

## Original Article

# Roles of *mycobacterium tuberculosis* ESAT-6 in the development of renal injury

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**Abstract:** Objective: The present study was designed to evaluate the role of *mycobacterium tuberculosis* early secretory antigen target-6 (MtbESAT-6) in the development of renal injury. Methods: PET42a (+) ESAT6 prokaryotic expression plasmid was constructed and the purified ESAT6 protein without endotoxin was obtained. Sixty healthy, clean, male Kunming mice were randomly divided into two groups: the experimental group (n = 30) and the control group (n = 30). Each mouse in the experimental group were injected with 0.5 ml ESAT-6 protein, and each mouse in the control group were injected with 0.5 ml sterile saline on the tail vein. Blood, urine and kidney tissues were collected. Serum creatinine (Scr), blood urea nitrogen (BUN), and urinary creatinine (Cr) were determined by HITACHI 7150 automatic biochemical analyzer and creatinine clearance rate (Ccr) was calculated. Renal tissues were conducted for hematoxylin-eosin (HE) staining and pathological scores of renal injury were recorded under the light microscope. Results: Using MTB H37Ra strains genome DNA as template, the ESAT6 gene amplified by Hieff Pfu DNA Polymerase using polymerase chain reaction (PCR) technique was consistent with the expected size. PET42a (+) ESAT6 vector plasmid was successfully obtained and ESAT6 recombinant protein was successfully expressed with the protein concentration of 1.69 mg/ml. BUN and Scr in the experimental group were gradually increased, Ccr was gradually decreased, and the pathological score of renal injury increased gradually, and all of which were significantly higher than that in the control group after the experiment of 12 h, 24 h and 48 h (all  $P < 0.05$ ). Conclusion: MtbESAT-6 might contribute to the development of renal injury.

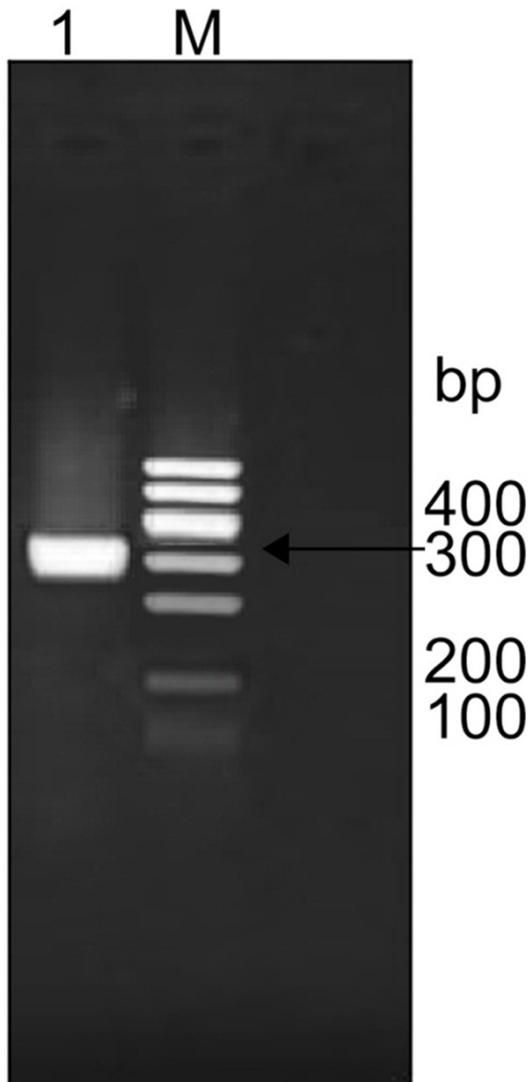
**Keywords:** Mycobacterium tuberculosis, early secretory antigen target-6, renal injury, serum creatinine, blood urea nitrogen, creatinine clearance rate

