

Expression and role of TNIP2 in multiple organ dysfunction syndrome following severe trauma

HUI GONG, XIAOMIN SHENG, JIANHUA XUE and DONGBO ZHU

Department of Emergency Surgery, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, P.R. China

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Abstract. Severe trauma can result in secondary multiple organ dysfunction syndrome (MODS) and death. Inflammation response and oxidative stress promote the occurrence and development of MODS. TNFAIP3-interacting protein 2 (TNIP2), which can repress the activation of nuclear factor- κ B (NF- κ B) and may be involved in MODS progression, has not been studied in regards to MODS. The present study aimed to investigate the expression, role and mechanism of TNIP2 in MODS following severe trauma. The expression level of TNIP2 was initially detected in the blood of patients with MODS using reverse transcription-quantitative polymerase chain reaction and western blot assay. Then, to investigate the role of TNIP2 in MODS, a MODS rat model was conducted by trauma and the model rats were treated with TNIP2-plasmid (intraperitoneal injection). Blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatine (Cr) and creatine kinase (CK); and tumor necrosis factor α (TNF- α), high-mobility group box 1 (HMGB-1), malondialdehyde (MDA) and total antioxidant capacity (TAC) in the different groups were assessed. In addition, activation of NF- κ B was assessed by detecting the level of phospho-p65. The results showed that TNIP2 was significantly decreased in the blood of patients with MODS. TNIP2 was also significantly downregulated in the blood and the pulmonary, renal and hepatic tissues of MODS rats. The levels of ALT, AST, LDH, BUN, Cr and CK were markedly increased in the blood of MODS rats, and these increases were inhibited by TNIP2-plasmid administration. Moreover, blood levels of TNF- α , HMGB-1 and MDA were significantly increased in MODS rats, while TAC was notably decreased, and these changes were prevented by TNIP2-plasmid administration. Furthermore, it was found that activation of NF- κ B induced by

MODS was eliminated by TNIP2-plasmid. In conclusion, the data indicated that TNIP2 is significantly decreased in MODS following severe trauma, and it plays a protective role in MODS development by inhibiting the inflammation response and oxidative stress by preventing NF- κ B activation.

Introduction

Severe trauma can cause local tissue damage, systemic inflammatory response syndrome, as well as multiple organ dysfunction syndrome (MODS), shock and even death (1,2). MODS is one of the major, serious complications following severe trauma and is the progressive dysfunction of one or more organ systems resulting from an exaggerated and long-term inflammatory response to severe illness and/or injury. Multiple factors including severe shock, infection, burns, trauma and severe pancreatitis are involved in the pathologic process of MODS (3-7). In recent years, the mechanism underlying MODS caused by trauma is under investigation, and there are a number of hypotheses to explain the pathological mechanism. However, the mechanisms underlying MODS caused by trauma have not yet been fully elucidated. At present, due to the lack of effective treatment, MODS is still a major cause of morbidity and mortality in intensive care units (8,9).

Nuclear factor (NF)- κ B is a protein complex that controls transcription of DNA, cytokine production and cell survival. NF- κ B is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines and free radicals (10-12). Abnormal expression of NF- κ B has been reported to be linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development (13,14). Recent studies have reported that NF- κ B is activated during the progression of MODS (15,16). TNIP2 (also termed ABIN2) interacts with several components of the NF- κ B signaling pathway and can both positively and negatively regulate NF- κ B-dependent transcription of target genes (17). The *TNIP2* gene encodes a protein that represses the activation of NF- κ B, and it is generally accepted that TNIP2 has an essential role in the NF- κ B signaling pathway (18). Moreover, studies have indicated that TNIP2 plays an important role in acute pancreatitis-induced myocardial injury and lupus nephritis via regulating the NF- κ B pathway (19,20).

To the best of our knowledge, no study has been conducted concerning the role of TNIP2 in the development and progression of MODS. Therefore, the aim of the present study was

Correspondence to: Dr Dongbo Zhu, Department of Emergency Surgery, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, Jiangsu 226001, P.R. China
E-mail: zhudongbo77@126.com

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to investigate the role and molecular mechanism of TNIP2 in MODS following severe trauma.

