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Immunohistological score of transcription factor 21 had a positive correlation with its urinary excretion and proteinuria in immunoglobulin a nephropathy

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Summary. Transcription factor 21 (TCF21) contributes to mammalian nephrogenesis, and especially to glomerular maturation. Our previous study suggested its influence on glomerular injury, showing that TCF21 expression in podocytes had a positive correlation with the urinary protein value and also with the urinary TCF21 concentration. We now focus on its influence on the clinical course of immunoglobulin A nephropathy (IgAN), as patients with IgAN constitute the largest population of individuals with primary chronic glomerulonephritis in the world. Twenty cases of IgAN were divided into two groups according to the immunohistological score (IHS) of glomerular TCF21 expression: group IHS1 (n=7) and group IHS2+3 (n=13). Sixteen of the 20 cases were followed up for 2 years. Group IHS2+3 had heavier urinary protein ($p=0.03$) and a greater urinary TCF21 level ($p<0.001$) compared to group IHS1 at baseline. None of the other factors including hematuria, estimated glomerular filtration rate (eGFR), or the Oxford classification showed a statistically significant difference between these two groups. At the 2-year follow-up, even though the rate of remission in urinary protein, hematuria and the eGFR decline were not statistically correlated to IHS, the IHS2+3 group had a slight tendency toward a steeper eGFR decline compared to IHS1 ($p=0.31$). The present study suggested that the higher IHS of TCF21 corresponded to heavier proteinuria and a higher urinary TCF21 level in IgAN. This could be the first step in determining the TCF21 value for predicting the prognosis for IgAN.

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Introduction

Transcription factor 21 (TCF21) is one of the transcription factors in the basic helix-loop-helix family, contributing to mesenchymal development in multiple organs (Robb et al., 1998). In the kidney, mammalian nephrogenesis, especially glomerular maturation, also owes a lot to this transcription factor, as represented in a previous report, which showed that *Tcf21* knockout mice presented with severely hypoplastic kidneys and that podocyte-specific *Tcf21* knockout mice showed focal segmental glomerulosclerosis (FSGS) (Quaggin et al., 1999; Maezawa et al., 2014). All the reports mentioned above were based on animal models, and no investigation has been found that examined human tissue and urinary samples. In this context, we reported the first study demonstrating the influence of TCF21 on human glomerular injury, investigating four glomerular diseases including immunoglobulin A nephropathy (IgAN), FSGS, minimal change disease, and membranous glomerulonephropathy (Usui et al., 2020). Our previous findings can be summarized with the following three points: First, TCF21 was expressed mainly in podocytes rather than kidney tubules. Second, TCF21 expression in glomeruli had a positive correlation with urinary protein level. Third, urinary TCF21 level was also correlated with TCF21 expression in glomeruli. All these findings suggested the possibility of TCF21 emerging as a marker for glomerular injuries. In this study, as a next step, we focused on the influence of TCF21 expression on the clinical course of IgAN, which accounts for most cases of chronic glomerulopathy, more common in Asian countries (Koyama et al., 1997).

Materials and methods

Study design

Twenty cases of IgAN diagnosed at University of Tsukuba Hospital were included in the study. Age, gender, urinary protein, hematuria, urinary TCF21, serum creatinine (Cre) level, serum IgA level, the MESTC histological scores according to Oxford classification, and immunohistological score (IHS) of TCF21 were collected as baseline data. Sixteen of the 20 cases (IHS1: 5cases, IHS2+3: 11cases) were followed up for 2 years, and the urinary protein, hematuria, and serum Cre level results and the treatment protocol were presented. Remission of the high urinary protein level was defined as less than 0.3 g/g Cre, and remission of hematuria was defined as less than 5 red blood cells per high-power field (RBC/hpf). The 20 cases were divided into two groups according to the IHS of TCF21, based on the scoring method described below in the "Scoring method" section. These groups were referred to as IHS1 and IHS 2+3.

This research protocol was approved by the Ethics Committee of University of Tsukuba Hospital (H20-273, H26-26). Additionally, the comprehensive permission system for the treatment of human biological samples at University of Tsukuba Hospital was utilized as a foundation for this research protocol (H26-26). Informed consent to participate in this study from each individual was required by the Institutional Review Board. An announcement of this study was simultaneously posted at the outpatient clinic of our institute.

