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Original Article

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Kidney Expression of Trefoil Factor 3 Is Not Associated with Kidney Survival in Immunoglobulin A Nephropathy: A Preliminary Study

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ABSTRACT

Background: Trefoil factor 3 (TFF-3) is a peptide that restores the epithelium as protection against injury. Recently, TFF-3 was shown to be expressed in kidney tubular cells and was associated with fibrosis. We aimed to determine whether TFF-3 is associated with kidney outcomes.

Methods: We evaluated TFF-3 expression and its relationship with mesangial hypercellularity, endocapillary hypercellularity, segmental glomerulosclerosis, tubular atrophy/interstitial fibrosis and crescents (MEST-C) score, tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β) expression in the kidney. We included 28 immunoglobulin A (IgA) patients with initial estimated glomerular filtration rate (eGFR) ≥ 30 mL/min/1.73m^2 and proteinuria ≥0.5 g/day. Kidney biopsies were scored using MEST-C and were stained for TFF-3, IL10, TGF-β, and TNF-α.

Results: Mean patient age was 37.0 ± 13.8 years and eGFR was 74.6 ± 41.2 mL/min/1.73 m^2. Patients were followed for 9.1 ± 3.6 years. Trefoil factor 3 was positive in 67.9% of patients exclusively in tubular cells. Tumor growth factor-β was also observed only in tubular cells in 71.4% of patients and was higher in TFF-3-positive patients (89.5% vs. 22.2%, $P < .05$). When patients with and without TFF-3 staining were compared, there was no difference in age, eGFR, albumin, or proteinuria. Yearly eGFR change was also similar (−0.5 ± 2.3 vs. −1.3 ± 2.4 mL/min/1.73 m^2, $P = .44$). Trefoil factor 3 was not associated with kidney survival. The MEST-C score was not correlated with TFF-3. Trefoil factor 3 staining showed correlation with TGF-β but was not associated with kidney survival in IgA patients.

Conclusion: Findings of the study imply TFF-3 is not a marker of permanent damage but might simply be a marker of the repair of ongoing tubular injury.

Keywords: Glomerulonephritis, IgA nephropathy, kidney biopsy, MEST-C score, trefoil factor 3

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INTRODUCTION

Trefoil factor 3 (TFF-3) is a peptide secreted by epithelial cells and plays an essential role in restitution and regeneration of mucosal surfaces, restoring the epithelium as protection against injury.[1] Recent evidence suggests that TFF-3 is also expressed in the kidneys, mainly in tubular epithelial cells.[2]The TFF-3 may play a key role in the repair of kidney damage and thus be used as a marker of tubulointerstitial damage. Several studies have already shown increased levels of TFF-3 in patients with drug-induced nephrotoxicity as well as chronic kidney disease (CKD).[3-5] However, there are scarce data regarding the expression of TFF-3 in glomerulonephritis where tubulointerstitial fibrosis is associated with long-term kidney outcomes.

Immunoglobulin A (IgA) nephropathy can cause CKD in about 20% of patients progressing to end-stage kidney

disease (ESKD) within 10 years. Mesangial IgA deposition is the most predominant finding on kidney biopsy. Pathological findings are classified using the MEST-C score, which include mesangial hypercellularity, endocapillary hypercellularity, segmental glomerulosclerosis, tubular atrophy/interstitial fibrosis, and crescents.[6] In IgA nephropathy, the MEST-C score is a reliable tool as a long-term prognostic factor and has been validated in large populations.[7,8]

Recently, a small study demonstrated TFF-3 expression in tubular epithelial cells in 12 IgA nephropathy patients, which was further associated with tubulointerstitial fibrosis.[9] The aim of the present study was to determine whether TFF-3 is associated with kidney outcomes such as the doubling of serum creatinine or ESKD in a larger cohort of IgA patients and evaluate the relationship between TFF-3 and the MEST-C score. We also aimed to determine whether there was any relationship between TFF-3 and tumor necrosis factor alpha (TNF-α), transforming growth factor-beta (TGF-β), and interleukin-10 (IL-10) expression in the kidney biopsy specimens.

