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# HMGB1, TGF-β and NF-κB are associated with chronic allograft nephropathy

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**Abstract.** The present study aimed to investigate the association between high mobility group protein B1 (HMGB1), transforming growth factor-β1 (TGF-β1), nuclear factor-κB (NF-κB) and chronic allograft nephropathy (CAN) and to identify the clinical significance of HMGB1, TGF-β1, NF-κB on patients with CAN. Between September 2012 and November 2014, 27 patients with CAN diagnosed by biopsy were enrolled in the present study and a further 30 patients that underwent nephrectomy following trauma were selected as the control group. Immunohistochemical staining with HMGB1, TGF-β1 and NF-κB expression in the renal tissues, and western blot analysis were used to measure the relative expression of HMGB1, TGF-β1 and NF-κB. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to estimate the relative expression of HMGB1, TGF-β1 and NF-κB mRNA. Statistical analysis was used to calculate the association between HMGB1, TGF-β1 and NF-κB expression and CAN grade. Immunohistochemical staining demonstrated that HMGB1, TGF-β1 and NF-κB had markedly positive expression rates in renal tubular epithelial cell cytoplasm and membranes in CAN renal tissues, and the positive rates of HMGB1, TGF-β1 and NF-κB increased with the aggravation of CAN pathological grade (I, II and III). The results of western blot analysis indicated that the expression levels of HMGB1, TGF-β1 and NF-κB were significantly higher in the CAN group, compared with the normal group ($P<0.05$), and the expression levels increased with the progression of CAN grade. A positive association among HMGB1, TGF-β1 and NF-κB expression was identified. RT-qPCR analysis demonstrated that the expression of HMGB1, TGF-β1 and NF-κB mRNA in the CAN group was significantly higher than in the normal group ($P<0.05$), and the relative expression level of HMGB1, TGF-β1 and NF-κB mRNA not only increased with the aggravation of CAN grade, but was also positively associated with the expression of HMGB1, TGF-β1 and NF-κB, respectively. The abnormal expression of HMGB1, TGF-β1 and NF-κB is therefore, an important manifestation of CAN and the expression of HMGB1, TGF-β1 and NF-κB mRNA in the renal tissues are significantly associated with CAN pathological progression. HMGB1, TGF-β1 and NF-κB may form a signaling pathway that leads to the occurrence of CAN, which induces renal interstitial fibrosis.

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*Key words:* chronic allograft nephropathy, high mobility group protein B1, transforming growth factor-β1, nuclear factor-κB, kidney transplant

## Introduction

Kidney transplantation has become the most efficient therapy for patients who suffer from end stage renal disease (1). However, despite the improvements of immunosuppressive methods on kidney transplantation, the long-term graft survival rate, such as the 5-year survival rate, has not improved markedly (2). Chronic allograft nephropathy (CAN) is one of the most common causes for loss of kidney graft function, which accounts for 50-80% of late graft loss after the first year of renal transplantation (3,4). The pathogenesis of CAN remains intricate and poorly understood, as the condition is caused by various immune and non-immune factors (5). For example, the immune factors include acute clinical rejection, human leukocyte antigen match, whereas non-immune factors consist of hypertension, glomerular hypertension, delayed graft function and acute calcineurin-inhibitor toxicity (6-8). These factors lead to cumulative damage to the kidney, which results in progressive allograft injury and consequently, dysfunction (9,10). A previous study also suggested that the effects of body mass index (BMI) 1 year after the kidney transplantation were associated with CAN (11). CAN is the process of fibrotic development with histological lesions, which can be characterized by atherosclerosis, glomerulopathy, interstitial fibrosis and tubular atrophy (6,9,12). Considering the negative effects that CAN causes on renal allograft, further insight on the pathology mechanisms is required and may provide potential therapeutics for CAN.

The high mobility group box 1 protein (HMGB1), which belongs to the most evolutionarily conserved proteins in the nucleus and cytoplasm, is one of the members of high mobility group nuclear protein family (13). HMGB1 has been demonstrated to act as a pro-inflammatory mediator and participate in human immune systems diseases (14,15). Studies

indicated that innate immune cells, including macrophages and monocytes, actively secreted HMGB1 (13,16). It has been demonstrated that HMGB1 not only induces various cytokines, such as interleukin (IL)-1, IL-6 and IL-8, but also stimulates necrosis-induced inflammation. It indicates that HMGB1 has an important role in renal diseases, including acute kidney injury, chronic kidney disease (CKD) and granulomatous nephritis (17-20). However, the association between HMBG1 and CAN remains to be clarified. Furthermore, HMGB1 acts as the endogenous ligand for toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4), and is associated with downstream translocation of nuclear factor-κB (NF-κB) (21,22). NF-κB is a protein complex, which has an important role in transcription, immune responses and inflammation developments (23,24). The NF-κB family is a family of inducible transcription factors that have been demonstrated to contribute to the process of renal inflammation and immunological disease development (25,26). Transforming growth factor β (TGF-β) is a multi-function cytokine complex with three isoforms, TGF-β1, TGF-β2, and TGF-β3 (27). Among them, TGF-β1 has an important role in interstitial fibrosis development (28-30). Multiple studies have suggested that the NF-κB pathway interacts with the TGF-β1/Smad pathway to form the NF-κB/TGF-β1/Smad signaling pathway, which is involved in the process of inflammation and renal tubulointerstitial fibrosis development (31-33). Furthermore, the association between TGF-β1 expression and CAN has been identified in animal models and patients, demonstrating that there is an overexpression of TGF-β1 in patients with CAN (3,27). However, to the best of our knowledge, the role of NF-κB in CAN has not been investigated previously.