## Introduction

Renal injury, also called kidney injury, has the characteristics of sudden and continued decline in glomerular filtration rate, which results in the accumulation of urea together with other chemicals in the blood [1]. The two main forms of renal injury are acute kidney injury (AKI) and chronic kidney disease (CKD), with the former often reversible with adequate treatment, and the latter not reversible [2]. The incidence of renal injury was 22.7% among the 19,249 hospitalizations in an analysis, and Blacks, older persons, as well as patients with reduced baseline kidney function were more susceptible to renal injury [3]. As a major form of renal injury, AKI patients who admitted to the pediatric intensive care unit (PICU) is related to a > 5 time increase in mortality [4]. Risk factors for renal injury include: age; sepsis; diabetes mellitus; hypovolaemia; hypotension; pre-exist-

ing hepatic, renal, or cardiac dysfunction; and exposure to nephrotoxins [5]. In renal injury, there may be problems with increased acid levels, increased fluid in the body, raised levels of potassium, increased levels of phosphate, decreased levels of calcium, and in later stages anemia [6]. The occurrence of renal injury has close associations with high financial costs economically, considerable morbidity, and also with excess early and late mortality [7]. Recently, early secreted antigenic target-6 (ESAT-6) was reported to be a novel and promising biomarker for renal injury [8].

ESAT-6, a low molecular weight protein secreted by *mycobacterium tuberculosis* (MTB), is a T cell antigen and a promising vaccine candidate [9]. ESAT-6 exists mainly in pathogenic mycobacteria, but not in all bacillus calmette Guerin strains or in most environmental mycobacteria [10] ESAT-6 has been suggested to play an



**Figure 1.** PCR amplification of ESAT6 gene (M: standard DNA Marker; 1: plasmid DNA PCR product) (PCR: polymerase chain reaction; ESAT, early secretory antigenic target).

essential role in the dissemination of MTB and it contributes to granuloma formation through induction of epithelial cell matrix metalloproteinase-9 (MMP-9) [11]. ESAT-6 also regulates cytokine production by immune cells, such as induction of IL-1 $\beta$  secretion by macrophages and inhibition of IFN- $\gamma$  production by T-cells, indicating that it may have important influence in the innate and adaptive immune responses to MTB infection [12]. In addition, gene encoding ESAT-6, together with its partner culture filtrate protein (CFP)-10, form heterodimer that is secreted by specialized ESAT-6 secretion system, called ESAT-6 secretion system 1 (ESX-1)

[13, 14]. Some observations indicated that ESAT-6 has become a diagnostic tool for monitoring active tuberculosis (TB) with great potential and high degree of specificity, suggesting that ESAT-6 become the target molecule for TB vaccine [15, 16]. However, there are still no reports on the expression of ESAT-6 in renal injury. Therefore, our study aims to observe the functions of ESAT-6 in renal injury, further explore the correlation between the antigen and renal injury, which provides new ideas for the early diagnosis of renal injury.

