Expression and significance of TGF-β1/Smad signaling pathway in children with IgA nephropathy

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Background: **IgA nephropathy (IgAN) exhibits an indolent but slowly progressive course, and about 30% of children with IgAN are found to deteriorate to end-stage renal failure characterized by overaccumulation of extracellular matrix, diffuse glomerular sclerosis, and tubulointerstitial fibrosis. The TGF-β/Smad signaling pathway plays an important role in glomerulosclerosis and tubulointerstitial fibrosis. The present study aimed to elucidate the significance of expressions of TGF-β1, phosphorylated Smad3 (p-Smad3), Smad7 and fibronectin (FN) in the renal tissue of children with IgAN.**

Methods: **Forty-six children with IgAN were divided into 3 groups according to their clinical features: isolated hematuria group (IH group, 8 patients), hematuria and proteinuria group (HP group, 24), and nephritic syndrome group (NS group, 14). Patients were also divided into three groups according to their pathologic grade: grade I+II (22 patients), grade III (12) and grade IV (12) groups. Five normal renal specimens were used as the control group. The expression of TGF-β1, p-Smad3, Smad7 and FN in renal biopsy specimens was detected by two-step PowerVision™. The degrees of renal tubular injury and interstitial fibrosis were scored according to the Katafuchi semi-quantitative criteria.**

Results: **The expression of TGF-β1, p-Smad3, Smad7 and FN in children with IgAN was significantly higher than that in the control group (in glomeruli: $P<0.05$, $P<0.01$, $P<0.05$ and $P<0.01$, respectively; in tubulointerstitium: $P<0.05$, $P<0.05$, $P<0.01$ and $P<0.05$, respectively) and the highest expression levels were found in the NS and grade IV groups ($P<0.05$, $P<0.01$). The expression levels of the four proteins were not only positvely correlated with each other, but also with the grade of renal tubular injury and renal interstitial fibrosis ($P<0.05$).**

Conclusion: **The TGF-β1/Smad signaling pathway plays an important role in the progress of glomerular sclerosis, renal tubular injury and interstitial fibrosis in children with IgAN.**

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Key words: **glomerulonephritis;**
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Introduction

IgA nephropathy (IgAN) is the most common glomerular disease in children and adolescents, accounting for 20% of the glomerular diseases diagnosed by renal biopsy and 30% to 40% of biopsies performed because of hematuria and/or proteinuria in children.[1] IgAN was formerly considered a mild disease with a favorable prognosis, but long-term clinical investigation indicated that 20%-50% of adult patients may progress to renal failure and need dialysis or kidney transplant at last.[2] IgAN is characterized by mesangial deposit of IgA accompanied by diffuse proliferation of mesangial cells and extracellular matrix (ECM). The accumulation of ECM can lead to progressive glomerulosclerosis and renal failure.

The pathogenesis of IgAN is not clear, but some immunological factors and signaling pathways are found to be related to IgAN in adults. For instance, the transforming growth factor-β1 (TGF-β1) presenting in glomeruli is positively correlated with matrix accumulation. The complex of TGF-β1 and its receptor can activate the signaling pathway mediated by cytoplasmic Smad proteins.[3] The role of the TGF-β1/Smad signaling pathway in children with IgAN, however, is still unknown. The present study aimed to investigate the expression of TGF-β1, phosphorylated Smad3 (p-Smad3), Smad7 and fibronectin (FN) in the

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pathway and to explore the role of the TGF-β/Smad signaling pathway in renal glomerulosclerosis and tubulointerstitial fibrosis of children with IgAN.

Methods

Patients

In the 46 IgAN children, 32 were boys and 14 were girls. Their median age was 9.42 years (range: 4.08-14.42 years). All the patients were in an active IgAN state with symptoms of hematuria, proteinuria, and nephrotic syndromes with different levels of edema, routine urine protein (3+ to 4+), 24-hour urine protein concentration (≥0.05 g/kg), and serum albumin <30 g/L. The serum IgA level was 2.06±0.07 g/L. These were confirmed by sole or predominant IgA presence in their biopsies. Secondary IgAN associated with Henoch-Schonlein syndrome, systemic lupus erythematosus and chronic hepatitis were excluded. Five normal renal specimens were used as the control group.

According to the nephropathy criteria of the Subspecialty Group of Nephrology, Chinese Society of Pediatrics,[4] the 46 IgAN children were divided into three clinical groups: isolated hematuria group (IH group, 8 patients), hematuria and proteinuria group (HP group, 24), and nephrotic syndrome group (NS group, 14). According to the WHO pathologic grade criteria,[5] the patients were divided into 3 pathologic groups: grade I+II group (22 patients), grade III group (12), and grade IV group (12).

