Early Diagnosis of Tuberculosis-Associated IgA Nephropathy with ESAT-6

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IgA nephropathy (IgAN) is the most common cause of primary renal diseases worldwide, and the early secreted antigenic target of 6 (ESAT-6) which was secreted by Mycobacterium tuberculosis (MTB) may be involved in the development and progression of IgAN. This study aimed to investigate the role of ESAT-6 for early diagnosis of IgAN caused by MTB infection. From 2011 to 2014, 21 patients with renal tuberculosis (RTB), 25 with IgAN, and 46 with IgAN infected with MTB (IgAN/MTB) were enrolled. Serum levels of antibodies against Mycobacterium tuberculosis antigen 85A (Ag85A) were measured by ELISA. Urine culture and phage amplified biologically assay were performed to detect MTB. HE staining was used to observe the morphological changes in kidney tissues. Immunohistochemistry was applied to detect the expression of ESAT-6. Immunofluorescence staining was conducted to detect IgA1. Positive rates of serum anti-Ag85A antibody and urine culture for MTB were higher in the RTB and IgAN/MTB groups than those in the IgAN group. The positive rates of plaques were also higher in RTB and IgAN/MTB groups than the positive rate in the IgAN group. By contrast, the positive rate of ESAT-6 was lower in the IgAN group than that in the RTB group or the IgAN/MTB group, whereas the expression levels of IgA1 were higher in the IgAN and IgAN/MTB groups, compared with the RTB group. Our findings suggest that ESAT-6 and IgA1 may be helpful for early diagnosis of IgAN caused by MTB infection.

Keywords: diagnosis; early secreted antigenic target 6; IgA nephropathy; mycobacterium tuberculosis; renal injury

Introduction

IgA nephropathy (IgAN) is the most common cause of primary renal diseases worldwide, and around one third of IgAN patients will suffer from renal failures within 10 years following diagnosis (Hodgin et al. 2014). Moreover, IgAN is found in 40% of renal biopsy specimens obtained from primary glomerulonephritis (GN) patients in China and Japan, and the number is 30% in Europe and 20% in the United States (Canetta et al. 2014). IgAN consists of primary IgAN and secondary IgAN, which is related to various diseases, including infections, immunological disorders and cancers (Khositseth et al. 2007).

Tuberculosis (TB) is a major public health issue, especially in developing countries. In 2010, about 9 million people developed TB and 1.5 million people died of it (Menzies et al. 2012). As the most common extra-pulmonary TB, genitourinary TB accounts for 15-20% of infections outside lungs (Anwar and Azher 2002). It is widely acknowledged that Mycobacterium tuberculosis (MTB) can infect the urinary tract and result in typical symptoms of cystitis, such as frequency, urgency, and flank pain. However, the effects of MTB on the kidney can be latent. Patients may experience glomerular disease, having advanced renal failure rather than symptoms of classic renal TB sometimes (Hsieh and Wen 2012; Liu et al. 2013; Solak et al. 2013; Waikhom et al. 2014). Furthermore, some IgAN patients should be treated with hormone and immunosuppressant, which may lead to the spread of TB and the deterioration of renal function (Bini et al. 2014). However, whether IgAN could be induced by MTB infection has not been reported in previous studies. Thus, it is important to explore the relationship between MTB infection and the development of IgAN for early diagnosis and treatment.

ESAT-6 (early secreted antigenic target of 6 kDa), secreted by MTB, is a T cell antigen and a promising vaccine candidate (Boggaram et al. 2013). Previously, ESAT-6 was used as a diagnostic reagent to discriminate TB infection from previous Bacillus Calmette-Guérin (BCG) vaccination (Kashyap et al. 2009). However, it also involves in the virulence and pathogenicity of MTB (Boggaram et al. 2013). Based on its ability of binding to laminin and causing lysis of lung epithelial cells, it is suggested that ESAT-6 plays a role in the dissemination of MTB (Kinhi kar et al. 2013).
ESAT-6 also regulates cytokine production through immune cells, such as induction of IL-1 secretion by macrophages (Mishra et al. 2010) and inhibition of IFN-γ production by T-cells (Peng et al. 2011), indicating that it may greatly influence the innate and adaptive immune responses to MTB infection. Gao et al. (2015) have demonstrated that ESAT-6 might contribute to the development of renal injury. In the present study, we aimed to investigate the role of ESAT-6 for early diagnosis of IgAN caused by MTB infection.