Immunohistochemistry of TCF21

Immunohistological analysis of TCF21 was provided by the following method, which was the same method we described in our previous study. Paraffin sections were stained with hematoxylin-eosin and periodic acid-Schiff for light microscopy. A microwave oven was used for antigen retrieval of paraffin sections. Briefly, each section was incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. The primary antibodies were polyclonal anti-TCF21 antibodies (Lot Number A75217, pAb; rabbit IgG: 1:250 dilution; Sigma-Aldrich, MO, USA). The secondary antibodies used for immunohistochemical studies were peroxidase-conjugated anti-rabbit IgG pAb (Nichirei Bioscience, Tokyo, Japan). The detection kits were DAB detection kits (Dako, Denmark). The sections were nuclear stained using hematoxylin.

Scoring method

IHS was evaluated using a semi-quantitative method, which was the same method we described in our previous study. Five glomeruli were selected in a random manner in each section, and the different staining patterns of each glomerulus were then evaluated

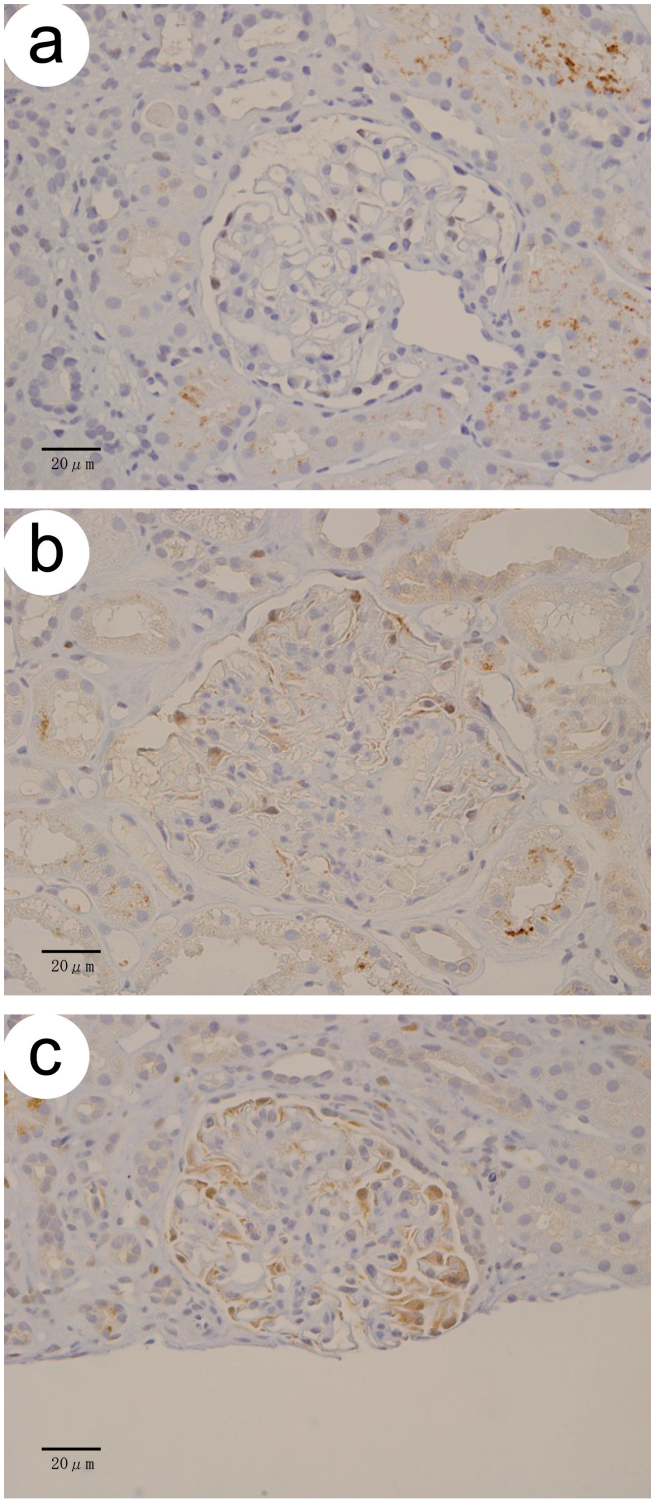

Fig. 1. Immunohistological analysis of TCF21 in human IgAN. **a.** In the sample defined as IHS1, TCF21 was weakly expressed in the nuclei of segmental podocytes. **b.** In IHS2, TCF21 was expressed in the nuclei of global podocytes. **c.** In IHS3, the cytoplasmic expression of global podocytes in diffuse glomeruli was observed.

and compared with representative glomeruli in the normal glomerular disease sample. Two expert renal pathologists made a diagnosis using the following levels: 0, the nuclear expression of all podocytes did not increase; 1, the nuclear expression of segmental podocytes (less than 50%) increased; 2, the nuclear expression of global podocytes (50% or more) increased; 3, the cytoplasmic expression of global podocytes in diffuse glomeruli (50% or more) was seen; 4, the cytoplasmic expression of global podocytes in most glomeruli (90% or more) was seen.