Measurement of serum IgA concentrations and the intensity of IgA deposits in renal biopsy specimens

The concentrations of serum IgA were tested by immunoturbidimetry with laser light scattering turbidimeter (BINDIND SITE Company). The intensity of IgA deposits in renal biopsy specimens was observed by immunofluorescence microscopy, and a semi-quantitative grading from 1+ to 3+ was given (1+: global or segmental and slight; 2+: global and moderate; 3+: global and intense).

Scale of renal tubular injury and interstitial fibrosis

The degree of renal tubular injury and interstitial fibrosis were scored according to the Katafuchi semi-quantitative criteria.[6] The tubular injury was graded as follows: 0 (no injury), 1+ (less than 25% of tubular injury); 2+ (25%-50% of tubular injury), 3+ (over 50% of tubular injury). The grades of interstitial fibrosis were as follows: 0 (no fibrosis), 1+ (less than 25% of fibrosis in cortical area), 2+ (25%-50% of fibrosis in cortical area), 3+ (over 50% of fibrosis in cortical area).

Immunohistochemistry

The renal tissue was fixed by formalin and then embedded by paraffin. The expression of TGF-β1, p-Smad3, Smad7 and FN was detected by the following two-step PowerVision™. All antibodies including rabbit and goat affinity-purified polyclonal antibodies of TGF-β1, p-Smad3, Smad7 and FN were purchased from Santa Cruz, USA. Three μm thick sections of renal tissue were deparaffinized in xylene and dehydrated with ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) at room temperature for 10 minutes. After washing with phosphate buffered saline (PBS), the sections were treated by steam heating or incubated with pepsin for 30 minutes for FN detection. To block nonspecific protein binding, these sections were incubated with non-immune goat or rabbit serum for 30 minutes, then with the PBS-diluted primary antibody overnight at 4ºC (The same amount of PBS solution served as the negative control). Washed with PBS, the sections were subsequently incubated with biotinylated host-specific secondary antibodies at room temperature for 30 minutes, and stained with diaminobenzidine (DAB). As the stained sections were dehydrated with ethanol, rinsed in hematoxylin and coversliped by mounting medium, the expression levels of TGF-β1, p-Smad3, Smad7, and FN were semi-quantitatively analyzed by the software of Image-Pro Plus 6.0.

Statistical analysis

The data were expressed as means ± SD, and analyzed with commercial statistical software SPSS for Windows, version 11.0. Unpaired Student's *t* test was used for comparison of the two groups. One-way ANOVA was used for multi-group means comparison, and the least significant difference was used for pairwise mean comparison. For correlation testing among the variables, Spearman's analysis was used. Two-sided $P<0.05$ was considered statistically significant.

Results

Expression and localization of TGF-β1, p-Smad3, Smad7 and FN in the IgAN and control groups

TGF-β1 immunostaining was noted in mesangial cells, endothelial cells and capillary walls in the IgAN group (Fig. A), and only sparse TGF-β1 scatted in mesangial cells in the control group (Fig. B). p-Smad3 immunostaining was noted in great abundance in the nuclei of glomerular and tubulointerstitial cells in the IgAN group (Fig. C), but no p-Smad3 immunostaining noted in the control group (Fig. D). Smad7 immunostaining was predominantly located in

the cytoplasm of the glomeruli and tubulointerstitium in the IgAN group (Fig. E), and less Smad7 immunostaining in the cytoplasm of glomerular cells and tubulointerstitial cells in the control group (Fig. F). FN immunostaining was mainly distributed in the glomerular mesangial region, capillary basement membrane, Bowman's capsule, crescents, tubular basement membrane and renal interstitium in the IgAN group (Fig. G), but mainly in the glomerular mesangial region, capillary basement membrane and glomeruler Bowman's capsule in the control group (Fig. H). The expressions of TGF-β1, p-Smad3, Smad7 and FN of renal tissue in the IgAN and control groups are also summarized in Table 1.

Expressions of TGF-β1, p-Smad3, Smad7 and FN in the renal tissue of three clinical groups

In glomeruli, the expression of TGF-β1 in the NS group was higher than that in the control and IH groups (both $P<0.01$); the expression in the HP group was higher than that in the control group ($P<0.05$). The expression of p-Smad3 in the NS group was higher than that in the control and IH groups (both $P<0.05$). The expression of Smad7 in HP and NS groups was higher than that in the control group (both $P<0.05$). The expression of FN in the NS group was higher than that in the control, IH and HP groups (all $P<0.05$).

