Early secretory antigenic target 6 and culture filtrate protein 10 as diagnostic indicators in IgA nephropathy associated with renal tuberculosis

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Research article

Keywords: Renal tuberculosis, IgA nephropathy, ESAT-6, CFP-10, Diagnostic value

Posted Date: January 24th, 2020

DOI: https://doi.org/10.21203/rs.2.21801/v1

Abstract

Background: This study evaluated the diagnostic value of early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) in immunoglobulin A nephropathy (IgAN) associated with renal tuberculosis (RT).

Methods: Between January 2013 and January 2016, 40 patients with IgAN (IgAN group), 32 patients with RT (RT group), and 52 patients with IgAN associated with RT (IgAN + RT group) were selected for this study. A Tuberculin skin test (TST) was conducted, and serum *Mycobacterium tuberculosis* (*MTB*) antibody levels were measured. Urine samples were collected to culture *MTB*. Immunohistochemistry and western blotting were used to determine the expression of ESAT-6 and CFP-10 in renal tissues. Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic values of ESAT-6 and CFP-10 in IgAN associated with RT.

Results: TST, serum *MTB* antibody, and urine *MTB* assessments were negative in the IgAN group. The positive rates of the TST and serum *MTB* antibody and urine *MTB* testing were higher in the RT group than in the IgAN + RT group. Among the three groups, expression levels of ESAT-6 and CFP-10 were found to be the highest in the IgAN + RT group and were found to be the lowest in the IgAN group. The ROC curves indicated that the area under curve (AUC) value of ESAT-6 protein for IgAN + RT diagnosis was 0.907 with a cut-off of 26.72 as the critical value. Detection by ESAT-6 protein levels achieved 75.0% sensitivity and 94.2% specificity. The AUC value of the CFP-10 protein for diagnosis of IgAN + RT was 0.800, with a cut-off of 25.665 as the critical value. Detection by the protein levels of CFP-10 showed 63.9% sensitivity and 84.6% specificity.

Conclusions: Our study provides evidence for the potential of the proteins ESAT-6 and CFP-10 as candidate markers for the diagnosis of IgAN associated with RT.

Background

Immunoglobulin A nephropathy (IgAN) is a type of glomerulonephritis that is common worldwide and usually affects people at a young age [1, 2]. IgAN is often associated with mucosal infections and is usually accompanied by recurrent episodes of gross haematuria, microscopic haematuria, or acute nephritic syndrome [3]. Current treatments for IgAN include antiplatelet medications, oral prednisolone, and tonsillectomy combined with steroid pulse therapy [4]. It is estimated that renal failure occurs in 40% of the IgAN patients within 20 years after diagnosis, and the long-term prognosis remains relatively poor [5]. Findings have also indicated that it is highly risky for patients to develop tuberculosis at an advanced stage of renal disease [6]. Renal tuberculosis (RT), caused by members of the *Mycobacterium tuberculosis* (*MTB*) complex, is a serious, localised genitourinary disease [7, 8]. Symptoms of RT include a high frequency of painless micturition and a series of pathological changes, from typical calcified lesions and papillary necrosis to autonephrectomy [9]. Other mild and non-specific symptoms include a small number of leukocytes in the urine and a low sedimentation rate [10]. Patients with RT do not

usually experience the typical symptoms of tuberculosis [8]. Therefore, clinicians often find RT diagnosis quite challenging and find it difficult to prescribe the most suitable treatment for patients with these two diseases, which can lead to more severe renal failure [4].

Early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are low-molecular-weight proteins that are respectively encoded by the *Rv3874* and *Rv3875* genes located in the region of difference-1 in the genome of virulent *MTB* strains [11, 12]. The ESAT-6 and the CFP-10 proteins can induce the malignant spread of *MTB* by participating in the cytolysis of alveolar epithelial cells and macrophages [13]. Recent evidence has also shown that when used together, the ESAT-6 and the CFP-10 antigens, two potential diagnostic markers, can also enhance the diagnostic specificity and sensitivity of tuberculosis testing [14]. Therefore, detecting the ESAT-6 and the CFP-10 proteins may allow early targeted diagnosis of RT and help to distinguish patients infected with *MTB* from individuals vaccinated with Bacillus Calmette Guerin [12]. Based on these findings, this study aimed to investigate the diagnostic value of the ESAT-6 protein and the CFP-10 protein in IgAN associated with RT, which will be highly significant for diagnosing patients with these two diseases.