#### Materials and methods

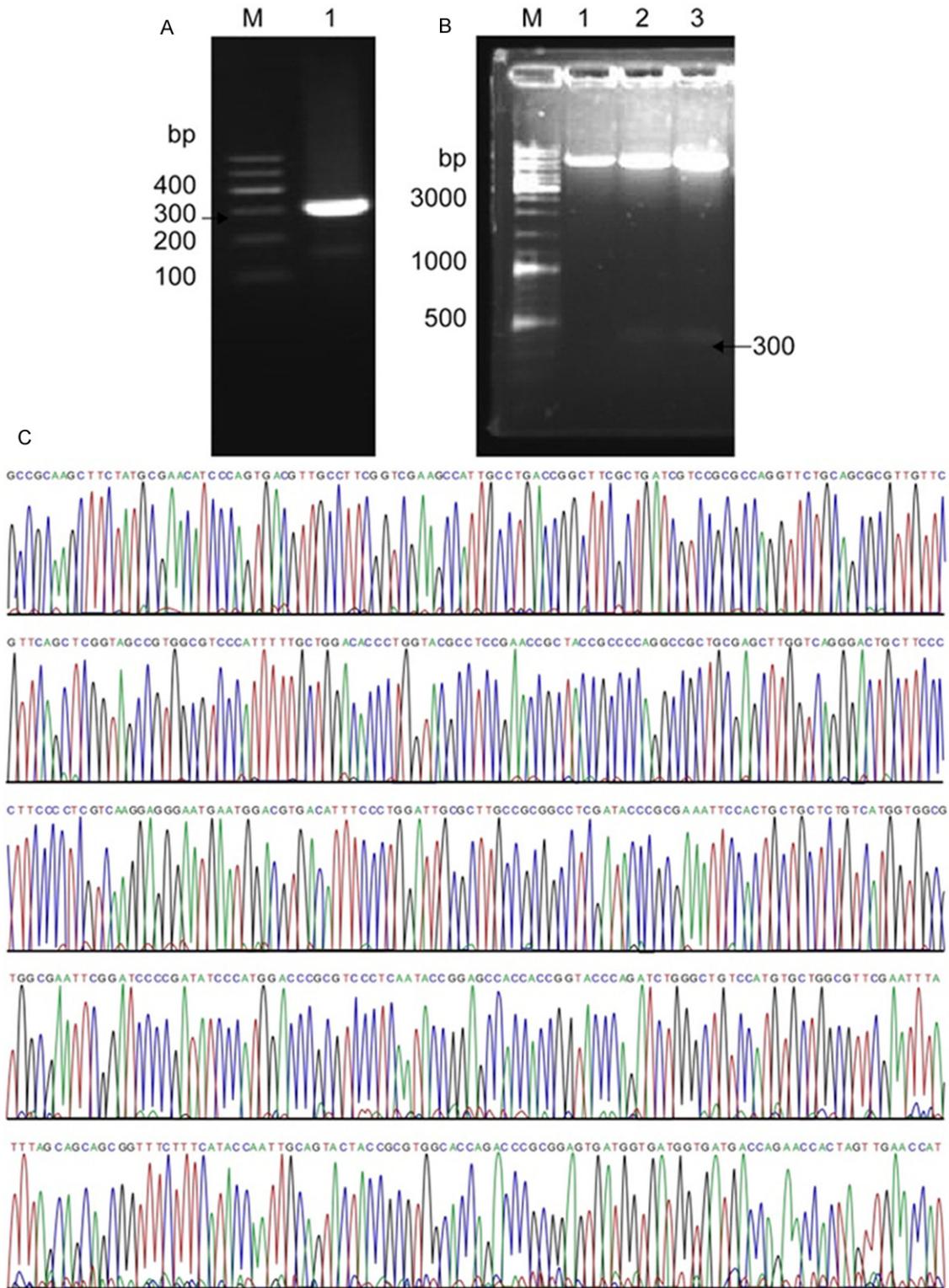
##### *Ethics statements*

All animal procedures were conducted based on the approval from the Medical Ethics Review Board of Shaanxi Provincial People's Hospital. All procedures in our study were conducted strictly based on the protocol of the Care and Use of Laboratory Animals of the National Institutes of Health [17].

##### *Culture of MTB H37Ra strain and extraction of genomic DNA*

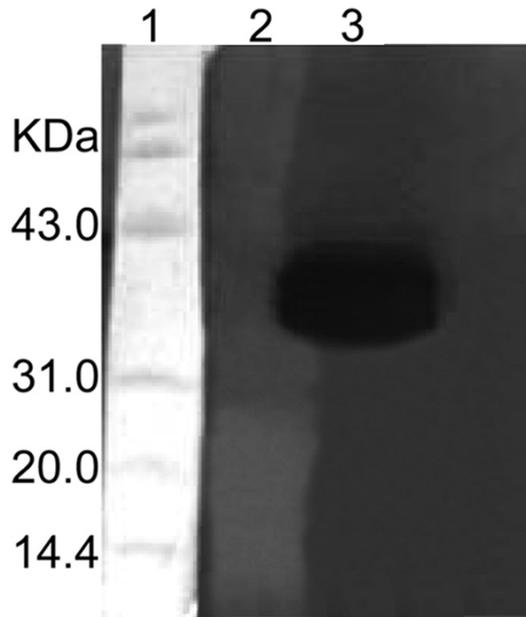
MTB H37Ra strain (lot no. 9302025), which taken from Beijing Strain Preservation Center of China Institute of Drug Control, conducted transfer culture at 37°C for 21 d by inoculating in solid 7H11 slant medium, and then conducted liquid fast enrichment culture at 37°C for 3-4 weeks by inoculating in 7H9 liquid medium. MTB H37Ra in appropriate amount were put into 1.5 ml evoked potential (EP) tube and centrifuged at 3800 rpm for 15 min for the collection of the MTB H37Ra. The tube was added with 600  $\mu$ L triethylene diamine tetraacetic acid (TE) buffer to suspend the MTB H37Ra, 60  $\mu$ L 10 $\times$  lysate, 30  $\mu$ L 10 mg/mL proteinase K, and 6  $\mu$ L 10 mg/mL RNAase, and water-bathed at 56°C for 1 h, boiled at 100°C for 10 min, and centrifuged at 13000 rpm for 10 min, and then the supernatant was transferred to another sterilized EP tube. The equal volume of phenol was added into the EP tube and the tube was centrifuged at 13000 rpm for 10 min, and then the supernatant was transferred to a third sterilized EP tube. The 0.1 times volume of 3 M sodium acetate and 2 times volume of pre-cooled anhydrous ethanol were added to fully mix with the supernatant, and the mixture was placed at -20°C for 1 h, centrifuged at 13000