Measurement of TCF21 concentration

Urinary TCF21 concentration was measured using a TCF21 enzyme-linked immunosorbent assay kit (ELISA, Cusabio Biotech, China). The procedure used was the standard sandwich method under a products-adjunctive protocol. Absorbance determination with a 3,3',5,5'-tetramethylbenzidine substrate was performed using the MTP-300 microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan). The urinary TCF21 concentration was corrected using the urinary Cre concentration, whose unit was titer pg/mg Cre.

Statistical analysis

The quantitative data were given as mean values ± standard deviations (SDs), and also median and range in each figure. To analyze the parameters, the quantitative variables between two groups were compared with Student's t test and the Mann Whitney U test, and the categorical variables were compared with Fisher's exact test. Statistical significance was established at $p<0.05$. Bell Curve for Excel version 3.00 (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for all of the statistical analyses.

Results

Immunohistological analysis of TCF21

Twenty cases of IgAN were included in the study. Seven cases were diagnosed as immunohistological score (IHS)1, 10 cases as IHS2, and the other 3 cases as IHS3 (Fig. 1). None of the cases were diagnosed as IHS0 or IHS4. Then, they were divided into 2 groups: IHS1 (7 cases) and IHS2+3 (13 cases) for analysis, as shown in Table 1.

Table 1. Comparison of clinical characteristics between cases with an immunohistological score of 1 and those with immunohistological scores of 2+3.

		Immunohistological score		
		1 (n=7)	2 + 3 (n=13)	p value
Age	(Average±SD years)	36.1±14.6	37.7±12.6	0.81
Gender	(Male / Female)	4 / 3	5 / 8	0.64
Urinary protein	(Median/range g/gCre)	0.74 (0.20-0.87)	1.46 (0.26-2.78)	0.03
Hematuria	(n, %)			
0, 1-4		0, 0.0	2, 15.4	0.91
5-9		3, 42.9	2, 15.4	
10-19		1, 14.3	3, 23.1	
20-99		1, 14.3	4, 30.1	
100-		2, 28.6	2, 15.4	
Urinary TCF21	(Median/range pg/mg Cre)	25.6 (0.0-158.8)	785.9 (80.6-3072.6)	<0.001
eGFR	(Average±SD mL/min/1.73m^2)	75.6±18.8	71.4±23.6	0.68
Serum IgA	(Average±SD mg/dL)	306.1±103.0	292.0±74.7	0.73
The Oxford classification	(n, %)			
M		4, 57.1	9, 69.2	0.65
E		4, 57.1	7, 53.8	1.00
S		6, 85.7	13, 100.0	0.35
T		1, 14.3	6, 46.2	0.33
C		3, 42.9	6, 46.2	1.00
Treatment				
Corticosteroids	(n, %)	4, 80.0	6, 54.5	0.59
RAS inhibitors	(n, %)	4, 80.0	7, 63.6	1.00
Tonsillectomy	(n, %)	2, 40.0	4, 36.4	1.00
Prognosis at 2-year follow-up				
Remission of urinary protein	(n, %)	4, 80.0	8, 72.7	1.00
Remission of hematuria	(n, %)	1, 20.0	7, 63.6	0.28
eGFR	(n, %)			
decline 5% or more		1, 20.0	6, 54.5	0.31

TCF21, transcription factor 21; IgA, immunoglobulin A; RAS: the renin-angiotensin system; eGFR, estimated glomerular filtration rate.