In the tubulointerstitial tissue, the expression of TGF-β1 in the NS and HP groups was stronger than that in the control group (both $P<0.05$) and the IH group (both $P<0.05$). The expression of p-Smad3 in the NS and HP groups was stronger than that in the control group (both $P<0.05$) and the IH group (both $P<0.05$). The expression of Smad7 in the NS and HP groups was stronger than that in the control group (both $P<0.05$) and the IH group (both $P<0.05$). The expression of FN in the NS group was higher than that in the control, IH and HP groups (all $P<0.05$). In the HP group it was higher than that in the control group (Table 2).

Expressions of TGF-β1, p-Smad3, Smad7 and FN in the renal tissue of IgAN patients with different pathomorphological grade

Both in glomeruli and in tubulointerstitial tissue, the expressions of TGF-β1 in the grade III and IV groups were higher than that in the control group (all $P<0.05$).

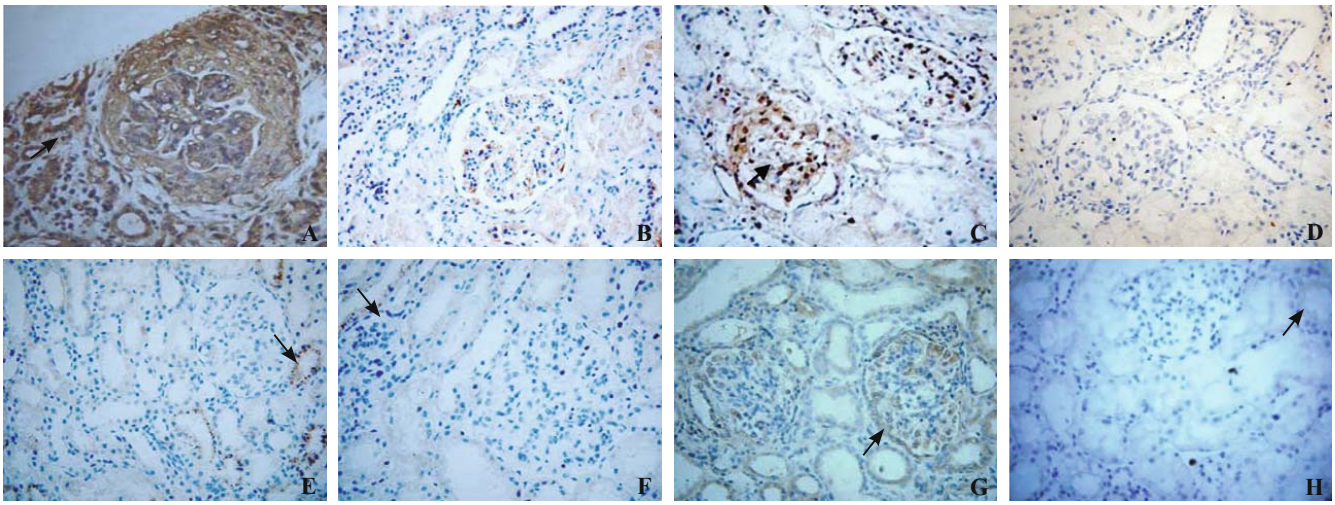

Fig. Immunostaining of TGF-β1, p-Smad3, Smad7, FN in the control and IgAN groups (original magnification × 400) (arrows indicating the positive immunostaining). **A, B**: the immunostaining of TGF-β1 in the IgAN (**A**) and control (**B**) groups; **C, D**: the immunostaining of p-Smad3 in the IgAN (**C**) and control (**D**) groups; **E, F**: the immunostaining of Smad7 in the IgAN (**E**) and control (**F**) groups; **G, H**: the immunostaining of FN in the IgAN (**G**) and control (**H**) groups.