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**Figure 2.** Identification of prokaryotic expression vector pET42a (+)-ESAT6 (A: PCR identification of pET42a (+)-ESAT6 vector plasmid; M: standard DNA Marker; 1: plasmid DNA PCR product; B: double enzyme digestion identification chart of pET42a (+)-ESAT6 vector plasmid; M: standard DNA Marker; 1: pET42a (+) double enzyme digestion product; 2-3: recombinant plasmid double enzyme digestion product; C: pET42a (+)-ESAT6 sequencing map (PCR: polymerase chain reaction; ESAT, early secretory antigenic target).

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**Figure 3.** Western blot analysis and identification plot (1: protein Marker; 2: non-induced bacterial protein; 3: purified protein).

rpm for 10 min, washed two times with 70% precooled ethanol and centrifuged at 5000 rpm for 5 min. After the precipitation was air dried at room temperature, 100  $\mu$ L TE buffer was used to dissolve the precipitation, and 2  $\mu$ L of the solution was taken to conduct agarose gel electrophoresis and DNA concentration.

### *PCR amplification of MTB ESAT6 gene*

The primer Premier5.0 software was used to design primer: forward primer: 5'-CGGAATTCGC-CACC ATGACAGAGCAGCAGTGG-3'; 5'-GCACGC-CCCAAGCTTCTATGCGAACATCCCAGT-3'. The primers were synthesized by Nanjing GenScript Biological Technology Co., Ltd. The reaction conditions were as follows: pre-denatured at 94°C for 5 min; denatured at 94°C for 1 min, annealed at 57°C for 1 min, and extended at 72°C for 1.5 min, totally 36 cycles; and extended at 72°C for 10 min after the last cycle. Purification of the product was in accordance with the manufacturer's instruction of the PCR product purification kit from the Sangon Biotech (Shanghai) Co., Ltd.

### *Construction and identification of eukaryotic expression vector of ESAT6 gene*

According to the manufacturer's instruction of plasmid DNA small extraction kit from BioMIGA

Company, the plasmid was extracted to further prepare plasmid vector pET42a (+). The DNA double enzyme digestion (restriction enzyme EcoR I and Hind III) was conducted with 20  $\mu$ L standard reaction system of DNA restriction digestion from Fermentas Company, and identification and recovery of double enzyme digestion products were conducted with the manufacturer's instruction of Ary Prep DNA gel recovery kit. Escherichia coli DH5 $\alpha$  and BL21 (DE3) competent cells were prepared, linear vector and inserted DNA fragments were connected with T4 DNA ligase at the molar ratio of 1:5. Five single bacterial colonies were randomly selected from the conversion plate, inoculated into 3 ml Luria Broth (LB) liquid medium containing kana+, and oscillated cultured overnight at the temperature of 37°C. The extraction of plasmid DNA was performed according to the plasmid DNA small extraction kit from BioMIGA Company. As for determination of the PCR and double enzyme digestion, the double enzyme digestion was performed with restriction enzyme EcoR I and Hind III from TaKaRa Company. The positive clones which correctly verified by enzyme digestion were sent to Nanjing GenScript Biological Technology Co., Ltd for sequencing. The correctly sequenced recombinant expression plasmid was transformed to BL21 E.coli (DE3), and the name of correctly identified recombinant expression plasmid was pET42a (+)-ESAT6.

### *Expression, purification and identification of MTB ESAT6 protein*

After the transfer of the recombinant expression plasmid pET42a (+)-ESAT6 to Escherichia coli expression host BL21 (DE3), positive clones were selected into LB culture medium containing 30 mg/L kanamycin, oscillated cultured overnight at the temperature of 37°C, inoculated after diluted at the ratio of 1:50 the next day, added with Isopropyl  $\beta$ -D-1-Thiogalactopyranoside (IPTG) (American Fluka Company, America) into bacteria liquid to the final concentration of 1 mmol/L when A600 was about 0.6, and respectively conducted induced expression for 6 h at 30°C and 37°C. Strains containing recombinant plasmid were cultured with large scale, and thalluses were collected when strains were centrifuged at 4000 rpm for 15 min at the temperature of 4°C after IPTG induction. The 0.1 times culture