MATERIAL AND METHODS

Study Populations

The study protocol was approved by the institutional review board of Marmara University Hospital (Date: July 22, 2022, protocol number: 09.2022.967) and carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent. In this retrospective study we included patients with biopsy-proven primary IgA nephropathy with at least 8 glomeruli and initial estimated glomerular filtration rate (eGFR) ≥ 30 mL/min per 1.73 m^2 and proteinuria ≥0.5 g/day at the time of biopsy. Patients with secondary IgA nephropathy, eGFR < 30 mL/min per 1.73 m2, proteinuria < 0.5 g/day, diabetes, malignancy, active infection, and less than 8 glomeruli on biopsy were excluded. Overall, 28 patients were included in the final analysis. Demographic, laboratory, and clinical data were recorded from patient charts. The GFR was estimated using CKD-EPI

MAIN POINTS

- Trefoil factor 3 (TFF-3) is a small peptide that restores the epithelium as protection against injury.
- Trefoil factor 3 may play a key role in the repair of kidney damage and thus be used as a marker of tubulointerstitial damage.
- In this study, we showed that about 68% of patients with IgA nephropathy stained positive for TFF-3 in the tubular cells, and TFF-3 staining showed a correlation with TGF-β expression. However, TFF-3 was not associated with kidney survival over a period of about 9 years.
- The findings of the study imply that TFF-3 is not a marker of permanent damage but might simply be a marker of ongoing injury.

Pathology

Kidney biopsies were scored using the MEST-C scoring system by a single pathologist who was blinded to the study. Details of histological classification have previously been described.[6] In brief, the MEST-C score is defined as follows: M0/M1 as a mesangial score ≤ or >0.5, E0/E1 as the absence or presence of endocapillary hypercellularity, S0/S1 as the absence or presence of segmental sclerosis, and T0/T1/T2 as the degree of tubular atrophy or interstitial fibrosis <25%, 25-50%, and >50%, respectively.

Serial 3-µm-thick formalin-fixed, paraffin-embedded sections from kidney biopsies were used for immunohistochemistry. Deparaffinized sections were treated with heat-induced antigen retrieval with citric acid buffer solution (pH 6.0) for 20 minutes at 98°C. Endogenous peroxidase was blocked with 3% hydrogen peroxidase for 20 minutes in order to reduce background staining. Rabbit antihuman TFF-3 antibody (Abcam, ab101099, UK) was used as the primary antibody. After incubation with primary antibody at 4°C overnight, sections were incubated with goat anti-rabbit (EXPOSE rabbit specific HRP/DAB detection IHC kit, Abcam, ab80437, UK) as secondary antibody, followed by treatment with diaminobenzidine (DAB) chromogen. After applying counter stain and dehydration, the samples were assessed under light microscopy (BX51 Olympos, Japan). The kidney biopsy specimens were further stained for IL10 (Abcam, ab34843, UK), TGF-β (Abcam, ab66043, UK), and TNF-α (Abcam, ab6671, UK) as per manufacturer's instructions. Immunostaining was scored using a scale of 0 to 3, where 0 means no staining and 3 means maximum staining.

Kidney Outcomes

The kidney outcomes were defined as the doubling of serum creatinine and/or reaching ESKD. The ESKD was defined as initiation of chronic dialysis or kidney transplantation.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences Statistics (SPSS) for Windows, version 20.0 software (IBM SPSS Corp.; Armonk, NY, USA). All variables that are distributed normally are presented as mean ± SD and those with non-normal distribution as median and range. The Student *t*-test was used to compare means between groups, and the chi-square test was used to compare proportions. For correlations between variables, the Pearson test was used for normally distributed data and the Spearman test for those with nonparametric distributions. We considered $P < .05$ as statistically significant.