Therefore, the present study aimed to investigate whether HMGB1 and NF-κB in renal tissues are associated with CAN. Due to the association between TGF-β1 and CAN, TGF-β1 was included in the present study to investigate the relationship of TGF-β1 with HMGB1 and NF-κB in CAN. Ultimately, the current case-control study was conducted to accumulate data and determine the association between HMGB1, NF-κB, TGF-β1 and CAN.

## Materials and methods

*Subjects*. A total of 27 patients (age range, 18-54 years old; mean age, 41±8 years; 15 males and 12 females) who were diagnosed with CAN by histological biopsy diagnosis between September 2012 and November 2014 at Linyi People's Hospital (Linyi, China) were enrolled into the study. All patients suffered graft kidney dysfunction between 1 and 10 years after allograft renal transplantation and the mean allograft survival time was 4.3 years. Every CAN patient received renal biopsy guided by B ultrasound to exclude other renal diseases and renal tissue specimens were harvested from each CAN patient. In addition, 30 cases (age range, 29-63 years old; mean age, 44±9 years; 16 males and 14 females) who received nephrectomy following trauma in the same time period were selected as the control group for the current study. Normal renal tissue specimens were collected through nephrectomy in the control group. Non-anticoagulant blood was collected under fasting conditions 1 day prior to renal biopsy to detect a number of physical indications, including hemoglobin, high sensitivity C-reactive protein (hs-CRP), IL-6, triglyceride (TG), low density lipoprotein (LDL-C), highdensity lipoprotein (HDL-C) and fasting plasma glucose (FPG). The present study was approved by the Ethics and Clinical Committee of Linyi People's Hospital and written informed consent was obtained from each patient.

*Immunosuppressive regimens*. Methylprednisolone (Pfizer Manufacturing Belgium, Puurs, Belgium; imported drug registration number: H20080285) was administered at a dose of 500 mg/day once a day by intravenous injection in the first 3 days following renal transplant. Subsequently, mycophenolate mofetil (Hangzhou Zhongmei Huadong Pharmaceutical Co. Ltd, Zhejiang Sheng, China; permission number approved by the state: H20052083) at 1.0-1.5 g/day was administered orally 48 h after the surgery. The concentration of serum creatinine (Cr) was assessed once a month using an automated biochemical analyzer (AU5800; Beckman Coulter, Inc., Brea, CA, USA). Cyclosporine A (CsA; Hangzhou Zhongmei Huadong Pharmaceutical Co. Ltd; permission number: H10960122) was administered at 8 mg/kg/day when the concentration of Cr was <250 μmol/l; and then adjusted to maintain the drug level at 300-350 ng/ml in the first 2 weeks following surgery, 250-300 ng/ml in weeks 2-4, 200-250 ng/ml in weeks 4-12 and 150-200 ng/ml in weeks 12-48 following surgery. Finally, prednisone (Zhejiang Xianju Pharmaceutical Co. Ltd., Zhejiang, China; permission number: H33021207) was used instead of methylprednisolones at 0.6 mg/kg/day and the dose was adjusted to maintain the blood drug concentration at 10-15 mg/day at 2 months after renal transplantation.

*Classification of chronic allograft nephropathy*. The diagnostic criteria and classification for all the specimens was assured according to the Banff 07 working classification (34). The classifications based on histopathological findings were as follows: Grade I (mild), mild interstitial fibrosis and tubular atrophy; Grade II (moderate), moderate interstitial fibrosis and tubular atrophy; and Grade III (severe), severe interstitial fibrosis and tubular atrophy and moderate tubular loss.

*Histomorphology*. For microscopic observation, tissues were fixed in 10% formalin at 37˚C for 24 h and conventionally dehydrated (70% ethanol for 1 day, 80% ethanol for 45 min, 90% ethanol for 1 h, 95% ethanol for 1 h, and ethanol 100% for 3 h). Tissues were embedded in paraffin and 2-μm tissue sections were stained with hematoxylin and eosin (HE). For electron microscopy observation, renal tissues were fixed in 2.5% glutaraldehyde at 4˚C for 4 h, and then fixed in 2% osmic acid (Seebio Biotech Co., Ltd., Shanghai, China) at 4˚C for 2 h. Tissues were then dehydrated by a graded acetone series. Epon812 (Sangon Biotech Co., Ltd., Shanghai, China) was used for saturating and embedding tissues, following the manufacturer's protocol. An Ultra Rapid Tissue Processor (Histra-QS; Jokoh Co., Ltd., Tokyo, Japan) was used to produce 2-μm sections and uranyl acetate and lead citrate were applied to stain the tissues. A H-7500 transmission electron microscopy (magnification, x7,000) was used to observe and capture images of the tissue sections.