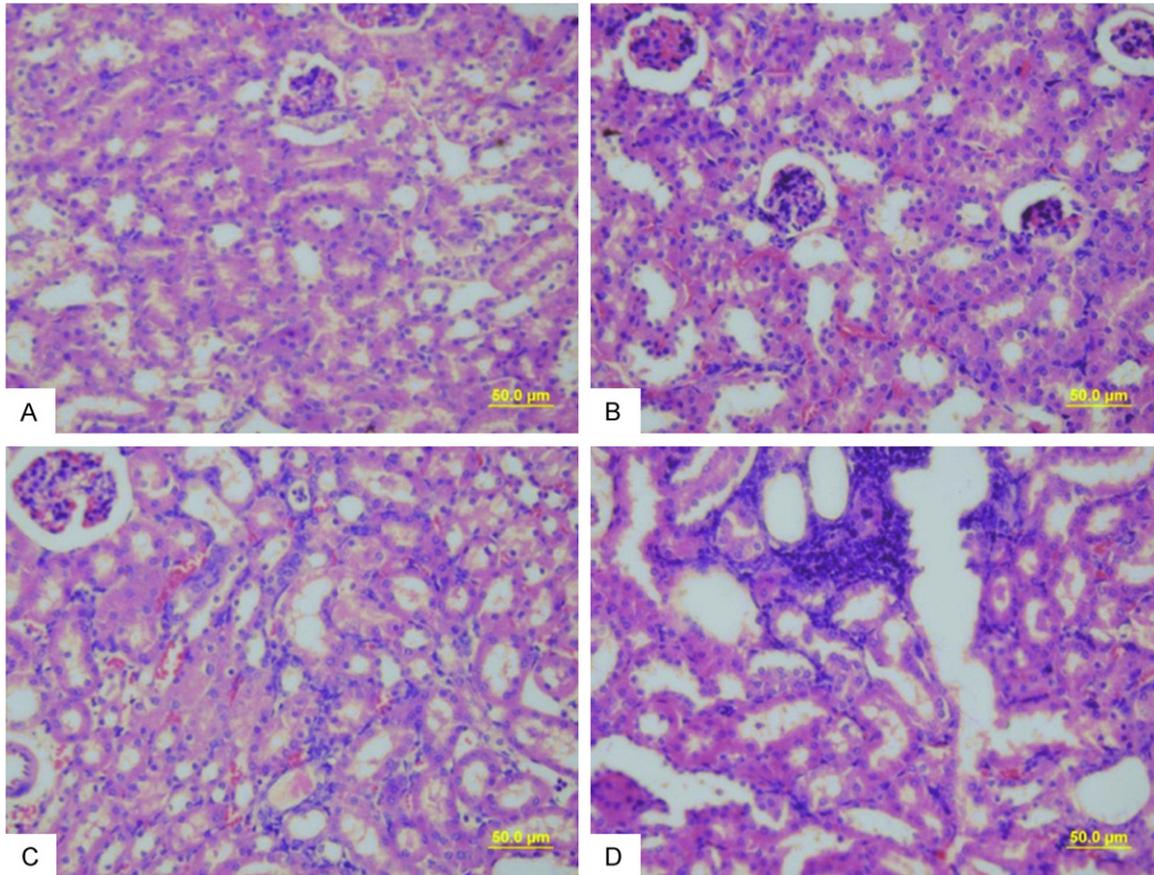
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**Table 1.** Comparisons of serum creatinine, blood urea nitrogen, and creatinine clearance rate in the experimental group and the control group

	Before experiment		12 h after experiment		24 h after experiment		48 h after experiment	
	Control group	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group	Experimental group
Ccr (mmol/L)	0.681 ± 0.035	0.684 ± 0.037	0.683 ± 0.036	0.545 ± 0.039*	0.682 ± 0.037	0.394 ± 0.025*	0.683 ± 0.036	0.284 ± 0.021*
BUN (mmol/L)	7.28 ± 1.05	7.31 ± 1.07	7.29 ± 1.06	12.61 ± 2.15*	7.30 ± 1.07	16.54 ± 2.43*	7.29 ± 1.05	19.25 ± 2.69*
Scr (μmol/L)	71.45 ± 6.95	72.36 ± 7.02	71.48 ± 6.99	91.25 ± 10.87*	71.50 ± 6.98	105.23 ± 15.79*	71.49 ± 7.01	121.64 ± 20.17*

\*Refers to  $P < 0.05$  when compared with the control group. Ccr, creatinine clearance rate; BUN, blood urea nitrogen; Scr, serum creatinine.