RESULTS

Sixteen female (57.1%) and 12 male (42.9%) patients with primary IgA nephropathy were included. The mean patient age was 37.0 ± 13.8 years and eGFR was 74.6 ± 41.2 mL/min per 1.73 m^2. Patients were mostly in stage 1 (39.3%) and 2 (25.0%)

Table 1. Demographic and Laboratory Data in 28 Patients with Immunoglobulin A Nephropathy at Baseline

Parameters	
Age (years)	37.0 ± 13.8
Gender (female), n (%)	16 (57.1)
BMI (kg/m^2)	23.6 ± 2.6
Smoker, n (%)	8 (28.6)
Hypertension, n (%)	13 (46.6)
Microscopic hematuria, n (%)	23 (82.1)
Macroscopic hematuria, n (%)	11 (39.3)
Creatinine (mg/dL)	1.6 ± 1.5
eGFR (mL/min/1.73 m^2)	74.6 ± 41.2
Total protein (g/dL)	6.7 ± 0.6
Albumin (g/dL)	4.0 ± 0.6
Uric acid (mg/dL)	6.0 ± 2.2
Proteinuria (g/day)	2.4 ± 1.9

Data presented as mean ± SD or number (percentage).
BMI, body mass index; eGFR, estimated glomerular filtration rate.

CKD, whereas 14.3%, 17.9%, and 3.6% were in stages 3, 4, and 5, respectively. The mean proteinuria at the time of kidney biopsy was 2.4 ± 1.9 g/day; 21.4% of patients had mild 0.3-1 g/day, 50% had moderate 1-3.5 g/day, and 33.3% had nephrotic range proteinuria. Thirteen (46.6%) patients were hypertensive. Demographic and laboratory data at baseline are shown in Table 1.

Patients were followed for 9.1 ± 3.6 (2-14) years. Twenty-six (92.8%) patients were on renin–angiotensin system blockers. During follow-up, 82% of patients received glucocorticoids, and 25% received fish oil. The yearly change in eGFR was −0.8 ± 2.3 (from −5.8 to 4.5) mL/min per 1.73m^2. The ESKD developed in 5 (17.9%) patients, and the combined endpoints of doubling of creatinine or ESKD occurred in 7 (25.0%) patients (Table 2).

Table 2. Clinical Data of the Patients during Follow-Up (n = 28)

Parameters	
Duration of follow-up (years)	9.1 ± 3.6
RASB use, n (%)	6 (92.8)
Glucocorticoids use, n (%)	23 (82)
Fish oil use, n (%)	7 (25)
eGFR change per year (mL/min/1.73 m^2)	−0.8 ± 2.3
ESKD, n (%)	5 (17.9)
Doubling of creatinine/ESKD, n (%)	7 (25.0)

Data presented as mean ± SD or number (percentage
eGFR, estimated glomerular filtration rate; ESKD, end-stage kidney disease; RASB, renin–angiotensin system blockers.

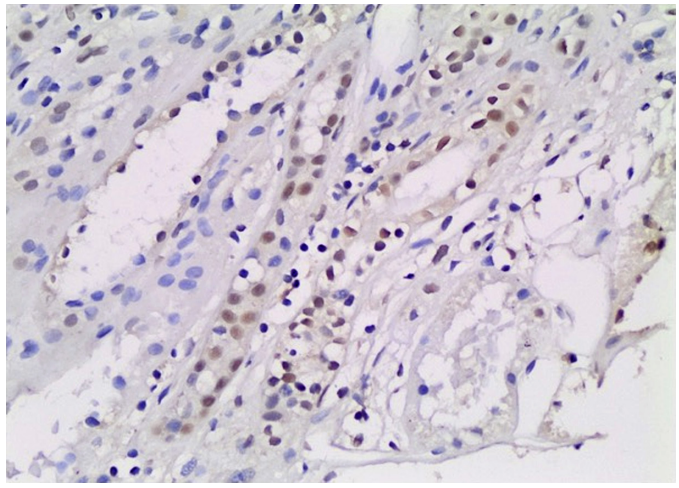

Figure 1. Trefoil factor 3 staining in the kidney tubular cells in a patient with IgA nephropathy. The figure shows nuclear TFF-3 expression in tubular cells (representing as brown color).