IHS and clinical characteristics

No statistical difference was found in age or gender, with results of 36.1±14.6 years (IHS1) versus 37.7±12.6 years (IHS2+3) for age (p=0.81), and 57% male (IHS1) versus 38.4% (IHS2+3) for gender (p=0.64) (Table 1). The urinary protein and urinary TCF21 levels showed significant differences between IHS1 and IHS2+3 (Fig. 2), with results of 0.74 (0.20-0.87) g/gCre and 1.46 (0.26-2.78) g/gCre for proteinuria (p=0.03), and 25.6 (0.0-158.8) pg/mg Cre and 785.9 (80.6-3072.6) pg/mg Cre for urinary TCF21 (p<0.001), respectively. Most of the cases in both IHS1 and IHS2+3 were positive for hematuria, more than 5 RBC/hpf, but no statistical difference was seen between these two groups; this was also the case for the serum IgA level. The Oxford classification had 5 categories: M, E, S, T, C, without showing a difference between the IHS1 and HIS2+3 groups, nor any correlation with IHS and urinary TCF21 level. The treatment protocol was divided into 3 groups: corticosteroids, renin-angiotensin system (RAS) inhibitors, and tonsillectomy, without showing a difference between the IHS1 and IHS2+3 groups. As for prognosis at the 2-year follow-up, even though the rate of remission in urinary protein and hematuria and the estimated glomerular filtration rate (eGFR) decline were not statistically correlated to IHS, the IHS2+3 group showed a higher tendency to have a rate of eGFR decline of more than 5% than the IHS1 group, suggesting that IHS2+3 had a tendency to show a steeper eGFR decline compared to IHS1 (p=0.31).

Summarizing the above, IHS2+3 had heavier urinary protein and greater urinary TCF21 levels compared to IHS1 at baseline, results that were different from those for other factors. At the 2-year follow-up analysis, IHS2+3 had a slight tendency to show declining renal function compared to IHS1.

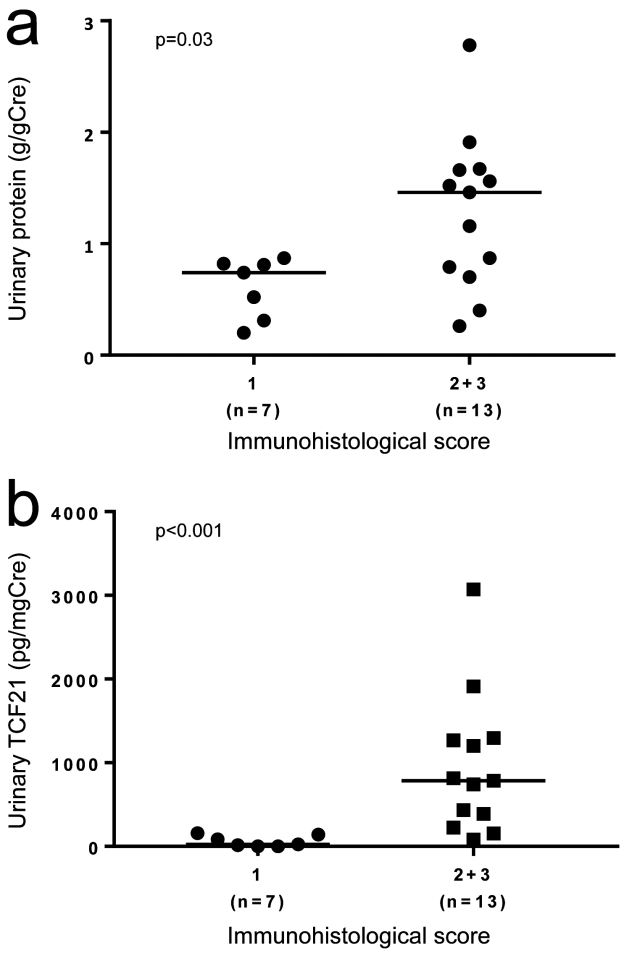

Fig. 2. The urinary protein and urinary TCF21 levels between IHS1 and IHS2+3. **a.** The urinary protein showed significant difference between IHS1 and IHS2+3, with results of 0.74 (0.20-0.87) g/gCre and 1.46 (0.26-2.78) g/gCre(p=0.03). **b.** The urinary TCF21 levels also showed remarkable difference between IHS1 and IHS2+3, with results of 25.6 (0.0-158.8) pg/mg Cre and 785.9 (80.6-3072.6) pg/mg Cre (p<0.001).