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**Figure 4.** Hematoxylin-eosin (HE) staining results for renal tissues in the experimental group and the control group (400 ×), A: kidney tissues in the control group; B: kidney tissues after the experiment of 12 h; C: kidney tissues after the experiment of 24 h; D: kidney tissues after the experiment of 48 h.

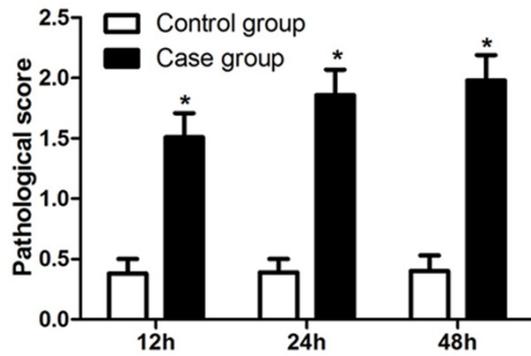
medium volume of PBS suspension was used, lysozyme was added to the final concentration of 100 μg/mL and 30% TritonX-100 was added to the final concentration of 1%, and the above mixture was water-bathed at 37°C for 30 min, ultrasonic processed in ice bath, and centrifuged at 13000 rpm for 20 min, and finally the supernatant was collected. The supernatant was diluted with PBS and filtered by 0.22 μm filter membrane, and then purified by glutathione S-transferase (GST) purification resin (Shanghai Rick Biological Technology Co., Ltd., Shanghai, China). The eluent which purified by GST was further purified by His affinity layer (General Electric company, fairfield, Connecticut, USA). After the purified protein was SDS-PAGE electrophoresed, wet transfer method was adopted to transfer protein bands on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to polyvinylidene fluoride (PVDF) membrane. The anti-

ESAT6 mouse monoclonal antibody was regarded as the primary antibody (Wuhan Boster Biological Engineering Co., Ltd, Wuhan, China) and HRP-goat anti mouse IgG as the secondary antibody (Wuhan Boster Biological Engineering Co., Ltd). Expression of recombinant target protein was identified with Western Blot. The removal of endotoxin was conducted with the manufacturer's instruction of endotoxin-removed kit from the Nanjing GenScript Biological Technology Co., Ltd.

### *Experimental animal and grouping*

Sixty healthy, clean, male Kunming mice (age: 12 weeks; body weight: 20 ± 2 g) were provided by Medical Laboratory Animal Center of China Medical University. These mice were adaptively fed for 2 days in different cages with the temperature of 15°C~25°C, the relative humidity of 40%~55%, natural alternation for day and night, and natural lighting conditions in the ani-

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**Figure 5.** Pathological score of renal injury for mice at the different time points in the experimental group and the control group (\*refer to  $P < 0.05$  when compared to the control group).

mal laboratory. These mice were fed with standard feedstuff which provided by the department of animal laboratory, and they intake food and drink water freely. The laboratory should have clean environment and good ventilation condition, and without noise. All the experiments were carried out in accordance with the experimental animal care and user guide. These mice were randomly divided into two groups: the experimental group ( $n = 30$ ) and the control group ( $n = 30$ ). Each mouse in the experimental group were injected with 0.5 ml ESAT-6 protein (20  $\mu\text{g}$  recombinant ESAT-6 protein was dissolved in 0.5 ml sterile saline) in the tail vein, and each mouse in the control group were injected with 0.5 ml sterile saline in the tail vein. Each group was divided into three subgroups at the time point of 12 h, 24 h and 48 h with each subgroup of 10 mice, respectively.

### *Collection and preliminary processing of samples*

All the mice intake food freely, and their urines were collected for 24 h. They were intraperitoneally injected with 2% pentobarbital sodium and were collected 0.8 ml blood by removing the right eye after anesthesia satisfaction. Blood samples were centrifuged, and the left serum was stored at  $-20^{\circ}\text{C}$  to prepare for the detection of renal function. After the blood was taken, the mice were fixed on the operation table, and followed with surgical skin preparation, 2% iodine disinfection, and asepsis hole-towel spreading. The upper abdomens of mice were cut in the middle, with their right kidneys revealed, the renal pedicles cut and the kidneys taken out. The kidneys were washed with

normal saline and placed in 4% paraformaldehyde after drying by filter paper. After the collection of specimens, mice were executed by spinal dislocation method. The kidney specimens were cut into slices after treated with dehydration, paraffin soaking, and paraffin embedding, etc.