Methods

Study subjects

Between January 2013 and January 2016, 40 patients with IgAN (IgAN group), 32 patients with RT (RT group), and 52 patients with RT associated with IgAN (IgAN + RT group) diagnosed by renal biopsy were enrolled in this study. The age ranges of the patients in the three groups were 27 years–54 years, 25 years–59 years, and 24 years–57 years old, respectively, and the male to female ratios were 21:19, 17:15, and 23:29, respectively. None of the patients had hepatitis, systemic lupus erythematosus, allergic purpura, or other secondary nephritis, and none had been administered any glucocorticoid and immunosuppressant before renal biopsy. Pure protein derivative of tuberculin (PPD) (the Shanghai Institute of Biological Products, Shanghai, China) was used for the tuberculin skin test (TST), which was conducted as follows: PPD was injected intracutaneously through the forearm-palmaris, and the injection site was observed for a reaction about 72 h after injection. Patients were considered to be PPD positive if the reaction was >5 mm [15]. Venous blood (3 mL) was collected from all patients after fasting. Serum was isolated from the blood samples and used for the detection of anti-tuberculosis antibody.

Urine culture for MTB

Urinary sediment (20 mL) was obtained from 24-h urine specimens and then centrifuged to obtain the precipitate. The sediment was mixed with 4% sodium hydroxide (NaOH) to facilitate the precipitation. The supernatant was removed and centrifuged under sterile conditions, and the precipitate was collected and mixed with 6% sulphuric acid. The specimens were then inoculated on a Roche slanted solid culture medium and incubated in a constant temperature incubator at 37°C and observed at regular time intervals. After 2 months of observation, bacterial growth on the culture medium indicated the presence of *MTB*.

Haematoxylin and eosin (HE) staining

Renal tissue specimens were fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS), dehydrated with graded ethanol, and cleared with xylene. After the remaining xylene was washed away with distilled water, the tissue specimens were embedded in paraffin, sliced into 4-μm sections, and deparaffinised with ethanol. Some sections were stained with HE. Briefly, prepared renal tissue sections were stained with haematoxylin for 5 min, washed with distilled water, stained with 0.5% eosin for 2 min, dehydrated with ethanol, cleared in xylene, sealed with neutral gum, and then observed with an optical microscope. The remaining sections were stored at -20°C until use.

Immunohistochemistry

After adding 30% H_2O_2 to block endogenous enzyme activity, renal tissue sections from the patients were heated in antigen retrieval buffer. After cooling for 5 min, the sections were heated and cooled twice more. After cooling to a temperature that coincided with the room temperature (about 25°5), the sections were blocked by adding 5% bovine serum albumin (BSA) blocking buffer and incubating at room temperature for 20 min. The excess blocking buffer was removed, and then mouse anti-human ESAT-6 primary antibody (ab26246, 1:3000; Abcam, Cambridge, MA, USA) and mouse anti-human CFP-10 primary antibody (ab64754, 1:3000; Abcam) were added to the sections and incubated at 4°C overnight. Then, the sections were incubated with biotinylated goat-anti mouse IgG (ab6789; Abcam) at 37°C for 40 min. The sections were washed with PBS, and the antibody-antigen reactions were visualised by incubation with 3,3’-diaminobenzidine; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). Images of the stained sections (three randomly selected fields from three sections of each specimen) were quantitatively analysed with the Image-ProPlus (Media Cybernetics, Rockville, MD, USA) to determine the integral optical density (IOD) values of the ESAT-6 protein and the CFP-10 protein.

Western blotting

The renal tissue sections were incubated with 1X sodium dodecyl sulphate buffer with mixing at 300 ×g until the sections were fully lysed. The lysed sections were then incubated on ice for 30 min and centrifuged for 4 min at 1200 ×g . The supernatant was removed and stored at -80°C. The concentration of the extracted proteins in the supernatant was determined using the BCA protein determination kit (AR0146; Boster Biological Technology Co., Ltd., Wuhan, China), which was then adjusted to 3 μg/μL. The protein samples were mixed with loading buffer and incubated for 10 min at 95°C, and then equal protein (30 μg) from each sample was separated by 10% polyacrylamide gel electrophoresis. The separated proteins were then transferred to a PVDF membrane (P2438; Sigma, St. Louis, MO, USA) by semi-dry electrotransfer and then blocked with 5% BSA for 1 h at room temperature. Then, the membrane was incubated with anti-ESAT-6 antibody (ab45073, 1:1000 in 5% BSA; Abcam) or rabbit anti-CFP-10 (ab45074, Abcam) and rabbit anti-α-smooth muscle actin (ab5694; Abcam), which was diluted to 0.2 μg/mL–1 μg/mL, in a refrigerator with shaking overnight. The membranes were washed three times with TBST for 10 min each and then incubated with the secondary antibody (ab6720, 1:10000; Abcam) in

TBST for 4 h–6 h at 4°C. After incubation, the membrane was washed with TBST three times for 15 min each. The membrane was then incubated with chemiluminescence reagents A and B, mixed at 1:1, followed by the addition of photographic developer solutions. GAPDH was used as an internal reference. All immunoblotting bands were subjected to densitometric analysis.