Materials and Methods

Subjects

The study was approved by the Ministry of Health Human Biomedical Research Ethics Committee, Cangzhou, China, and was carried out in accordance with the Declaration of Helsinki (M 2014). Informed consent was obtained from each subject, and patient anonymity was also preserved.

A total number of 92 patients (mean age of 46.12 ± 17.52 years, range 34-60 years) hospitalized at Cangzhou Central Hospital from 2011 to 2014 were enrolled. Subjects were divided into three groups: subjects with renal tuberculosis (RTB), subjects with IgAN nephropathy (IgAN) and groups with IgAN infected with MTB into IgAN/MTB group. We enrolled 21 patients (male: 11 cases; female: 10 cases) who had unilateral nephrectomy due to RTB into RTB group, which was confirmed by pathological diagnosis. The IgAN group consisted of 25 patients (male: 13 cases; female: 12 cases) who had no pulmonary or extra-pulmonary tuberculosis (TB). Tuberculin skin tests (TST) and serum TB antibody titers were negative. The IgAN/MTB group had 46 patients (male: 24 cases; female: 22 cases) who met at least two of such three criteria as follows: (1) the subject had history of TB; (2) the TST and serum TB antibody titers were positive; (3) urine culture for acid-fast bacilli in urinary sediment were positive.

Detection of antibodies against Mycobacterium tuberculosis antigen 85A (Ag85A)

The venous blood of patients in each group was collected on admission, placed at the angle of 45-60 degrees in a 4°C refrigerator for 1 h, centrifuged at 3,500 r/min for 3-5 min with a low speed centrifuge and isolated with the supernatant for further use. The titer of serum anti-Ag85A antibody was measured by enzyme-linked immunosorbent assay (ELISA). The serum samples were diluted at 1/50, 1/100, 1/200, 1/400 and 1/800 respectively, and 100 µl Ag85A solution (10 µg/ml) was added to each well to coated the plates and the plates were covered and incubated at 4°C overnight. After blocking with 5% non-fat dry milk solution (BSA) and 1% bovine serum (FCS) at 37°C, the ELISA plates were washed and added the serum samples with different dilutions. The secondary antibodies, goat anti-mouse IgG-HRP (Wuhan Boster Biological Co. Ltd., China), were stained by dianinobenzidine (DAB); and then the optical density at 490 nm (OD490) was read and the absorbance values were calculated. The titer of serum was detected using the ELISA kit (Shanghai Crystal BioTECH Co. Ltd., China) with a 35-kDa recombinant antigen of MTB according to the instructions. The ratio of absorbance of the samples measured by enzyme-labeled fluorescent substrate method and the IgAN group ≥ 2.1 was considered as positive. The titer of the antibody was measured according to the positive samples at the maximum dilution, with the dilution higher than 1/800 counted as 1/800, lower than 1/50 considered as not detected (0).

Urine cultures for MTB

Urine sample was collected from each patient. After removing the supernatant, 20 ml remaining sediment, after centrifugation, was added into 4% sodium hydroxide. A sterile environment, after centrifugation of the supernatant, 6% sulphuric acid solution was added and blended with the sediment. Samples were inoculated on the slant of Roche solid medium, cultured in the incubator at 37°C for a maximum incubation period of 8 weeks. The results were recorded weekly. Ziehl Neelsen (ZN) staining was performed to detect the presence of mycobacteria when colonies were noted.