The kidney biopsy specimens were evaluated using the MEST-C scoring. Twenty-five percent of kidney biopsies showed diffuse mesangial hypercellularity (M1), and 32.1% showed segmental glomerulosclerosis (S1). All biopsies had less than 25% tubular atrophy/interstitial fibrosis (T0). None showed endocapillary hypercellularity. Crescent formation was seen in only a single patient (3.6%). Immunostaining for TFF-3 was positive in 19 (67.9%) patients where staining was exclusively present in the tubular epithelial cells (shown in Figures 1 and 2). Localization of staining within the tubular epithelial cells was further characterized. Fifteen (78.9%), 3 (15.8%), and 1 (0.05%) showed staining in the cytoplasm, nucleus, and both, respectively. There was no reaction for TFF-3 in the glomeruli or the interstitial space in any of the patients. Intensity of TFF-3 staining was 1+ in 16 (57.1%) of the patients, and 2+ in 3 (10.7%) of the patients. Similar to TFF-3, TGF-β was also observed only in the tubular space in 20 (71.4%) patients. Twenty-five (89.3%) patients were

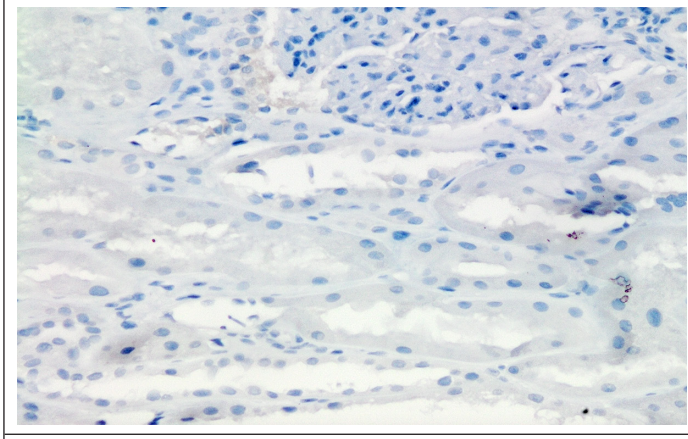

Figure 2. Negative staining for trefoil factor 3 in the kidney tubular cells in a patient with immunoglobulin A nephropathy.

Table 3. Comparisons of the Patients According to Trefoil Factor 3 Staining in Kidney Tissue Spacements

Parameters	TFF-3 (+) (n = 19)	TFF-3 (−) (n = 9)	*P*
Age (years)	35.8 ± 13.7	39.6 ± 14.5	>.05
Baseline eGFR (mL/min/1.73 m^2)	74.2 ± 44.8	75.6 ± 34.9	>.05
Albumin (g/dL)	3.9 ± 0.6	4.1 ± 0.7	>.05
Proteinuria (g/day)	2.2 ± 1.7	3.1 ± 2.4	>.05
TGF-β positive staining, n (%)	17 (89.5)	4 (22.2)	**<.05**
IL-10 positive staining, n (%)	18 (94.7)	8 (88.9)	>.05
TNF-α positive staining, n (%)	4 (21.0)	3 (33.3)	>.05
eGFR change per year (mL/min/1.73 m^2)	−0.5 ± 2.3	−1.3 ± 2.4	>.05
Doubling of creatinine/ESKD, n (%)	5 (26.3)	2 (22.2)	>.05

Data presented as mean ± SD or number (percentage).
ESKD, end stage kidney disease; eGFR, estimated glomerular filtration rate; IL-10, interleukin-10; TFF-3, trefoil factor 3; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor alpha.
The bold values are indicate statistical significance of *P* value

positive for IL-10 staining in both the tubular and the glomerular areas. Tumor necrosis factor-α staining was scarce with only being positive in 7 (25%) patients.