*Immunohistochemistry*. Expression of HMGB1, TGF-β1 and NF-κB in renal tissue was detected by immunohistochemical staining (streptavidin-perosidase). Polyclonal antibodies against HMGB1 (#BA2361; 1:200), TGF-β1 (#BM4876; 1:200) and NF-κB p65 (#BM3946; 1:200) were purchased from Wuhan Boster Biological Engineering Co., Ltd., (Wuhan, China) The procedure was performed following the manufacturer's protocol of the VECTASTAIN® ABC kit (#PK-6100; Vector Laboratories, Inc., Burlingame, CA, USA). Tissues were fixed in 10% formalin at 37˚C for 24 h and conventionally dehydrated (70% ethanol for 1 day, 80% ethanol for 45 min, 90% ethanol for 1 h, 95% ethanol for 1 h, and ethanol 100% for 3 h). Tissues were embedded in paraffin and 3-$\mu$m tissue sections were prepared. Following deparaffinization and rehydration to block endogenous peroxidase, sections were soaked in 0.003 (volume ratio) $H_2O_2$ in 80% methanol and washed with phosphate-buffered saline (PBS) three times. Normal non-immune serum was used to prevent unspecific binding. Subsequently, the primary antibody was added to sections overnight at 4˚C, followed by overlaying with appropriate secondary antibody (#BM3895; 1:1,000; Wuhan Boster Biological Engineering Co., Ltd.) for 15 min at 37˚C. PBS was used as the negative control instead of primary and secondary antibodies. Following 3,3'-diaminobenzidine (DAB) staining and hematoxylin counterstaining, a section of renal tissue was fixed with ethanol (75% for 1 min, 85% for 1 min, 95% for 1 min and 100% for 4 min). A light microscope (magnification, x100) was used for observation and 10 high-power fields were randomly selected in each section to record the number of dying cells and calculate the positive signal rate. If there were no positive cells in each of the high power field on average, a '-' grade was given. Positive cell numbers of <25%, 25-50% and >50% were graded as '+', '++' and '+++', respectively.

*Western blot analysis*. Western blot analysis was used to detect the protein expression levels of HMGB1, TGF-β1 and NF-κB. The procedure was performed with the EasySee Western Blot kit (#DW101; Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. Polyclonal antibody rabbit anti-human HMGB1 (#BA2361; 1:200), TGF-β1 (#BM4876; 1:200) NF-κB p65 (#BM3946; 1:200), and goat anti-rabbit IgG conjugated to horseradish peroxidase (#BM3895; 1:1,000) were purchased from Wuhan Boster Biological Engineering Co., Ltd. Samples were treated with RIPA reagent (#P0013B; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 4˚C for protein extraction. A total of 10 $\mu$g cell plasma protein was separated by 10% SDS-PAGE. This was transferred onto a polyvinylidene fluoride membrane under a steady electric current (160 mA) for 20 min. The membrane was blocked with TBS-Tween-20 (TBST; Beyotime Institute of Biotechnology) and 5% skim milk at 37˚C for 1 h. Then the samples were treated with primary antibodies at 4˚C overnight. After washing with TBST 3 times, samples were treated with secondary antibodies at 37˚C for 1 h. ChemiDocTM XRS gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for capturing images of the gel following DAB staining, and Image J software v1.48 (National Institutes of Health, Bethesda, MD, USA) was used to read integral absorbance values. Each sample was assessed with three replicates, and β-actin was used as an internal reference.

*Reverse transcription-quantification polymerase chain reaction (RT-qPCR) analysis*. RT-qPCR was used to detect the relative expression level of HMGB1, TGF-β1 and NF-κB mRNA relative to β-actin. Total RNA was isolated from renal tissues using an RNAprep Pure Tissue kit (#DP431; Tiangen Biotech Co., Ltd., Beijing, China), following the manufacturers protocol. In a 25-$\mu$l total reaction volume, 2 $\mu$g RNA was reverse transcribed into cDNA using an OligdT primer (Takara Bio, Inc., Otsu, Japan), following the protocol of 37˚C for 15 min and 85˚C for 5 sec. All PCR primers sequences are presented in the Table I. The full reaction components were: 0.4 $\mu$l PCR forward primer (10 $\mu$M), 0.4 $\mu$l PCR reverse primer (10 $\mu$M), 2 $\mu$l cDNA template, 7.2 $\mu$l RNase free $dH_2O$ and 10 $\mu$l SYBR Premix Ex Taq TM II (2x) (Takara Bio, Inc.). The amplification reaction was completed as follows: Pre-degeneration for 5 min at 95˚C; degeneration for 1 min in 94˚C; annealing for 60 sec at 59˚C and extension for 60 sec at 72˚C (35 cycles); and final extension for 8 min at 72˚C. PCR product (5 $\mu$l) was used for electrophoresis on 1% agarose gel, and ethidium bromide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for DNA stain. ChemiDocTM XRS gel imaging system (Bio-Rad Laboratories, Inc.) was used to image the agarose gel and Image-J software v1.48 was used to read its integral absorbance values. The relative expression level of mRNA was acquired by the ratio of relative intensity between the PCR products electrophoresis banding and the internal reference gene, β-actin.