Table 1. The expression of TGF-β1, p-Smad3, Smad7 and FN in the IgAN and control groups (means±SD)

Group	Glomeruli				Tubulointerstitium			
	TGF-β1	p-Smad3	Smad7	FN	TGF-β1	p-Smad3	Smad7	FN
Control	14.96±2.91	1.84±0.07	16.90±1.09	8.54±1.31	17.76±0.32	1.78±0.14	18.32±0.87	8.72±0.55
IgAN	36.15±21.21*	3.52±2.28†	24.41±6.62*	14.45±7.06†	44.45±21.54*	3.97±2.18*	27.59±6.85†	16.66±7.38*
t	-2.197	-3.530	-2.495	-3.728	-2.730	-2.213	-2.976	-2.369
P	0.037	0.002	0.019	0.001	0.011	0.036	0.006	0.026

*: $P<0.05$, †: $P<0.01$, compared with the control group. TGF: transforming growth factor; FN: fibronectin; IgAN: IgA nephropathy.

Table 2. Expression of TGF-β1, p-Smad3, Smad7 and FN in patients with different IgAN clinical groups (means±SD)

Groups	*n*	Glomeruli				Tubulointerstitium			
		TGF-β1	p-Smad3	Smad7	FN	TGF-β1	p-Smad3	Smad7	FN
Control	5	14.96±2.91	1.84±0.07	16.90±1.09	8.54±1.32	17.76±0.32	1.78±0.14	18.32±0.87	8.71±0.55
IH group	8	19.67±0.24	2.04±0.09	19.57±0.43	9.79±0.31	20.02±0.26	2.00±0.09	20.02±0.11	9.92±0.24
HP group	24	34.06±15.44*	3.41±2.23*	23.74±6.09*	13.16±5.62*	42.69±16.70*‡	3.60±2.02*‡	26.76±5.63*‡	15.73±5.73*
NS group	14	49.14±28.75†§	4.56±2.69*‡	28.32±7.62*	19.31±8.90*‡‖	61.45±21.18*‡	5.75±1.84*‡	33.32±6.00*‡	22.11±8.55*‡‖
F		4.646	2.621	4.783	4.344	11.283	7.689	12.614	6.947
P		0.005	0.046	0.005	0.007	<0.000	<0.000	<0.000	0.001

*: *P*<0.05, †: *P*<0.01, compared with the control group; ‡: *P*<0.05, §: *P*<0.01, compared with the IH group; ‖: *P*<0.05, compared with the HP group. IH: isolated hematuria; HP: hematuria and proteinuria; NS: nephrotic syndrome.

Table 3. Expression of TGF-β1, p-Smad3, Smad7 and FN in IgAN patients with different pathological grade (means±SD)

Groups	*n*	Glomeruli				Tubulointerstitium			
		TGF-β1	p-Smad3	Smad7	FN	TGF-β1	p-Smad3	Smad7	FN
Control	5	14.96±2.91	1.84±0.07	16.90±1.09	8.54±1.32	17.76±0.32	1.78±0.14	18.32±0.87	8.71±0.55
Grade I+II	22	24.86±6.42	2.19±0.40	20.74±2.13	10.39±1.54	34.52±19.28	2.63±1.29	23.62±5.48	11.78±2.54
Grade III	12	41.69±23.18*	4.66±2.65*‡	25.84±5.84†§	15.82±5.83†§	50.86±17.44*	5.00±2.44*‡	29.95±5.54†§	20.83±6.64†§
Grade IV	12	51.29±27.72*‡	4.81±2.85*‡	29.71±9.14†§	20.51±9.89†§	56.26±23.66*‡	5.43±1.96*‡	32.51±6.73†§	21.44±9.07†§
F		5.509	5.053	7.023	6.448	5.069	7.113	8.259	8.737
P		0.005	0.007	0.001	0.002	0.007	0.001	0.001	<0.000

*: *P*<0.05, †: *P*<0.01, compared with the control group; ‡: *P*<0.05, §: *P*<0.01, compared with the grade I+II group.

The expression of TGF-β1 in the grade IV group was higher than that in the grade I+II group (*P*<0.05).

Both in the glomeruli and tubulointerstitial tissues, the expression of p-Smad3 was higher in the grade III and IV groups than in the control group (all *P* <0.05), and was higher in the grade III and IV groups than in the grade I+II group (all *P*<0.05). The expression of Smad7 in the grade III and IV groups was higher than that in the control group (all *P*<0.01), and it was higher in the grade III and IV groups than in the grade I+II group (all *P*<0.01). The expression of FN in the grade III and IV groups was higher than that in the control group (all *P*<0.01), and it was higher in the grade III and IV groups than in the grade I+II group (all *P*<0.01) (Table 3).