We compared patients with and without TFF-3 staining (Table 3). There was no difference between the groups in terms of age, baseline eGFR, albumin or proteinuria. The yearly eGFR change between the groups during follow-up was also similar (−0.5 ± 2.3 vs. −1.3 ± 2.4 mL/min per 1.73 m^2, $P = .44$). The TGF-β staining was significantly higher in patients positive for TFF-3 (89.5% vs. 22.2%, $P < .05$); however, there was no difference in IL-10 or TNF-α staining. The MEST-C score was not correlated with TFF-3. Combined endpoint of a doubling of creatinine or ESKD was similar in both groups (2 patients with TFF-3 negative vs. 5 patients with TFF-3 positive, $P > .05$) (Table 3).

DISCUSSION

In this study we showed that about 68% of patients with IgA nephropathy stained positive for TFF-3 in the tubular cells, and TFF-3 staining showed a correlation with TGF-β expression. However, TFF-3 was not associated with kidney survival over a period of about 9 years. It also plays a crucial role in the defense and repair of epithelial barriers, restoring the epithelium as protection against injury.[1] It is the predominant trefoil factor family peptide that is synthesized in the urinary tract; however, its exact function in the kidney is unknown.[2]

Animal studies have shown that while TFF-3 immunostaining is visible in the tubular system of developing kidneys, predominantly in the cortical tubules, the glomeruli are negative for TFF-3 throughout all the developmental stages.[10] Du et al[11] have shown the expression of TFF-3 in tubular epithelial cells in 23 patients with CKD, while there was no staining in the glomeruli, peritubular capillaries, or interstitium. Recently, urinary TFF-3 has been evaluated as a marker of nephrotoxicity in several studies. Interestingly, urinary TFF-3 levels decrease in animals after drug-induced tubular toxicity, whereas they increase in humans.[12,13] The reasons for this discrepancy are not clear. In view of this evidence, TFF-3 has been accepted as an indicator of acute drug-induced kidney toxicity by the US Food and Drug Administration and the European Medicines Evaluation Agency. Levels of TFF-3 also increase in CKD.[11] Astor et al[14] followed 143 patients over a period of about 8 years and showed that higher TFF-3 was strongly associated with incident CKD. However, there are scarce data regarding TFF-3 expression in glomerulonephritis where tubulointerstitial fibrosis is associated with long-term kidney outcomes.

Pathologically, the MEST-C score is a prognostic factor and has been validated in large populations.[6-8] Recently a small study involving 12 IgA nephropathy patients showed for the first time that TFF-3 mRNA was expressed in kidney biopsies and was further correlated with tubulointerstitial fibrosis but not inflammation.[9] The staining was observed only in tubular epithelial cells, while there was no reaction in the glomeruli. Urinary TFF-3 was also associated with urinary markers of tubular injury such as α1-microglobulin and β2-microglobulin. In our study, we investigated whether TFF-3 is associated with kidney survival and the MEST-C score. Ideally, such a biomarker would be invaluable in guiding treatment and reassuring patients about their long-term kidney prognosis. We included 28 IgA nephropathy patients and found that TFF-3 staining was exclusively seen only in the tubular epithelial cells, which is in agreement with the previous literature. None of the glomeruli was positive for TFF-3, and TFF-3 staining was not related to baseline proteinuria. There was no correlation between TFF-3 and MEST-C scoring or the tubulointerstitial fibrosis. This is in contrast to the study by Tanaka et al;[9] however, there may be several explanations for this. It is possible that TFF-3 is simply a marker of repair and not irreversible damage. Relationship between TFF-3 and fibrosis pathophysiology is not known. Trefoil factor-3 might be expressed as a response to hypoxia since it has been shown that hypoxia induces TFF-3 transcription in vitro.[15] It may also be involved in the repair of ongoing injury similar to that observed in the gastrointestinal tract. A recent study showed that exogenous trefoil factor 2 dramatically reversed epithelial thickening and subepithelial fibrosis in allergic airway disease.[16]

In our study, we found that the yearly eGFR change in patients staining positive or negative for TFF-3 was similar in both groups, and TFF-3 staining was not associated with kidney survival over 9 years. Similar to our findings, Ascher et al[17] investigated urinary biomarkers in 198 patients with HIV after tenofovir initiation and found that even though there was a significant increase in TFF-3 levels, it was not related to changes in eGFR. In another study by O'Seaghdha et al,[4] including more than 2000 patients, TFF-3 was not associated with rapid eGFR

decline, which was defined as loss of >3 mL/min/1.73 m^2 eGFR per year. Together, these findings imply that TFF-3 is not a marker of permanent damage per se but might simply be a marker of ongoing injury.