*Statistical analysis*. SPSS 19.0 (IBM SPSS, Armonk, NY, USA) statistical software was used to analyze the data. Measurement data was expressed as mean ± standard deviation. The $\chi^2$ test was applied for enumeration data and Student's t-test and analysis of variance were applied for measurement data. Pearson correlation analysis was used to assess the protein expression level and mRNA relative expression level of HMGB1, TGF-β1 and NF-κB. Spearman correlation analysis was applied to analyze the association between CAN grade and HMGB1, TGF-β1 and NF-κB. A two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Baseline characteristics*. Clinical characteristics of CAN patients and controls are presented in Table II. No statistically significant differences were observed for age, sex, BMI index, hs-CRP, TG, LDL-C, HDL-C or FPG between CAN patients and controls ($P>0.05$). The glomerular filtration rate (GFR) and hemoglobin of CAN patients were significantly lower when compared with controls ($P<0.05$), whereas IL-6 was significantly higher in CAN patients than in controls ($P<0.05$; Table II).

*Histomorphology*. In microscopic observations of paraffin sections with HE staining, no characteristic changes were observed, including abnormally elevated Cr induced by acute rejection or CsA toxicosis. However, interstitial fibrosis and tubular atrophy were observed based on the Banff 07 criteria,

Table I. Primer sequences for quantitative polymerase chain reaction analysis.

| Gene | Primers | Product size (bp) |
|---|---|---|
| HMGB1 | Forward: 5'-TCATCTGCTGCAGTGTTGTT-3'<br>Reverse: 5'-CTCAGAGAGGTGGAAGA-3' | 285 |
| TGF-β1 | Forward: 5'-TCCTGTGACAGCAGGGATAA-3'<br>Reverse: 5'-TCCTGTGACAGCAGGGATAA-3' | 298 |
| NF-κB | Forward: 5'-CTGAACCAGGGCATACCTGT-3'<br>Reverse: 5'-GAGAAGTCCATGTCCGCAAT-3' | 197 |
| β actin | Forward: 5'-AACCCTAAGGCCAACCGTGAAAAG-3'<br>Reverse: 5'-TCATGAGGTAGTCTGTCAGGT-3' | 240 |

HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy; bp, base pairs.

Table II. Clinical characteristics of CAN patients and controls.

| Characteristic | CAN (n=27) | Controls (n=30) | $\chi^2$/t | P-value |
|---|---|---|---|---|
| Age, years | 41±8 | 44±9 | 1.324 | 0.19 |
| Male/female | 15/12 | 16/14 | 0.028 | 0.87 |
| GFR | 27±10 | 87±20 | 14.540 | <0.05 |
| BMI | 24±4 | 26±4 | 1.885 | 0.07 |
| Hemoglobin, g/l | 132±15 | 141±9 | 2.710 | <0.01 |
| Hs-CRP, mmol/l | 4.7±1.2 | 4.3±1.2 | 1.257 | 0.21 |
| IL-6, ng/l | 27.4±8.5 | 15.1±4.7 | 6.659 | <0.05 |
| TG, mmol/l | 2.1±1.4 | 1.9±0.9 | 0.634 | 0.53 |
| LDL-C, mmol/l | 2.9±1.3 | 2.6±1.1 | 0.943 | 0.35 |
| HDL-C, mmol/l | 1.3±0.6 | 1.4±0.5 | 0.686 | 0.50 |
| FPG, mmol/l | 5.5±0.5 | 5.3±0.6 | 1.358 | 0.18 |

Data are presented as the mean ± standard deviation. CAN, chronic allograft nephropathy; GFR, glomerular filtration rate; BMI, body mass index; Hs-CRP, high sensitivity C-reactive protein; IL-6, Interleukin-6; TG, triglyceride; LDL-C, low density lipoprotein; HDL-C, high density lipoprotein; FPG, fasting plasma glucose.

which were the primary characteristics of chronic allograft nephropathy. Among 27 renal tissues from CAN patients, there were 5 cases of CAN grade I, 12 cases of CAN grade II and 10 cases of CAN grade III. Observation results of light microscopy and electron microscopy are presented in Figs. 1 and 2, respectively.

*Immunohistology*. In the renal tissues of patients with CAN, HMGB1, TGF-β1 and NF-κB were markedly positively expressed in the cell cytoplasm and membrane of renal tubular epithelial cells (Fig. 3). HMGB1, TGF-β1 and NF-κB in the renal tissues of the CAN group revealed markedly higher positive rates than normal renal tissues (Table III). The positive rates of HMGB1, TGF-β1 and NF-κB in CAN grade III were markedly higher than in CAN grade I (Table III).