Phage amplified biologically (PhaB) assay

Colonies grown better in Roche solid medium were scraped, added 1 ml liquid culture medium and several glass balls each 3 mm in diameter. With 0.5 min-oscillation, 5 ml liquid culture medium was added and 0.1 ml supernatant was extracted after 10 min precipitation. Then 0.1 ml phage (1×10⁹ PFU/ml) and the supernatant were placed at 37°C for 60 min, added 0.1 ml antivirus agent and mixed well at room temperature for a period of time. Finally, 5 ml culture medium and 1 ml (1×10⁹/ml) cells indicating MTB were well-mixed with the equal amount of melted agar, which was poured into a plate, cooled and cultured at 37°C for 24 h. The number of plaques was calculated. The positive result was that there appeared plaques with different size and quantity, or multiple plaques fused to each other in a transparent form. While the negative result was that the cells grew evenly in the agar medium and no plaques appeared.

Renal histopathology

Patients in the IgAN and IgAN/MTB groups underwent renal biopsy. Both kidney tissues from each patient were obtained and cut into two halves. Each tissue was cut into 4-µm sections, and then stained with hematoxylin and eosin (H&E). Procedures for renal histopathological examination were conducted as previously described (Jiang et al. 2010). Other specimens were used for ESAT-6 and IgA1 detection.

Immunohistochemistry

Paraffin sections were routinely dewaxed and hydrated, treated with 3% H₂O₂, 5-10 min at room temperature to block endogenous enzyme and washed three times in H₂O₂. Then the sections were put in citrate buffer (pH 6.0, 0.01 M) and then heated to boiling by microwave, repeated 1-2 times with an interval of 5-10 min. After washed 2-3 times in PBS (pH 7.0, 0.1 M), sections were blocked with normal goat serum, and incubated with mouse anti-human primary antibody anti-ESAT-6 (mouse monoclonal antibody HYB 076-08, AntibodyShop, Denmark) at room temperature for 1 h. Then biotinylated goat anti-mouse IgG (Wuhan Boster Biological Co. Ltd., China) was added and incubated at 37°C for 30 min. The sections were added with streptavidin-biotin complex (SABC) (Wuhan Boster Biological Co. Ltd., China), and incubated at 37°C for 30 min. Washed with PBS for 5 min, the sections were stained by DAB four times and also hematoxylin. After dehydration and transparency, samples were sealed and placed under microscope at high magnification. Five high magnifications (400 ×) were randomly selected from
each section for following observation. The integrated optical density of ESAT-6 positive staining was measured by Image pro-plus version 6.0 software (Media Cybernetics, Silver Spring, USA).

**Immunofluorescence**

After heated at 72°C for 1.5 h, the 4-μm thick paraffin sections were dewaxed in dimethylbenzene. Then the sections were put in citrate buffer (pH 6.0, 0.01 M) and then heated to boiling by microwave, repeated 1-2 times with an interval of 5-10 min. After washed 1-2 times in PBS (0.1 M), mouse-anti-human IgA1-FITC antibody (mouse monoclonal antibody 9130-02, SBA Co. Ltd., Germany) was added and the sections were incubated at 37°C for 30 min in wet box. Washed 3 times in PBS, once for 5 min, the sections were sealed with glycerol after drying out at room temperature. Observations with fluorescence microscope and photograph were conducted, with bright green revealing positive IgA1 (wavelength of exciting light was 490 nm). Microscopic image analyzer was used to quantitatively analyze the microscopy images. Each sample was placed under microscope at high magnification (400 ×) for observation. Gray values of IgA1 immunofluorescence staining was also obtained by Image pro-plus version 6.0 software (Media Cybernetics, Silver Spring, USA).

**Statistical analysis**

The SPSS 17.0 software was used for statistical analysis. Continuous data of ESAT-6 IOD value and IgA1 positive staining results were expressed as mean ± standard deviation (Mean ± SD). Comparisons between groups were tested by one-way analysis of variance (ANOVA), and inter-group comparisons were carried out using t test. MTB culture positive rate, renal biopsy pathology results, ESAT-6 positive rate, the accuracy of MTB and ESAT-6 in diagnosing MTB infection were compared using χ² test. IOD value of ESAT-6 and gray values of IgA1 was analyzed by Pearson correlation analysis. P < 0.05 was considered statistically significant.