Immunofluorescence

Renal tissue sections from patients were heated to a boil in antigen retrieval buffer. After cooling for 5 min, the sections were heated and cooled two more times, and then cooled to room temperature. Then, the sections were incubated with mouse anti-human IgA1-FITC (ab99793, 1:50; Abcam) in a humidified chamber at 37°C for 30 min. The sections were washed with PBS (pH 7.2– pH 7.6) three times for 5 min each. After air drying, the sections were sealed with buffered glycerol and observed with a fluorescence microscope under a glass coverslip. Five high-power fields (400X) of each section were randomly selected, and the corresponding mean gray value (MGV) of the positively-stained areas was measured.

Statistical analysis

All data were analysed with SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). Data have been presented as the mean ± standard deviation. The *t*-test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparisons of multiple groups. Counted data have been expressed as a rate or percentage, and two groups were compared using the chi-square test. For multiple groups with equal variance, the *q* test was performed for pairwise comparisons, and the non-parametric rank test was performed for group comparisons. A receiver operating characteristic (ROC) curve ($\alpha = 0.05$) was plotted to evaluate the diagnostic value of the ESAT-6 and the CFP-10 proteins. *P* values less than 0.05 were regarded as statistically significant.

Results

More IgAN + RT patients had a positive TST and were positive for serum MTB antibody

There was no significant difference in the gender or age of the patients in the IgAN, RT, and IgAN + RT groups ($P > 0.05$ for both). Patients in the IgAN group had no history of tuberculosis, and patients in the RT group had no history of renal disease. No significant difference was noted in the history of tuberculosis between the RT and IgAN + RT groups ($P = 0.390$) or the history of renal disease between the IgAN and IgAN + RT groups ($P = 0.124$). The TSTs and serum tuberculosis antibody tests of patients in the IgAN group were negative. There were 22 (68.75%) patients and 38 (73.08%) patients in the RT and IgAN + RT groups, respectively, who had a positive TST; and 23 (31.94%) patients and 41 (78.85%) patients in the RT and IgAN + RT groups, respectively, with positive serum tuberculosis antibody tests. The positive rates for the TST and serum tuberculosis antibody test in the RT and IgAN + RT groups were both higher than those in the IgAN group ($P < 0.05$ for both; Table 1).

More IgAN + RT patients were positive for MTB in urine culture

There were 10 (31.2%) patients and 30 (57.7%) patients who showed positive results for *MTB* in the urine culture test in the RT and IgAN + RT groups, respectively (Table 2). Compared with the IgAN group, the positive rates in both the RT and IgAN + RT groups were significantly higher. However, the rate in the IgAN + RT group was found to be significantly higher than that in the RT group ($P < 0.05$).

Pathological changes in the renal tissues of patients in the IgAN, RT, and IgAN + RT groups

Figure 1 shows the following pathological changes in the patient groups. In the IgAN group, cellular crescents had formed in the renal tissues, the glomerular mesangium was found to be thickened, and the amount of mesangial matrix and glomerular mesangial cells were found to be increased. In the RT group, tubercles were observed with caseous necrosis at the centre, along with Langerhans cells, epithelioid cells, and a large number of lymphocytes. In the IgAN + RT group, segments, moderate mesangial cell proliferation, and increased mesangial matrix were observed.

IgAN + RT patients showed high IOD values for the ESAT-6 and the CFP-10 proteins

In the RT and IgAN + RT groups, yellowish-brown and brown positive deposits were observed in the renal-interstitium, glomerular mesangial region, and cytoplasm of the kidney tubule cells. In the IgAN group, only two sections had light-brown deposits in the renal-interstitium. Pairwise comparisons were performed of the IgAN, RT, and IgAN + RT groups, which showed that the IOD values of the ESAT-6 protein and the CFP-10 protein in the renal tissues of patients were found to be the highest in the IgAN + RT group and the lowest in the IgAN group ($P < 0.05$ for both; Figure 2).

IgAN + RT patients showed high levels of the ESAT-6 and the CFP-10 proteins

Pairwise comparisons of the IgAN, RT, and IgAN + RT groups showed that the EAST-6 and the CFP-10 protein levels in the renal tissues were found to be the highest in the IgAN + RT group and the lowest in the IgAN group ($P < 0.05$ for both; Figure 3).

Enhanced IgA1 expression (visible as fluorescence) was detected in the renal tissues of patients in the IgAN and IgAN + RT groups

Immunofluorescence detection showed that there were yellow-green deposits of IgA1 in the glomerular mesangium, capillary loop, and a portion of the renal tubular epithelial cells in the IgAN and IgAN + RT groups. In contrast, the renal tissues of patients in the RT group were found to be negative for IgA1 expression. The MGVs in the IgAN, IgAN + RT, and RT groups were 57.83 ± 7.16, 55.74 ± 6.08, and 10.02 ± 1.38, respectively. Compared with the RT group, both the IgAN and IgAN + RT groups showed significantly higher IgA1 expression ($P < 0.05$ for both). However, IgA1 expression levels in the renal tissues of patients did not differ significantly between the IgAN and IgAN + RT groups ($P > 0.05$; Figure 4).