*Western blot analysis*. As demonstrated by western blot analysis (Fig. 4A), the expression of HMGB1, TGF-β1, NF-κB was significantly higher in the renal tissues of patients in the CAN group, compared with normal tissues from the control group (P<0.05; Fig. 4B-D). Furthermore, statistically significant differences in the expression of HMGB1, TGF-β1 and NF-κB were identified among CAN grades I, II and III (P<0.05; Fig. 4). The expression of HMGB1 and NF-κB in the CAN grade I demonstrated no statistical difference when compared with the controls (P>0.05; Fig. 4B and C, respectively), whereas TGF-β1 expression was significantly higher compared with the control group (P<0.05; Fig. 4D). Furthermore, expression of HMGB1, NF-κB and TGF-β1 in CAN grade III was significantly higher than grade II (P<0.05; Fig. 4B-D, respectively).

*RT-qPCR analysis*. Compared with the control group, the relative expression of HMGB1, TGF-β1 and NF-κB mRNA in the CAN group was significantly elevated (P<0.05; Fig. 5A-C, respectively). Notably, there was significantly increased expression of HMGB1, TGF-β1 and NF-κB mRNA in CAN grade II, compared with grade I (P<0.05). Similarly, the expression of HMGB1, TGF-β1, NF-κB mRNA in CAN grade III were significantly higher compared with grade II (P<0.05; Fig. 5; Table IV).

Table III. Expression of HMGB1, TGF-β1 and NF-κB in the CAN and control groups.

| | | HMGB1 | | | | TGF-β1 | | | | NF-κB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | N | - | + | ++ | +++ | - | + | ++ | +++ | - | + | ++ | +++ |
| Controls | 30 | 19 | 11 | 0 | 0 | 18 | 12 | 0 | 0 | 18 | 12 | 0 | 0 |
| CAN I | 5 | 0 | 2 | 2 | 1 | 0 | 2 | 2 | 1 | 0 | 1 | 2 | 2 |
| CAN II | 12 | 0 | 2 | 4 | 6 | 0 | 2 | 6 | 4 | 0 | 2 | 7 | 3 |
| CAN III | 10 | 0 | 1 | 3 | 6 | 0 | 1 | 2 | 7 | 0 | 0 | 4 | 6 |

HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy; +, rate of positive cell number <25%; ++, rate of positive cell number 25-50%; +++, rate of positive cell number >50%.

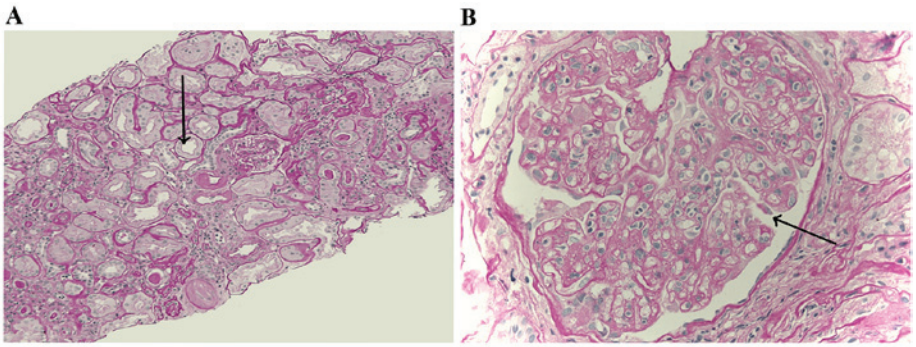


Figure 1. Light microscopic observation of (A) tubular atrophy, interstitial fibrosis (arrow) and (B) glomerulosclerosis (arrow). Cytoplasm was stained as red, nucleus was stained as blue. Magnification in (A), x100; in (B), x200.

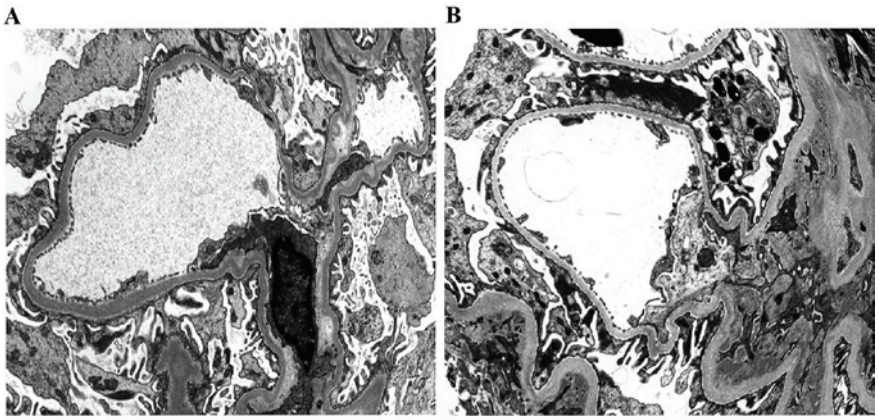


Figure 2. Electron microscopy observation of (A) a normal glomerulus and (B) transplant glomerulopathy with a well-developed basement membrane along the entire capillary circumference, mesangial expansion, and accumulation of subendothelial deposit. Magnification, x7,000.