Correlation analysis

In the glomeruli and tubulointerstitial tissue, the expression of FN was positively correlated with the expression of TGF-β1, p-Smad3, and Smad7 (*r*=0.965, 0.927, 0.934, respectively; all *P*<0.01). There was a positive relationship between the score of renal tubular injury and interstitial fibrosis with the expression of TGF-β1, p-Smad3, Smad7 and FN (*P*<0.05). There was no correlation between the serum concentration of IgA, and the intensity of IgA deposits in renal biopsy specimens with the expression of TGF-β1, p-Smad3, Smad7 and FN (*r*=-0.333, -0.246, -0.250, -0.207; *r*=0.311, 0.223, 0324, 0.209, respectively, *P*>0.05).

Discussion

TGF-β1 is a multifunctional cytokine, discovered by De Larco and Todaro from murine sarcoma virus transformed cells in 1978.[7] As an initial factor of the TGF-β1/Smad signaling pathway, TGF-β1 is involved in the regulating process of FN gene expression by binding to its receptor on cell surface to regulate multimerization and phosphorylation of the downstream factors such as R-Smads (Smad1, Smad2, Smad3, Smad5 and Smad8) in the cytosol and transmembrane, and formation of a transcription-regulating complex in the nuclei. The other two Smads, Smad6 and Smad7, can serve as the negative regulators (I-Smad) of R-Smads activity to keep a balanced activity of the TGF-β1/Smad signaling pathway. Recent studies of renal diseases have supported that the TGF-β1/Smad signaling pathway is involved in the progressive process of renal fibrosis. For example, TGF-β1, Smad2/3, Smad4 and Smad7 are expressed widely in pathological tissues and normal kidneys of adults.[8] In this study, TGF-β1, Smad7 and FN were expressed in the control group, indicating that the TGF-β1/Smad signaling pathway plays a role in the renal growth and development. The expression of TGF-β1, p-Smad3, Smad7 and FN in renal tissues of IgAN children was much higher than that in the control group. It indicates over-expression of TGF-β1, p-Smad3, Smad7 and FN relating to renal injury.

Glomerulosclerosis with a feature of excessive ECM accumulation is the final stage in a variety of

kidney diseases. Yamamoto et al[9] found that there was no difference in positive expression of TGF-β1 between normal renal tissue and renal diseases with weak ECM accumulation such as thin basement membrane nephropathy, minimal change nephropathy, whereas the expression of TGF-β1 was higher in diseases with significant ECM accumulation such as focal segmental glomerulosclerosis, crescentic glomerulonephritis and lupus nephritis. Ruan et al[10] found that TGF-β1 and its signaling transduction molecule Smad2 were involved in the excessive deposition of glomerular ECM. So it played an important role in the development of glomerulosclerosis. The results of this study suggested that the TGF-β1/Smad signaling pathway plays an important role in glomerular sclerosis, renal tubular injury and interstitial fibrosis in children with IgAN.

Smad7 is an inhibitory protein in the TGF-β1/Smad signaling pathway in glomerulosclerosis. It can be induced by TGF-β1 or other cytokines, and an intracellular negative feedback response that limits TGF-β1 effects is mediated. Chen et al[11] reported that Smad7 mRNA was induced by TGF-β1 in a dose-dependent manner in the transformed murine mesangial cell line. In this study, the expression of Smad7 was higher in the IgAN group, especially in the NS group, grade III and IV groups. Thus the increased expression of Smad7 was considered to be a feedback response. The over-expression of Smad7 might alter the pathophysiologic balance between R-Smads with I-Smads and TGF-β1 induced renal fibrosis. A previous study[12] showed that the high expression of murine mesangial cell Smad7 could be stimulated by TGF-β1 in a dose-dependent manner. The changes of Smad7 activity play important roles in the mesangial cell fibrogenesis. Endogenous Smad7 could not inhibit mesangial cell fibrogenesis, because the stimulation of TGF-β1 on the endogenous Smad7 expression is not enough to overcome its simultaneous stimulation on fibrogenesis. Possibly the essential expression level (even under the stimulation) of Smad7 may prevent the TGF-β1 effects mediated by Smad2/3. By this interpretation, endogenous Smad7 expression creats a threshold of stimulation, by which a fibrogenic response will occur.[12] But the detail of the threshold needs to be further explored.

Our results showed that there was no significant correlation between the increased expression of TGF-β1, p-Smad3, Smad7 and FN with serum IgA level, and with the intensity of IgA deposit in renal tissues, although the renal expression of these four proteins correlated positively with the renal tubular injury and interstitial fibrosis of children with IgAN. It may be due to different types and forms of IgA that present in the serum and renal tissues of IgAN children.

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