The ESAT-6 and the CFP-10 proteins showed good diagnostic performance for IgAN associated with RT

Based on the ROC curves, the area under curve (AUC) of the ESAT-6 protein for IgAN + RT diagnosis was 0.907 (95% CI = 0.852–0.963, $P < 0.001$), the cut-off value was 26.72, and the sensitivity and specificity were 75.0% and 94.2%, respectively; the AUC of the CFP-10 protein for IgAN + RT diagnosis was 0.800 (95% CI = 0.721–0.880, $P < 0.001$), the cut-off value was 25.665, and the sensitivity and specificity were found to be 63.9% and 84.6%, respectively (Figure 5).

Discussion

RT is an infectious disease caused by *MTB* strains [7]. IgAN, which is one of the most common primary glomerular diseases in young males worldwide, is particularly prevalent in Asia [16]. Although many proteins have been used to improve the diagnosis and prognosis of RT over the past few years, and a renal biopsy is necessary to diagnose IgAN, numerous clinical studies have suggested that new tools are still urgently needed to distinguish IgAN and RT [14, 17-19]. In this study, we investigated whether the expression of the ESAT-6 and CFP-10 proteins could serve as diagnostic indicators of IgAN associated with RT. The results showed that ESAT-6 and CFP-10 have high value for the diagnosis of IgAN associated with RT.

Initially, our study showed that, compared with the IgAN group, the RT and IgAN + RT groups had higher *MTB* urine culture positive rates, and the positive rate in the IgAN + RT group was higher than that in the RT group. This result indicated that the rates of *MTB* detection were much higher in patients with RT than in patients without RT. This corresponded to a study showing that, under most circumstances, RT was caused by *MTB* [8]. However, the IgAN + RT group showed a higher *MTB* positive rate than the RT group. Previous studies have suggested that the immune complexes, such as IgA antibodies, in IgAN patients might contribute to the response to *MTB*, which was further confirmed by our results [20, 21].

Our immunohistochemistry analysis showed that the IOD values of The ESAT-6 and the CFP-10 proteins were highest in the IgAN + RT group, followed by the RT and IgAN groups, respectively. T cell-mediated immunity is critical to control *MTB* infections. The ESAT-6 and the CFP-10 proteins are good candidates for stimulating T cells in *MTB* infected tissues to stimulate the production of interferon-γ and other cytokines, such as IL-2 [22, 23]. It has been reported that cells harvested from the lungs and Peyer's patches of immunised mice stimulated with the CFP-10 protein exhibit significant IFN-γ levels and these mice produce increased levels of serum IgG and lung IgA anti-CFP-10 responses [24]. In addition, and this research supported the potential of an IgA response against the ESAT-6/CFP-10 antigens for discriminating clinical TB from healthy *MTB*-infected and uninfected cases. The kidney is one of the main sites of tuberculosis infection, as it precisely meets the stringent growth requirements of *M. tuberculosis* due to its high oxygen tension. Genitourinary tuberculosis, including RT, is the second most common extrapulmonary tuberculosis infection after lymphatic tuberculosis [25, 26]. Therefore, the two proteins showed stronger reactions in the IgAN + RT and IgAN groups. In light of the fact that renal insufficiency is a risk factor for *MTB* infection [27], it is not surprising that patients in the IgAN + RT group with more severe renal damage had a higher level of *MTB*. Furthermore, a previous study demonstrated that both ESAT-6 and CFP-10, which are produced by the ESX-1 system in *MTB*, play a significant role in

the development and spread of *MTB* infection [13]. Thus, the presence of *MTB* in the RT and IgAN + RT groups facilitated the response to the ESAT-6 and the CFP-10 antibodies, thus leading to higher IOD values.

Furthermore, our results showed that IgA1 expression levels were much higher in the IgAN and IgAN + RT groups than in the RT group. IgA1 is a unique serum glycoprotein, which is a subclass of IgA that exclusively exists in the mesangial deposits in patients with IgAN [28, 29]. Another study mentioned that galactose-deficient IgA1 is one of the most accurate indicators for the pathogenesis of IgAN [30]. In terms of the relevant mechanism, deposits of IgA1 molecules likely play an active role in causing renal damage by interacting directly with resident glomerular cells and regulating complement activation [31]. Hence, IgA1 expression was weaker in the RT group than in the IgAN and IgAN + RT groups.

Conclusions

In summary, the present study provides evidence that both the ESAT-6 protein and the CFP-10 protein are of high value for diagnosing IgAN accompanied by RT. The expression levels of these two proteins, as demonstrated by our results, were found to be the highest in IgAN + RT patients, indicating their great potential for the diagnosis of renal diseases and exploring the pathogenesis of these diseases. Since this study involved a relatively small sample, further large-scale trials will be required to confirm this conclusion.