**Results**

**Clinicopathological features of patients among three groups**

A total number of 92 patients (mean age of 46.12 ± 17.52 years, range 34-60 years) were enrolled in our study. There were no significant differences in age and sex among three groups (all P > 0.05), while there were significant differences in pathology and disease symptoms among three groups (all P < 0.05) (Table 1).

**Comparison of the positive rates of serum anti-Ag85A antibody among three groups**

As shown in Table 2, significant difference was observed in positive rates among three groups (χ² = 11.050, P = 0.026). The positive rates of serum anti-Ag85A antibody in RTB and IgAN/MTB groups were higher than that in IgAN group (χ² = 10.290, P = 0.006; χ² = 8.016, P = 0.018), while there were no significant difference between RTB group and IgAN/MTB group (χ² = 0.342, P = 0.843).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RTB group (n = 21)</th>
<th>IgAN group (n = 25)</th>
<th>IgAN/MTB group (n = 46)</th>
<th>χ²</th>
<th>P</th>
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RTB group, patients with renal tuberculosis; IgAN group, patients with IgA nephropathy; IgAN/MTB group, patients with IgA nephropathy infected with MTB; PPD, purified protein derivative.
Differences in the positive rates of urine culture for MTB among three groups

In RTB group, 7 patients were found to be urine MTB culture positive with a positive rate of 33.33%, while no positive outcomes were observed in IgAN group (Fig. 1). Eight patients in IgAN/MTB group were MTB culture positive (a positive rate of 17.39%). Significant difference was observed in positive rates of MTB culture among three groups ($\chi^2 = 9.373$, $P = 0.009$). The positive rates in RTB and IgAN/MTB groups were higher than that in IgAN group ($\chi^2 = 9.829$, $P = 0.002$; $\chi^2 = 4.900$, $P = 0.027$), while no significant difference was found between RTB group and IgAN/MTB group ($\chi^2 = 2.109$, $P = 0.146$) (Fig. 1).

Comparison of the positive rates of plaques among three groups

In RTB group, 8 patients were found to be urine MTB culture positive with a positive rate of 38.10%, while no positive outcomes were observed in IgAN group (Fig. 2). Twelve patients in IgAN/MTB group were MTB culture positive (a positive rate of 23.91%). There were significant differences in the positive rate of MTB culture among three groups ($\chi^2 = 10.700$, $P = 0.005$). The positive rate of plaques in RTB and IgAN/MTB groups was higher than that in IgAN group ($\chi^2 = 11.530$, $P < 0.001$; $\chi^2 = 7.070$, $P < 0.001$), while no significant difference was found between RTB group and IgAN/MTB group ($\chi^2 = 1.430$, $P = 0.232$).

Morphological changes in kidney tissues of patients in IgAN/MTB and IgAN groups

Among the 46 patients in IgAN/MTB group, 29 (63.04%) patients were diagnosed with IgAN, 9 (19.57%) with non-IgA mesangial proliferative glomerulonephritis (MesProGN), 5 (10.87%) with sclerosing glomerulonephritis (SGN), 2 (4.35%) with minimal change glomerulonephritis (MCGN), and 1 (2.17%) with membranous nephropathy (MN). In IgAN group, 1 (4.00%) patient was diagnosed with IgAN, 2 (8.00%) with MN, 3 (12.00%) with SGN, 5 (20.00%) with MCGN and 14 (56.00%) with MesProGN. The morbidity rate of IgAN in IgAN/MTB group was significantly higher than that in IgAN group ($\chi^2 = 23.141$, $P < 0.001$) (Table 3).