### *Biochemical assay*

Serum creatinine (Scr), blood urea nitrogen (BUN), and urinary creatinine (Cr) were determined by HITACHI 7150 automatic biochemical analyzer. Creatinine clearance rate (Ccr) were calculated with urine volume per minute ( $\text{ml}/\text{min}$ )  $\times$  urinary Cr concentration ( $\text{mg}/\text{L}$ )/Scr concentration ( $\text{mg}/\text{L}$ ).

### *HE staining and pathological score of renal injury*

The slices were dewaxed with dimethylbenzene, dehydrated with 95%-75% absolute ethyl alcohol, dyed with haematine for 10 min, soaked in water for 5 min, rested in 1% hydrochloric acid alcohol for 3 s, rinsed with water for 20 min, dyed with eosin solution for 10 min, dehydrated with gradient alcohol, added with half a drop of neutral balsam to sealing the slices. HE stained slices were observed under the optical microscope. Each slice under the light microscope (400 times) was randomly selected five non-overlapping fields of view, and double blind observation was conducted by two pathological experts, respectively. The degree of degeneration, cloudy swelling, necrosis and interstitial hyperemia, and edema of vacuole in the renal tubular epithelial cell were observed. The pathological score of renal injury was performed under the light microscope referring to the methods reported by Leelahavanichkul [18].

### *Statistical analysis*

The statistical analysis was performed with SPSS 19.0 statistical software. Using  $t$  test, the measurement data were expressed in the form of mean  $\pm$  standard deviation (SD).  $P < 0.05$  was showing a statistical significance.

## **Results**

### *PCR amplification of ESAT6 gene*

Using MTB H37Ra strain genome DNA as template, the *ESAT6* gene was amplified by Hieff

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Pfu DNA Polymerase using PCR technique. The agarose gel electrophoresis showed a target band of about 300 bp, which was consistent with the expected size (**Figure 1**).

### *Identification of prokaryotic expression vector pET42a (+)-ESAT6*

The positive clones of extracted plasmid were selected for PCR identification, and agarose gel electrophoresis showed a specific target band of about 300 bp (**Figure 2A**); double restriction enzyme EcoR I and Hind III cataphoresis showed two specific target bands of about 3000 bp and 300 bp, which was consistent with the expected sizes (**Figure 2B**). The positive clones which correctly verified by enzyme digestion was sent to Nanjing GenScript Biological Technology Co., Ltd for sequencing (**Figure 2C**). The positive clones were completely consistent with the standard strain H37Ra genome sequence and standard strain H37Ra ESAT6 gene sequence via the online BLAST comparison, showing PET42a (+)-ESAT6 vector plasmid was successfully obtained.

### *Expression of ESAT6 protein analyzed and identified by Western blot*

Western blot analysis and identification conducted on expression product was used mouse anti ESAT6 monoclonal antibody, there showed specific positive bands at the relative molecular weight of about 33 kDa, and the specific positive band wasn't found in the non-induced thal- lus, suggesting the successful expression of ESAT6 recombinant protein (**Figure 3**). The OD595 value of endotoxin-removed protein samples was measured by Coomassie brilliant blue staining, and the protein concentration was 1.69 mg/ml.

### *Comparison of renal function in two groups of mice at different time points*

As shown in **Table 1**, there was no significant difference for Ccr, BUN and Scr between the experimental group and the control group before the experiment (all  $P > 0.05$ ). The Ccr in the experimental group was gradually decreased and was significantly lower than that in the control group after the experiment of 12 h, 24 h and 48 h (all  $P < 0.05$ ), while BUN and Scr in the experimental group were gradually increased and were significantly higher than

that in the control group after the experiment of 12 h, 24 h and 48 h (all  $P < 0.05$ ).

### *Pathological changes of renal injury in two groups of mice*

**Figure 4** shows the pathological changes of renal injury in two groups of mice. In the control group: the glomerular was densely distributed, round or oval with regular peripheral; Bowman's space was normal, renal tubules had coarse diameter and small lumen and there was no hyperemia, hemorrhage and inflammatory cell infiltration for peripheral capillary of renal tubules. In the experimental group: different degrees of expansion of Bowman's capsule, narrowing of renal tubular, hyperemia, hemorrhage and inflammatory cell infiltration for peripheral capillary of renal tubule, degeneration, swelling, atrophy for tubular cells were found at different time points. The degree of renal injury was aggravated over time.