List Of Abbreviations

CFP-10, culture filtrate protein 10; ESAT-6, early secretory antigenic target 6; IgAN, immunoglobulin A nephropathy; MTB, Mycobacterium tuberculosis; TST, Tuberculin skin test; RT, renal tuberculosis; ROC, Receiver operating characteristic; AUC, area under curve; HE, Haematoxylin and eosin; PBS, phosphate buffered saline; BSA, bovine serum albumin; IOD, integral optical density; ANOVA, analysis of variance; MGV, mean gray value.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Shanghai Pulmonary Hospital, and all patients provided informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

None.

Authors' contributions

Xiao-Fang You designed the experiments. Yong Fang carried out the majority of experiments and wrote the article. Qing-hui Wang and Jun-hui Li revised the article. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Tables

Table 1 Comparisons of baseline characteristics of patients in IgAN, RT and IgAN + RT groups

Characteristic		IgAN group (n = 40)	RT group (n = 32)	IgAN + RT group (n = 52)
Gender				
	Male	21	17	23
	Female	19	15	29
Age (year)		40.70 ± 9.23	40.66 ± 10.70	42.27 ± 8.51
History of tuberculosis (month)		0^{a}	4.17 ± 1.93^{b}	4.60 ± 1.85^{b}
History of renal disease (month)		2.10 ± 0.99^{a}	0^{b}	1.73 ± 0.82^{b}
Tuberculin skin test				
	Positive	0	22	38
	Negative	40^{a}	10^{b}	14^{c}
Serum tuberculosis antibody				
	Positive	0	23	41
	Negative	40 ^{a}	9^{b}	11^{c}

Notes: IgAN, immunoglobulin A nephropathy; RT, renal tuberculosis; a, b, and c letters means $P < 0.05$ when pair comparisons were performed among the IgAN, RT, and IgAN + RT groups.

Table 2 Positive rates of urine *MTB* in IgAN, RT and IgAN + RT groups

Group	*MTB* in urine		Total	Positive rate
	Negative	Positive		
IgAN group	40	0	40	0[a]
RT group	22	10	32	31.2%[b]
IgAN + RT group	22	30	52	57.7%[c]

Notes: *MTB*, *mycobacterium tuberculosis*; IgAN, immunoglobulin A nephropathy; RT, renal tuberculosis; a, b, and c letters means $P < 0.05$ when pair comparisons were performed among the IgAN, RT, and IgAN + RT groups.

Figures

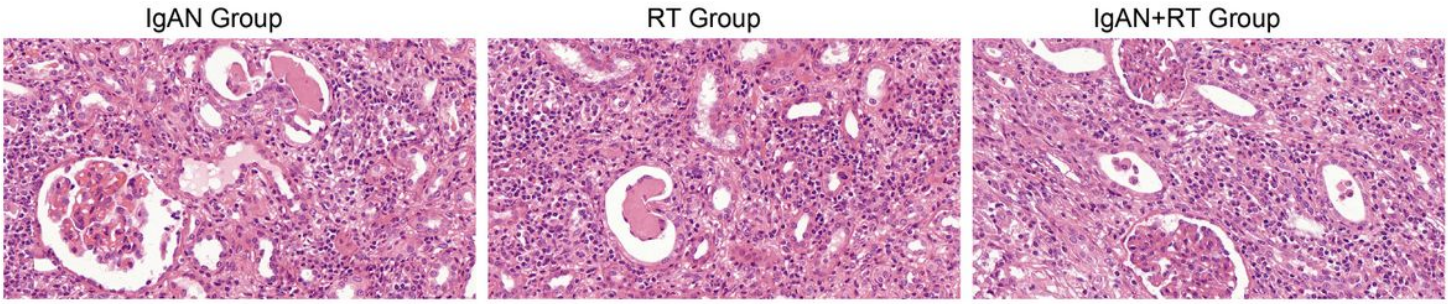

Figure 1

Pathological changes in the renal tissues of patients in the IgAN, RT, and IgAN + RT groups by HE staining (400X). Abbreviations: IgAN, immunoglobulin A nephropathy; RT, renal tuberculosis; HE, haematoxylin and eosin.