Comparison of ESAT-6 expressions of patients among three groups

In RTB group, renal tissues of all patients were ESAT-6 positive. Brown granular positive deposits were observed in renal interstitium, cytoplasm of renal tubular epithelial cells, glomerular mesangial area and capillary basement membrane (Fig. 3A). In IgAN group, light-brown schistose deposits were observed in renal interstitium in only 2 of the patients (Fig. 3B). In IgAN/MTB group, 30 (65.22%) patients showed ESAT-6 positive.
Brown granular deposits in renal interstitium, cytoplasm of renal tubular epithelial cells, glomerular mesangial areas and capillary basement membranes, and more positive deposits were observed in severely injured areas (Fig. 3C). There were significant differences in ESAT-6 positive rates among three groups ($\chi^2 = 41.742, P < 0.001$) (Table 4), and the positive rates of ESAT-6 in RTB and IgAN/MTB groups were significantly higher than that in IgAN group ($\chi^2 = 38.641, P < 0.001; \chi^2 = 21.421, P < 0.001$).

Integrated optical density (IOD) values of the RTB group, IgAN/MTB group and IgAN group were $53.24 \pm 11.52$, $49.79 \pm 10.26$, and $6.23 \pm 1.35$, respectively (Table 4), and the results showed significant difference among three groups ($F = 218.2, P < 0.001$). Compared to IgAN group, the IOD value were significantly higher in RTB and IgAN/MTB groups ($t = 20.280, P < 0.001; t = 21.060, P < 0.001$).
Comparison of IgA1 expression among three groups

In the IgAN group, yellow-green granular deposits of IgA1 were observed in mesangial areas, capillary loops and part of renal tubular cells (Fig. 4A). In the RTB group, IgA1 expression was negative (Fig. 4B). In the IgAN/MTB group, IgA1 was detected in 39 patients (29 with IgAN, 9 with MesProGN, and 1 with SGN), and the yellow-green granular deposits were mainly observed in the mesangial areas, capillary loops and part of renal tubular cytoplasm (Fig. 4C), similar to the IgAN group (Fig. 4A). No IgA1 was detected in the renal tissues from 7 patients, including 4 patients with SGN, 2 with MCGN and 1 with MN. The average gray values of patients in the IgAN/MTB, RTB, and IgAN groups were 59.91 ± 4.35, 10.13 ± 1.41, and 58.05 ± 4.12, respectively (Fig. 4D), which showed the significant statistical differences (F = 1585, P < 0.001). The average gray values in the IgAN and IgAN/MTB groups were markedly higher than that in the RTB group (t = 54.57, P < 0.001; t = 55.50, P < 0.001). However, there was no significant difference between the IgAN group and the IgAN/MTB group (t = 1.650, P = 0.104).

Diagnostic values of urine culture for MTB and ESAT-6 detection for renal MTB infection

The diagnostic values of MTB cultured with urine and ESAT-6 detection for renal MTB infection are shown in Table 5. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of urine cultures for MTB and ESAT-6 in diagnosing MTB infection in RTB group and IgAN group were 33.33%, 100.00%, 100%, 64.10%, 69.56% and 100.00%, 92.00%, 91.30%, 100.00%, 95.65%, respectively. The sensitivity (χ² = 21.000, P < 0.001), negative predictive value (χ² = 10.661, P = 0.001) and accuracy (χ² = 10.891, P = 0.001) of ESAT-6 were significantly higher than those of MTB cultured with urine in diagnosing renal MTB infection. However, there were no significant differences observed in the specificity and positive predictive value (both P > 0.05).

Correlation between ESAT-6 expression and the pathogenesis of IgAN

Among the 30 patients with positive ESAT-6, 29 were positive for IgA1 with the positive rate of 96.7% (Fig. 5), while 10 patients were positive for IgA1 (the positive rate = 62.5%) among 16 patients with negative ESAT-6. The χ² test confirmed that the positive rates of IgA1 between the ESAT-6-positive patients and the ESAT-6-negative patients showed a significant difference (χ² = 9.442, P = 0.002) (Fig.
The results of Pearson correlation analysis showed that there was a positive correlation between the IOD value of ESAT-6 and the gray values of IgA1 ($r = 0.71$, $P < 0.001$) (Fig. 6).