Kidney biopsy specimens were also stained with TNF-α, IL-10, and TGF-β. Of them, TNF-α is primarily upregulated in inflammatory glomerulonephritis, such as lupus and Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; however, increased expression has also been shown in IgA nephropathy.[18] Previous studies showed that TNF-α acting via NF-κB negatively regulates TFF-3 gene transcription, and the application of intraperitoneal TFF-3 is accompanied by a reduction in TNF-α expression.[19,20] In our study, TNF-α was positive in only 7 patients and was not related to TFF-3. This difference may be due to the low number of patients. Increased immunoreactivity for IL-10 in mesangial proliferative glomerulonephritis including IgA nephropathy was shown in previous reports.[21,22] We also found that IL-10 staining was present in 89% of patients; however, it was not related to TFF-3. The lack of a relationship can be due to the fact that IL-10 is mainly secreted by mesangial cells and associated with proteinuria, while TFF-3 is mainly secreted by the tubular epithelial cells and has been shown to be related to tubulointerstitial fibrosis.[9] On the contrary, TGF-β was observed in about 71% of patients and staining for TGF-β was significantly higher in patients also positive for TFF-3. Transforming growth factor-β is a key mediator of fibrosis and accumulation of extracellular matrix in the kidney, causing glomerulosclerosis and tubulointerstitial fibrosis.[23] Tubulointerstitial fibrosis is a prognostic factor for kidney survival including IgA nephropathy patients. Interestingly even though we did not find a correlation between TFF-3 and tubular atrophy/interstitial fibrosis component of the MEST-C score, TFF-3 was associated with TGF-β staining. Lack of a relationship between the T score and TFF-3 can be explained by the fact that all our patients had a T score of 0, less than 25% tubular atrophy/interstitial fibrosis, on kidney biopsy. The finding that TFF-3 expression is associated with TGF-β supports the notion that TFF-3 is up regulated in tubular injury. However, it is not clear whether this up regulation causes fibrosis or acts as a repair mechanism against further insult.

There are several limitations in our study. First of all, the sample size was small with only 28 patients. Second the patients constituted a heterogeneous group where there were different clinical presentations, treatments, and response to therapy. We did not investigate urinary TFF-3 levels. However, our study is the largest study to investigate TFF-3 staining in IgA nephropathy with a relatively long follow-up period of about 9 years.

There is abundant TFF-3 staining in the tubular epithelial cells in patients with IgA nephropathy. The staining is associated with TGF-β expression, supporting the notion that TFF-3 is a marker of tubular injury. Larger studies are needed to determine the exact role of TFF-3 in the pathophysiology of IgA nephropathy.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of University of Marmara University Hospital (Approval no: 09.2022.967, Date: July 22, 2022).

Informed Consent: Informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – D.Ö., H.A., M.K.; Design – D.Ö., H.A., M.K.; Supervision – E.A., D.Ö., D.B.A., A.V., M.K., H.A.; Resources – E.A., D.Ö., D.F., A.V., M.K., H.A.; Materials – E.A., D.Ö., D.F., A.V., M.K., H.A.; Data Collection and/or Processing – E.A., D.Ö, D.B.A., D.F., M.T., A.V., M.K., H.A.; Analysis and/or Interpretation – E.A., D.Ö., D.B.A., M.T., A.V., M.K., H.A.; Literature Search – E.A., D.Ö., D.B.A., D.F., M.T., A.V., M.K., H.A.; Writing Manuscript – E.A., D.Ö., D.B.A., D.F., M.T., A.V., M.K., H.A.; Critical Review – E.A., D.Ö., D.B.A., D.F., M.T., A.V., M.K., H.A.

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