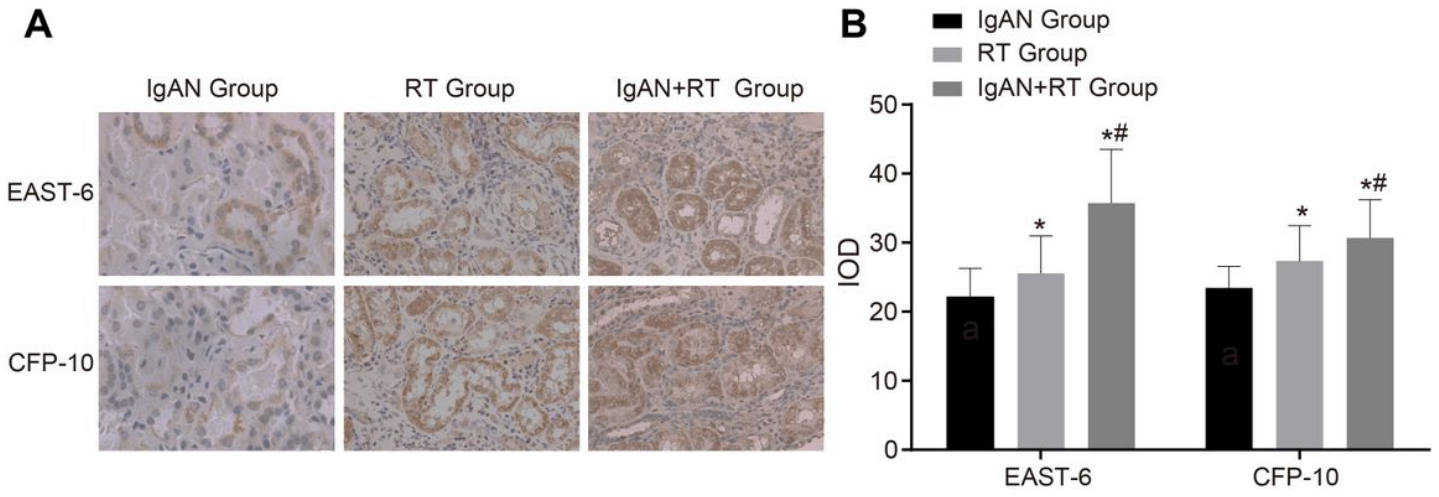

Figure 2

2 Immunohistochemical detection of the ESAT-6 protein and the CFP-10 protein in the renal tissues of patients in the IgAN, RT, and IgAN + RT groups. A, Immunohistochemistry map of expressions of the ESAT-6 protein and the CFP-10 protein in each group; B, histogram of the IOD of the ESAT-6 protein and the CFP-10 protein in each group. Abbreviations: ESAT-6, early secretory antigenic target 6; CFP-10, culture filtrate protein 10; IgAN, immunoglobulin A nephropathy; RT, renal tuberculosis; IOD, integral optical density; a, b, and c indicate significant differences ($P < 0.05$) when pairs of groups (IgAN, RT, and IgAN + RT) were compared.

