manner.[29] In the study of model mice, the intraperitoneal application of recombinant TFF3 promoted a protective effect against colitis and was accompanied by a reduction in the expression of TNF-α in the colonic endothelium.[30,31] Clinical studies have revealed that TFF3 is a promising peptide for the treatment of inflammatory bowel disease.[32] Few studies have reported the antifibrotic effects of TFF; however, in the setting of chronic allergic airway disease, it was shown that the intranasal delivery of human recombinant TFF2 could reduce epithelial thickening, subepithelial collagen deposition and bronchial epithelium apoptosis by inhibiting the actions of TGF-β1 and PDGF.[33] TFF3 may play a role in the protection and repair of the renal tubular epithelial cells in patients with kidney disease, similarly to its role in the gastrointestinal tract and respiratory system.

This is the first report to describe the detection of TFF3 mRNA in the tissue of human kidney disease patients. However, the present study is associated with some limitations. First, the OGN group included various causes of glomerulopathy with tubulointerstitial injury. Second, TFF3 might not be a specific biomarker for the tubulointerstitial injury of IgAN; however, the results of our study, at least in part, suggest an association between the renal TFF3 mRNA expression and tubulointerstitial fibrosis in patients with IgAN in the current setting. Third, we could not show the distribution of the TFF3 mRNA expression in the tissue by techniques such as *in situ* hybridization.

In conclusion, the increased expression of TFF3 mRNA in the renal tissue is associated with an increase in the urinary TFF3 level; thus, it may reflect the tubulointerstitial fibrosis in IgAN patients. Further studies are required to elucidate the involvement of the renal TFF3 expression in the progression of tubulointerstitial fibrosis in IgAN patients.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website:

Fig. S1 Immunohistochemical staining of TFF3 antibodies in renal tissue specimens (glomeruli). Representative micrographs of the normal part of the surgical specimen from a patient with renal cell carcinoma (RCC) (61-year-old, male) (a), and renal biopsy specimens from patients with minor glomerular abnormalities (MGA) (34-year-old, male) (b) other glomerulonephritis (OGN) (microscopic polyangiitis, 69-year-old, female) (c), and IgA nephropathy (IgAN) (43-year-old, female) (d) are shown. Scale bars indicate 100 μm.

Table S1. (a) Semi-quantitative analysis of the renal tubular expression and localization of TFF3 by immunohistochemistry.

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