Discussion

The present study focused on the IHS of TCF21 in IgAN, and its clinical characteristics. A remarkable difference was found in the correlation between the IHS of TCF21 with the urinary protein level and the urinary TCF21 level, whereas other factors including hematuria, eGFR, and Oxford classification were not statistically significantly different depending on IHS. In our previous study, we used semi-quantitative immunostaining of human kidney biopsies and ELISA measurements of TCF21 in urine to establish that TCF21 was increased in podocytes in a variety of glomerular diseases. Also, to look at the consequence of this up-regulation, we generated *Tcf21*-over-expressing murine cultured podocytes, performed transcriptomic analysis of these cells, and demonstrated that these cells were significantly protected from adriamycin-induced injury. We verified the high-level expression of *Tcf21* in podocytes rather than tubulointerstitium, and mentioned the correlation between the IHS of TCF21 and urinary protein level, and also urinary TCF21 in IgAN and nephrotic syndrome, including FSGS, minimal change disease, and membranous glomerulonephropathy. In nephrotic syndrome, TCF21 was widely expressed in the nucleus, cytoplasm, and foot processes of podocytes, whereas IgAN with a mild level of proteinuria presented TCF21 expression that was mainly limited to the nuclei of podocytes. The urinary TCF21 level was also remarkable in nephrotic syndrome compared to IgAN, showing a positive correlation with the severity of proteinuria. Thus, proteinuria and urinary TCF21 were less remarkable in IgAN than in nephrotic syndrome, but they were both more prominent than in normal controls, suggesting that they could function as tools with which to determine the existence of glomerular diseases. The present study confirmed the result of our previous report and suggested that IHS differed depending on the level of proteinuria, not only in nephrotic syndrome with

heavy proteinuria but also in IgAN with proteinuria in the non-nephrotic range.

The mechanism of this difference in urinary protein level and urinary TCF21 level depending on IHS could be explained by the extent of podocyte damage observed in glomerular lesions. In glomerulonephritis, proteinuria is a representative characteristic of podocyte injury, and there are various explanations of the mechanism by which this injury occurs (Shankland, 2006). In IgAN, podocyte damage might result from complement activation, which first occurs in the mesangium (Lemley et al., 2002). Based on the results of our previous *in vitro* analysis, TCF21 expression might increase to protect podocyte function, depending on the podocyte damage (Usui et al., 2020). This could explain the association between the IHS of TCF21 and proteinuria. As for urinary TCF21, it was hypothesized that the excretion of TCF21 was increased in urine, reflecting the increase of its expression in podocytes. We carefully examined whether urinary TCF21 was from podocytes or not by investigating the expression of TCF21 in renal tissue, including both glomeruli and renal tubules. The expression was mainly found in glomeruli, not in renal tubules. Also, we checked for supernatants containing urinary TCF21 after the removal of microvilli or exosomes by ultra-centrifugation, possibly from injured podocytes. These results led us to the hypothesis that the urinary TCF21 originates in glomeruli rather than tubular segments and that urinary TCF21 might derive from podocytes after cell membrane damage.

In IgAN, clinical features such as proteinuria and eGFR level at the time of the renal biopsy could be risk factors for an unfavorable renal outcome (Koyama et al., 1997; D'Amico, 2000). Also, emphasis has been placed on the pathological findings (Trimarchi et al., 2017), as represented in the Oxford classification, meaning that invasive procedures have been necessary to evaluate the activity of IgAN. Even though our present study did not show any correlation between histological findings and IHS or urinary TCF21, it is possible that some pathological findings such as sclerotic lesions might correlate with IHS or renal outcome, when analyzed with a higher number of cases. In addition, eGFR at 2 years had a tendency to show a lower decline in the IHS2+3 group compared to IHS1. This may be not sufficient to prove usefulness of TCF21 in predicting the prognosis of IgAN, and so the development of a tool to predict prognosis in a less invasive way is still needed to monitor the patients.

Three limitations of this study should be mentioned. First, the sample size was small. However, we think it was worthwhile to evaluate both histopathological findings and urinary excretion in these human samples. Second, the TCF21 expression level could be influenced by histopathological features in IgAN. Since the expression of TCF21 depends on the podocyte number, and the expression level of TCF21 was weak in FSGS (Usui et al., 2020), it is possible that segmental lesions including sclerotic lesions in IgAN also influenced the expression level. Third, the follow-up period was relatively short. Longer-term follow up may reveal greater differences in renal prognosis depending on the HS of TCF21.

The IHS of TCF21 in IgAN was positively correlated with the urinary protein and urinary TCF21 levels. The study alone is not sufficient to identify a tool for predicting the prognosis with a less invasive procedure, but it suggests that TCF21 could serve as a biomarker with which to identify glomerular diseases and perhaps monitor progression of disease. Further studies are required to highlight the value of TCF21 in defining the severity and predicting the prognosis of IgAN and other glomerular diseases.

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A conflict of interest statement. Nothing to declare.

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