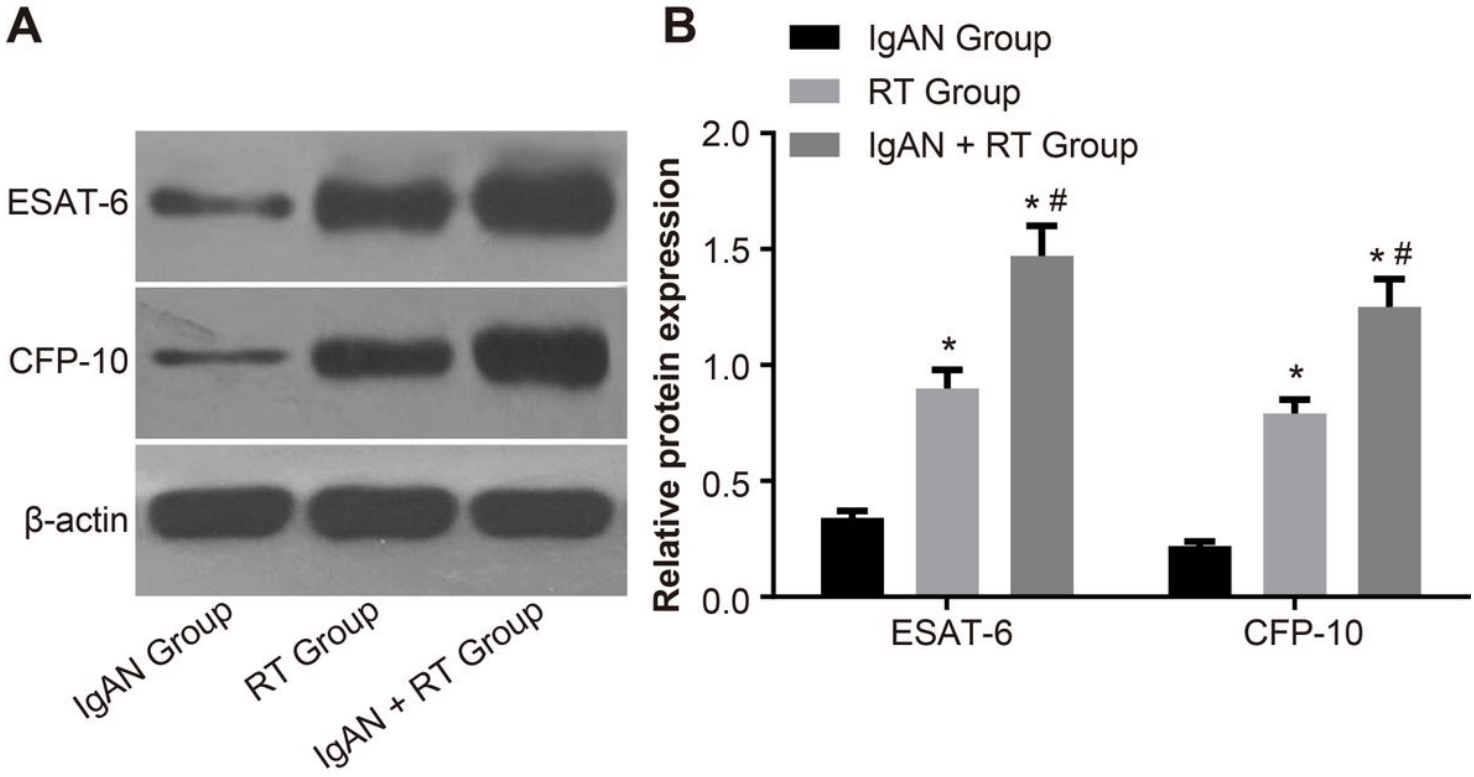

Figure 3

Expressions of the ESAT-6 protein and the CFP-10 protein in the renal tissues of patients in the IgAN, RT, and IgAN + RT groups as detected by western blotting. A, grey value of the protein bands of the ESAT-6 protein and the CFP-10 protein; B, protein levels of ESAT-6 and CFP-10. a, b, and c indicate significant differences ($P < 0.05$) when pairs of groups (IgAN, RT, and IgAN + RT) were compared.

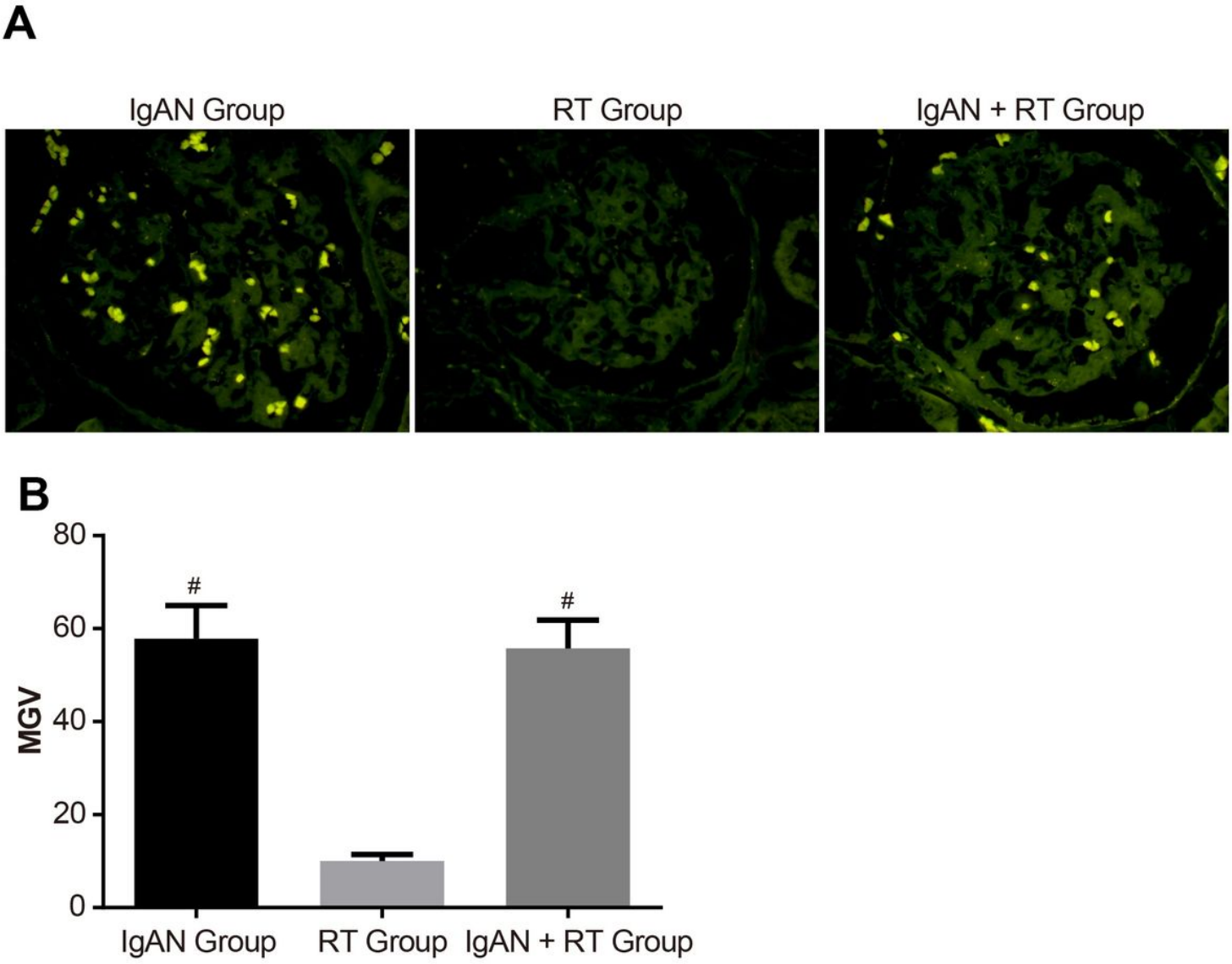

Figure 4

IgA1 expression in the renal tissues and the MGV in the IgAN, RT, and IgAN + RT groups. A, immunofluorescence images of IgA1 expression in the renal tissues of each group; B, histogram of the MGV in each group. Abbreviations: IgAN, immunoglobulin A nephropathy; RT, renal tuberculosis; MGV, mean gray value; IgA 1, immunoglobulin A 1. a, b, and c indicate significant differences ($P < 0.05$) when pairs of groups (IgAN, RT, and IgAN + RT) were compared.