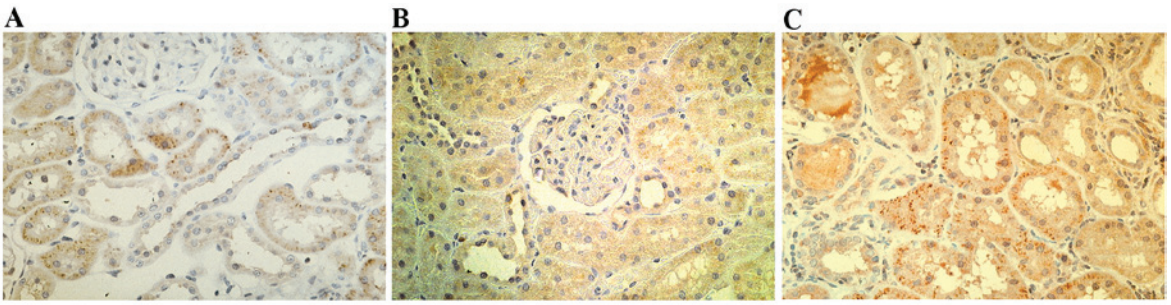


Figure 3. Expression of (A) HMGB1, (B) TGF-β1 and (C) NF-κB in the renal tissue of patients in the CAN group. Protein was stained as brown. Magnification, x100. HMGB1, high mobility group protein B1; TGF-mo transforming growth factor-B1 NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy.

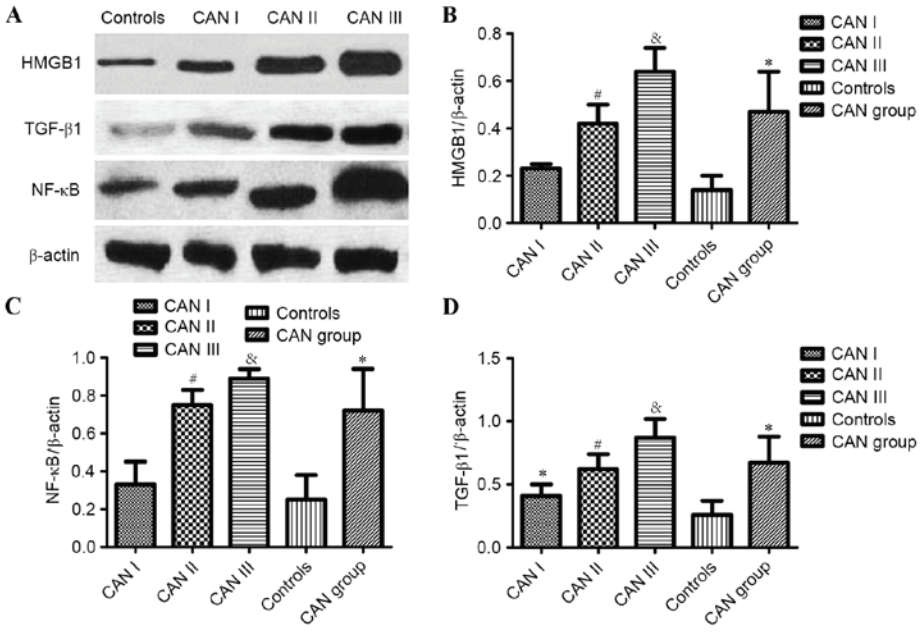


Figure 4. Western blot analysis of HMGB1, TGF-β1 and NF-κB. (A) Immunobloting of HMGB1, TGF-β1 and NF-κB proteins, with β-actin as the reference gene. Expression levels of (B) HMGB1 (C) TGF-β1 and (D) NF-κB in renal tissues. *P<0.05 vs. control; #P<0.05 vs. CAN grade I; &P<0.05 vs. CAN grade II. HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy.

*Correlation analysis among HMGB1, TGF-β1 and NF-κB and CAN grade*. Statistical analysis indicated positive associations between the expression levels of HMGB1, TGF-β1 and NF-κB in renal tissues (HMGB1 vs. TGF-β1: r=0.860, P<0.01; HMGB1 vs. NF-κB: r=0.899, P<0.01; TGF-β1 vs. NF-κB: r=0.835, P<0.01). In the renal tissues of patients with CAN, expression of HMGB1, TGF-β1 and NF-κB was positively associated with CAN pathological grade (HMGB1: r=0.894, P<0.01; TGF-β1: r=0.867, P<0.01; NF-κB: r=0.853, P<0.01) and the expression level of HMGB1, TGF-β1 and NF-κB tended to increase with the aggravation of the CAN pathological grade. Furthermore, a positive association between the protein and mRNA expression of HMGB1, TGF-β1 and NF-κB (r=0.904, P<0.01; r=0.858, P<0.01; r=0.885, P<0.01, respectively) was identified and the mRNA expression level of HMGB1, TGF-β1 and NF-κB in renal tissues also increased with the progression of the CAN pathological grade (Tables V and VI).