Discussion

The main results of this study showed that the MTB could be a crucial marker for renal diseases, while ESAT-6 might serve as a more accurate and effective biomarker for renal injury compared with it. Moreover, the correlation was observed in the positive detection of ESAT-6 and IgA1 showed, indicating that MTB infection-induced renal injury was associated with the IgAN pathogenesis. Histologically, renal involvement in TB includes epithelioid granuloma, with or without caseation, and general Langhans-type giant cells (Daher Ede et al. 2013). MTB can cause renal diseases through multiple pathways, among which genitourinary TB via direct invasion by the bacilli is most common (Eastwood et al. 2001). Immune response to MTB infection is primarily mediated by T cells (Lockhart et al. 2006). In previous research of human pulmonary TB, T cells responding to ESAT-6 are observed in approximately half of the patients (Guinn et al. 2004; Reiley et al. 2008). ESAT-6, a vital immune-dominant antigen encoded by region of difference-1 (RD1) and RD2, has been reported to have the potential for human MTB diagnosis (Sang and Zhang 2012; Li et al. 2014). Consistent with the study results of Macedo et al. (2011), our results demonstrated that the positive rate of ESAT-6 in RTB group and IgAN/MTB group was significantly higher than that in IgAN group, suggesting that ESAT-6 could be of diagnostic value for patients with RTB, because of its important role in the

<table>
<thead>
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<th>MTB cultured with urine</th>
<th>ESAT-6</th>
<th>$\chi^2$</th>
<th>$P$</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>33.33%</td>
<td>100.00%</td>
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<tr>
<td>Specificity</td>
<td>100.00%</td>
<td>92.00%</td>
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<tr>
<td>Positive predictive value</td>
<td>100.00%</td>
<td>91.30%</td>
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<tr>
<td>Negative predictive value</td>
<td>64.10%</td>
<td>100.00%</td>
<td>10.661</td>
</tr>
<tr>
<td>Accuracy</td>
<td>69.56%</td>
<td>95.65%</td>
<td>10.891</td>
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Table 5. The diagnostic values of urine cultures for MTB and ESAT-6 detection in the renal tissues for renal MTB infection.
innate and adaptive immune responses (Macedo et al. 2011). Further comparison between urine culture for MTB and detection of ESAT-6 showed that the sensitivity, negative predictive value, and accuracy of ESAT-6 were significantly higher than those in urine cultures for MTB in diagnosing RTB.

We also assessed the association between IgA1 positive rate and RTB in the present research. The results revealed that, similar to ESAT-6, IgA1 positive rate were higher in IgAN/MTB group than that in IgAN group. Furthermore, the average gray values were significantly different among three groups, indicating that IgA1 might also serve as a biomarker for the RTB diagnosis. Due to its role in humoral immune system, acute MTB infection is correlated with a marked increase in serum IgA levels, which includes the presence of IgA antibodies against A-60 mycobacterial antigen, IgA immune complexes and mycobacterial antigens in the serum of patients with active TB (Solak et al. 2013). Deposition of these complexes in turn may activate the alternative complement and the lectin pathway (Roos et al. 2006), with resultant local injuries leading to IgA nephropathy.

In our study, it was observed that positive detection result of ESAT-6 might be associated with IgA1 positive, and the IOD value of ESAT-6 was positively related to the gray values of IgA1, suggesting the degree of IgA1 deposition might be associated with the deposition degree of ESAT-6, and it might be also related with the severity of MTB infection induced RTB, for that ESAT-6 is a dominant antigen for cell-mediated immunity and a major target for memory T cells (Horwitz et al. 2009). In this study, we found that the positive rates of MTB and ESAT-6 as well as the average gray values of IgA1 deposition in RTB group and IgAN/MTB group were significantly higher than those in the IgAN group, meaning that the MTB, ESAT-6, and IgA1 deposition could be of potential diagnostic value in patients with RTB.

In conclusion, our findings suggested that the renal injury induced by MTB may be associated with the pathogenesis and progression of IgAN. ESAT-6 and IgA1 may be helpful for early diagnosis of IgAN caused by MTB infection. In view of the relatively small sample size in our study, the results of this study will need to be further confirmed by studies with larger sample sizes.

**Conflict of Interest**

These authors declare no conflict of interest.

**References**