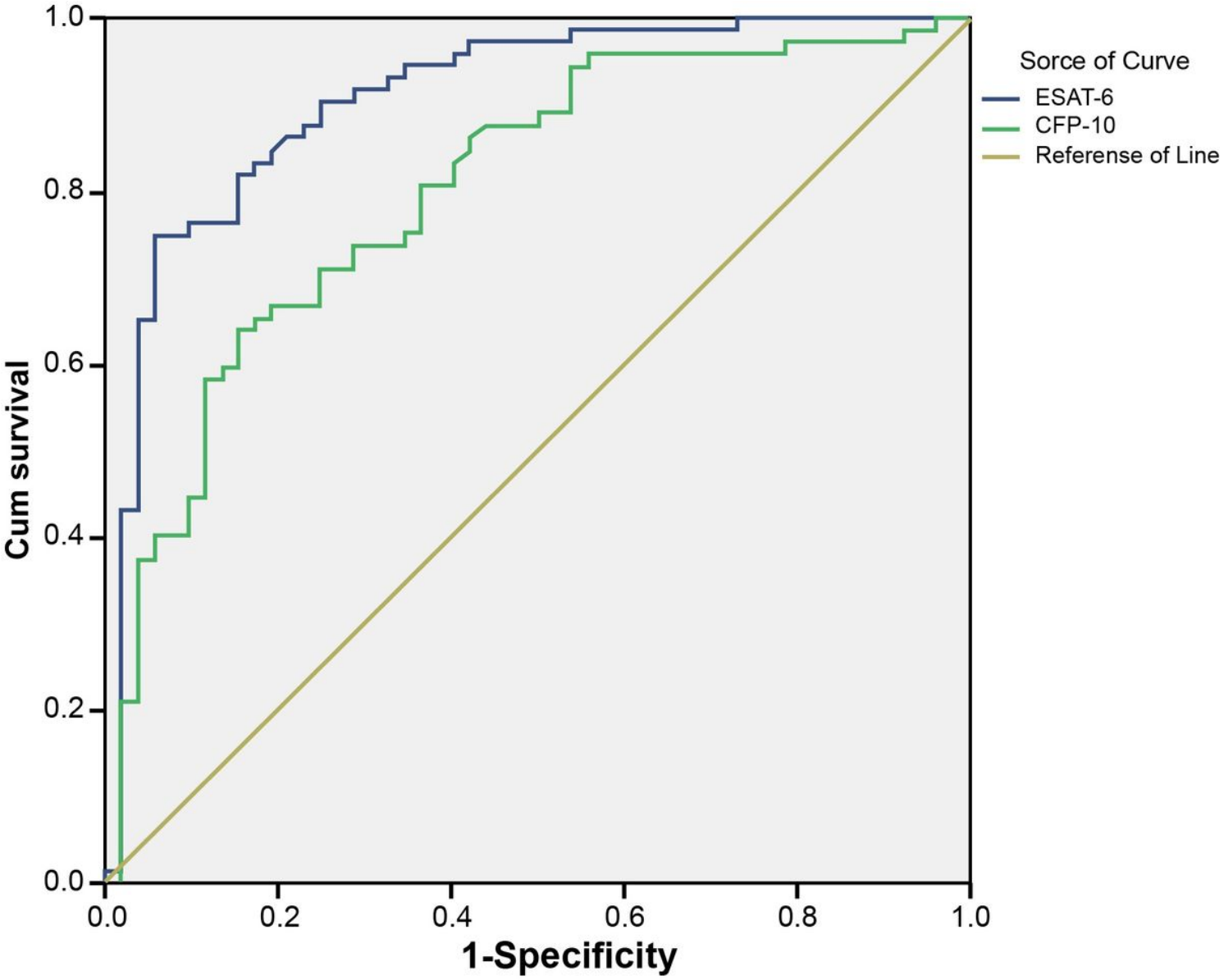

Figure 5

Diagnostic values of the ESAT-6 protein and the CFP-10 protein, as suggested by ROC curves, for the diagnosis of RT with IgAN. Abbreviations: ROC, receiver operating characteristic; ESAT-6, early secretory antigenic target 6; CFP-10, culture filtrate protein 10; IgAN, immunoglobulin A nephropathy; RT, renal tuberculosis.