## Discussion

With the great developments made in transplantation immunology, the short-term graft survival rate has markedly improved, while the long-term survival rate has remained low (34). Studies have revealed that CAN is a leading factor, causing 50-80% of allograft loss in renal function in the late period following kidney transplantation (3,35). As CAN is caused by various immune and non-immune factors, and is easily affected by a series of donor-related factors including donor age, brain death and consequences of ischemia-reperfusion injury, the pathogenesis and mechanisms remain unclear (36-40).

Previous studies suggested that HMGB1, which is a pro-inflammatory cytokine released from dying cells, would accumulate as renal function deteriorates and have an important

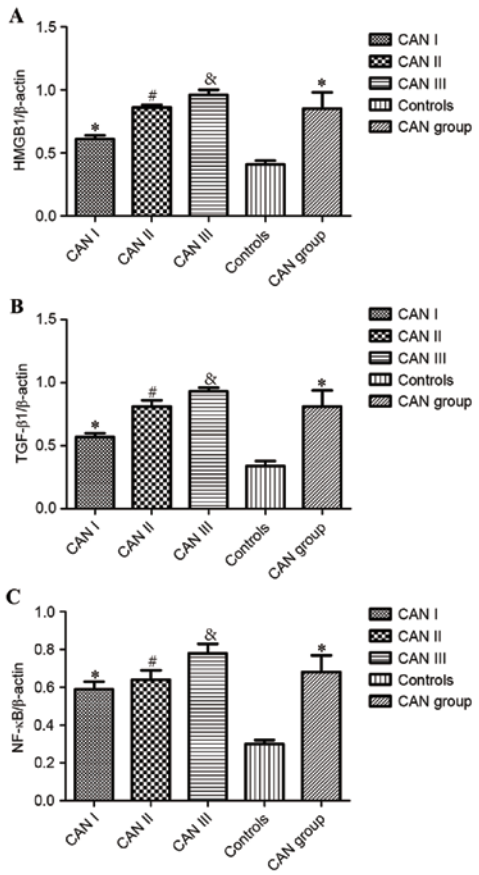


Figure 5. Reverse transcription-quantitative polymerase chain reaction analysis of (A) HMGB1 (B) TGF-β1 and (C) NF-κB mRNA expression levels in renal tissues. *P<0.05 vs. control; #P<0.05 vs. CAN grade I; &P<0.05 vs. CAN grade II. HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy.

Table IV. Relative expression of HMGB1, TGF-β1 and NF-κB mRNA in the CAN and control groups.

| | | CAN | | | |
|---|---|---|---|---|---|
| Cytokine | CAN (n=27) | Grade I (n=5) | Grade II (n=12) | Grade III (n=10) | Controls (n=30) |
| HMGB1 | 0.85±0.13[a] | 0.61±0.03[b] | 0.86±0.02[c] | 0.96±0.04 | 0.41±0.03 |
| TGF-β1 | 0.81±0.13[a] | 0.57±0.03[b] | 0.81±0.05[c] | 0.93±0.03 | 0.34±0.04 |
| NF-kB | 0.68±0.09[a] | 0.56±0.04[b] | 0.64±0.05[c] | 0.78±0.05 | 0.30±0.02 |

[a]P<0.05 vs. controls; [b]P<0.05 vs. CAN grade II; [c]P<0.05 vs. CAN grade III. Data are presented as the mean ± standard deviation. HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy.

Table V. Pearson correlation analysis of the association between the protein and mRNA expression levels of HMGB1, TGF-β1 and NF-κB (n=57).

| Analysis | | HMGB1 | TGFB1 | NF-κB | HMGB1 mRNA | TGF-1 mRNA | NF-κB mRNA |
|---|---|---|---|---|---|---|---|
| HMGB1 | Pearson correlation | 1 | 0.860 | 0.899 | 0.904 | - | - |
| | P-value (2-tailed) | | <0.001 | <0.001 | <0.001 | | |
| TGF-tai | Pearson correlation | 0.860 | 1 | 0.835 | - | 0.858 | - |
| | P-value (2-tailed) | <0.001 | | <0.001 | | <0.001 | |
| NF-κB | Pearson correlation | 0.899 | 0.835 | 1 | - | - | 0.885 |
| | P-value (2-tailed) | <0.001 | <0.001 | | | | <0.001 |
| HMGB1 mRNA | Pearson correlation | 0.904 | - | - | 1 | 0.979 | 0.962 |
| | P-value (2-tailed) | <0.001 | | | | <0.001 | <0.001 |
| TGF-ailed)A | Pearson correlation | - | 0.858 | - | 0.979 | 1 | 0.961 |
| | P-value (2-tailed) | | <0.001 | | <0.001 | | <0.001 |
| NF-κB mRNA | Pearson correlation | - | - | 0.885 | 0.962 | 0.961 | 1 |
| | P-value (2-tailed) | | | <0.001 | 0.000 | <0.001 | |

HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB.