### *Pathological score of renal injury for mice*

As shown in **Figure 5**, the pathological scores of renal injury were gradually increased at the time points of 12 h, 24 h and 48 h in the experimental group, and the scores in each time points were significantly higher than those in the control group (all  $P < 0.05$ ).

## Discussion

Our study confirmed that MtbESAT-6 might contribute to the development of renal injury, and MTB infection can cause TB-induced renal injury, suggesting that ESAT-6 secreted by MTB might be a significant antigen leading to renal injury. ESAT-6, a highly immunogenic protein secreted by MTB via ESX-1, is capable of lysing destabilize phagolysosomes, alveolar macrophages and epithelial cells, and activating the inflammation complex which can induce immune reaction [19]. ESAT-6 protein has a plurality of T and B cell epitopes, and it can be widely identified by infected TB cells of different species of animals or with different genetic backgrounds in the early stage of infection, which play significant roles in the protection of cellular immune responses, and mediated long-lasting immune memory [20]. ESAT-6, recognized by specific IFN- $\gamma$ -secreting T cells, is an immunodominant T cell stimulatory antigen, which is present in lots of patients who have

infections in comparisons with those who are uninfected with MTB [8]. TB-induced renal injury is the mainly form of renal injury caused by MTB which can trigger allergic reaction and immune response inducing the release of inflammatory factors and lymphocytotoxin, as well as the infiltration of inflammatory cells in early phase of invading the kidney [21, 22]. The formation of antigen-antibody complexes of MTB involves the antibodies binding to homologous native protein, and these antigen-antibody complexes then are released into the sub-epithelial space after being capped [23]. If not entirely eliminated, that antigen-antibody complex could be firmly attached to the glomerular basement membrane (GBM) in kidneys, and persist as immune deposits which can induce the formation of the complement membrane attack complex and then result in immune injury of kidney [24]. Furthermore, the antigen-antibody complexes might be accumulated on glomerular capillary walls, which lead to glomerular inflammatory mediated by immune complex [25]. Additionally, in our study, the pathological score of renal injury in the experimental group increased gradually, which was significantly higher than that in the control group at the time point of 12 h, 24 h and 48 h, which also provide evidence for the confirmation of MtbESAT-6 contributing to the development of renal injury. Therefore, in view of the relatively higher sensitivity and specificity, we recommended the new modality for diagnosing renal injury through detecting the expression of ESAT-6 in renal tissues.

Another important finding in this study was that BUN and Scr in the experimental group were gradually increased and Ccr was gradually decreased in the experimental group after the experiment of 12 h, 24 h and 48 h. Under normal circumstances, glomerular acts as the molecular sieve, and only allows water and small molecules passing by, and only 3% of hemoglobin molecule (molecular weight 64500) and less than 1% of plasma albumin (molecular weight 69000) can be filtered [26, 27]. While in pathological conditions, any lesions that can cause increased permeability of the GBM will show urinary albumin, revealing that there appears a subclinical increase in urinary albumin [28]. In addition, BUN is the main end products in the metabolism of human protein, which was identified as the first biochemi-

cal index by the medical community for the evaluation of renal function [29]. But in clinical, only when the glomerular filtration rate in patients decreased to less than half of normal values, the concentration of BUN would be increased slowly [30]. SCr is the most commonly used index for detecting renal function, but also the required inspection item in health examination [31]. SCr can accurately reflect the degree of renal parenchyma damage, while it cannot be describes as a sensitive indicator for the diagnosis of renal injury, for the reason that SCr was significantly increased only when glomerular filtration rate decreased to 1/3 of normal values [32, 33]. In clinical practice, Ccr or estimates of creatinine clearance, a composite index of kidney function, based on the SCr level are used to measure GFR [34]. In the case of a fixed amount of urine, Ccr not only reflects the function of glomerular filtration, but also shows the function of renal tubules [35]. In our study, the changes of these three indexes of patients in the experimental group show that they can be used to evaluate whether patients underwent renal injury.

In conclusion, these data indicated that increased concentration of ESAT-6 could contribute to renal injury, and MTB infection can cause TB-induced renal injury, indicating that the detection of ESAT-6 can provide a molecular basis for early diagnosis of renal injury.

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### Disclosure of conflict of interest

None.

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