Table VI. Spearman correlation analysis of the association between the protein and mRNA expression levels of HMGB1, TGF-β1 and NF-κB and CAN grade (n=57).

| Variable | HMGB1 | TGFB1 | NF-κB | HMGB1 mRNA | TGF-1 mRNA | NF-κB mRNA |
|---|---|---|---|---|---|---|
| Association | 0.894 | 0.867 | 0.853 | 0.915 | 0.917 | 0.913 |
| P-value (2-tailed) | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

HMGB1, high mobility group protein B1; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; CAN, chronic allograft nephropathy.

role in CKD (17). The levels of HMGB1 may be markedly overexpressed during the progression of CKD. It was also demonstrated that HMGB1 functions are associated with NF-κB in the inflammatory and immunostimulation response (41-43). NF-κB has also been implicated in kidney diseases and is associated with renal inflammatory injury and fibrosis (44). It is speculated that HMGB1-NF-κB may function as a signaling pathway in renal diseases. Previous studies revealed that TGF-β1 was regulated by NF-κB in certain signaling pathways, which indicates a potential role for NF-κB (45,46). Therefore, the present study aimed to investigate the association between HMGB1, NF-κB, TGF-β1 and CAN.

The current study analyzed the HMGB1, NF-κB and TGF-β1 staining intensity within graft kidneys. The results of immunohistological staining indicated that HMGB1, NF-κB and TGF-β1 were predominantly expressed in the mesangium and glomerular epithelium. The positive expression rate of HMGB1, NF-κB and TGF-β1 in CAN tissues was much higher when compared with controls, indicating potential associations between HMGB1, NF-κB, TGF-β1 and CAN. In the present

study, the results of western blot analysis and RT-qPCR also demonstrated that HMGB1, NF-κB and TGF-β1 were significantly associated with CAN and specifically to the grade of CAN. HMGB1, NF-κB and TGF-β1 were significantly overexpressed in renal tissues of patients with CAN, compared with controls. The expression of HMGB1, NF-κB and TGF-β1 increased with the progression of CAN. Following the statistical analysis performed in the current study, a significant association between HMGB1, NF-κB and TGF-β1 in CAN progression was revealed.

TGF-β1 is one of the critical cytokines, which leads to renal fibrosis, causing renal interstitial fibrosis in end-stage renal disease (47). It has been proven to be involved in CAN pathogenesis (28,48,49). To the best of our knowledge, no studies have assessed the association between NF-κB and CAN. However, NF-κB was indicated to have a function in kidney diseases, including CKD (50). Furthermore, it has been demonstrated that NF-κB was associated with hepatic stellate cell in the apoptosis process, in which NF-κB activation stimulated TGF-β1, leading to serious renal interstitial fibrosis (51-53). These findings suggested that NF-κB and TGF-β1 may constitute an important pathway in the pathogenesis of CAN, which may explain the positive association between NF-κB and TGF-β1 in the current study. HMGB1 has been studied in other renal diseases (54,55); however, it is another novel inflammatory marker, which, to the best of our knowledge, has not been studied in regard to CAN. The concentration of HMGB1 serum levels in CKD was elevated in a previous study (17). The overexpression of HMGB1 in tissues in the present study also demonstrated a marked association between HMGB1 and CAN. A potential explanation for the positive association between HMGB1 and NF-κB in the present study is that HMGB1 induces the downstream translocation of NF-κB by interacting with TLR2, TLR4 and RAGE (41,56-58). TLR2, TLR, HMGB1/NF-κB/TGF-β1 may form a signaling pathway to induce the pathogenesis of CAN. The overexpression of HMGB1 stimulates NF-κB activation, leading to the overexpression of TGF-β1, which causes renal interstitial fibrosis and a series of immunostimulatory and inflammatory responses through the pathway, resulting in CAN. With the accumulation of these factors in renal tissues, the conditions of CAN progressively deteriorate. Therefore, these factors (HMGB1, NF-κB and TGF-β1) in the tissues of CAN Grade III would be higher than those observed in CAN Grade I. Although this assumption is based on the present results and other studies, more details regarding the function of HMGB1, NF-κB and TGF-β1 in CAN pathogenesis are required to validate these results in further studies.

In conclusion, the present study identified that the levels of HMGB1, NF-κB and TGF-β1 in renal tissues are significantly associated with CAN. Furthermore, expression of HMGB1, NF-κB and TGF-β1 increases with CAN progression. Finally, the positive associations among HMGB1, NF-κB and TGF-β1 indicate that the HMGB1/NF-κB/TGF-β1 pathway may be one of the leading causes of CAN. The present study therefore provides a novel way to understand the mechanisms of CAN. Novel targets, including HMGB1 and NF-κB, were revealed in relation to CAN, and the HMGB1/NF-κB/TGF-β1 pathway may be the foundation of a novel target treatment for CAN. However, further studies are required to validate the roles of HMGB1, NF-κB and TGF-β1 in CAN and to investigate the mechanism of action in CAN pathogenesis. The present study may lead to the identification of effective methods to treat CAN and elevate the long-term graft survival rate following kidney transplantation.

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