Materials and methods

Clinical specimens. Peripheral blood samples (5 ml per individual) from 16 patients with MODS and peripheral blood samples from 16 healthy individuals were collected at the Affiliated Hospital of Nantong University (Nantong, China) from August 2014 to April 2017. The inclusion criteria were as follows: i) No history of injury; ii) injury severity score (ISS), ≥ 16 ; iii) age, ≥ 18 years; and iv) time interval between trauma and admission, < 90 h. The exclusion criteria were as follows: i) Mortality within 24 h after trauma; ii) main diagnosis, cardiac trauma; iii) intracranial hemorrhage; iv) heart, liver, kidney or endocrine history. Following admission, the age, sex, cause of trauma and medical history of the patients were recorded. Blood samples were collected from MODS patients 48 h after severe trauma and stored at -85°C . Informed consent was obtained from every patient enrolled and the present study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

Animal model of MODS. A total of 40 male Sprague-Dawley (SD) rats (~ 200 g) were purchased from Vital River Company (Beijing, China). All rats were fed *ad libitum* and maintained under standard conditions at $22\text{--}30^{\circ}\text{C}$ and a 12-h light/dark cycle. The experimental protocol was approved by the Animal Care Committee of Nantong University, and experiments were performed under the guidelines of guide for the care and use of laboratory animals (21). The rat model of MODS was designed and established according to a previous study (22). To investigate the role of TNIP2 in MODS, the rats were randomly assigned into 4 groups: Control, MODS model, MODS model + control-plasmid [C; intraperitoneal (i.p.) injection] and MODS model + TNIP2-plasmid (T; i.p. injection). The rats in the control and MODS model groups received saline (0.9% NaCl) solution i.p. instead of the plasmids. All rats were anesthetized with 30 mg/kg pentobarbital and handled at certain time points (6, 12, 24 and 48 h). Following the specific treatment, the subsequent experiments were conducted.

To confirm multiple organ injury/dysfunction, the following biochemical indicators in the plasma were measured. Liver injury was assessed by detecting the enhanced levels of alanine aminotransferase (ALT; a specific marker for hepatic parenchymal injury), aspartate aminotransferase (AST; a nonspecific marker for hepatic injury) and lactate dehydrogenase (LDH) in the plasma. Renal dysfunction was assessed by the increased levels of creatine (Cr) and blood urea nitrogen (BUN) (indicator of reduced glomerular filtration rate and hence renal failure) in the plasma (23).

Serum biochemical analyses. At 6, 12, 24 and 48 h after treatment, 3 ml venous blood was collected from the rats in all groups. Serum was prepared through centrifugation (4°C , $1,000 \times g$, 15 min) and stored at -80°C . To detect the levels of ALT, AST, LDH, BUN, CK and Cr, a Hitachi Automatic Analyzer 7170 (Hitachi, Ltd., Japan) was utilized.

Enzyme-linked immunosorbent assay (ELISA). At 6, 12, 24, and 48 h after treatment, the serum of rats were harvested by

centrifugation ($1,000 \times g$, 10 min) to determine the secretion of TNF- α , HMGB-1, MDA and TAC using an ELISA kit (Abcam, Cambridge, MA, USA) following the manufacturer's protocols. Every sample was detected at least three times by utilizing a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. Western blot analysis was carried out as previously described (24). Total proteins were extracted from tissues/blood using radioimmunoprecipitation assay lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate] supplemented with phenylmethylsulfonyl fluoride at a final concentration of 1 mM. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of protein (30 μg /lane) were resolved using SDS-PAGE on 10% gels, and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% skimmed milk in TBS-Tween at room temperature for 1.5 h, the samples were probed with antibodies against TNIP2 (cat no. sc-271850; dilution 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p-p65 (cat no. 3033; dilution 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), p65 (cat no. 8242; dilution 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), and β -actin (cat no. 4970; 1:1,000; Cell Signaling Technology, Inc.). After three times of washing, the blots were then incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG; 1:2,000; cat no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Appligen Technologies, Inc., Beijing, China). ImageJ 1.38X (National Institutes of Health, Bethesda, MD, USA) was used to perform densitometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as previously described (24). In brief, total RNA was extracted from tissues/blood samples using RNAiso Plus (Takara Bio, Inc., Otsu, Japan) following the manufacturer's instructions. RT was performed to synthesize cDNAs using the ThermoScript RT-PCR system (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed to analyze the synthesized cDNA using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a 7900 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The conditions of qPCR used for amplification were as follows: 95°C for 5 min, 40 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, then 72°C for 10 min. The primer sequences used for qPCR are as follows: TNIP2, forward 5'-CTAAAG AGCGGCAGGTCCCTC-3' and reverse 5'-CAAGATGAC CTTCCAGTGAC-3'; GAPDH, forward 5'-CTTTGGTAT CGTGAAGGACTC-3' and reverse 5'-GTAGAGGCAGGG ATGATGTTCT-3'. GAPDH served as an internal control. The relative gene expression was assessed by using the $2^{-\Delta\Delta\text{Ct}}$ method (25). The experiment was repeated at least three times.

Statistical analysis. All data analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Results

are expressed as mean \pm standard deviation. Student's t-tests and one-way analysis of variance followed by Student-Newman-Keuls tests were performed to analyze the differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Basic patient characteristics. The present study included 16 patients. The basic characteristics of the patients are presented in Table I.

TNIP2 is significantly reduced in MODS patients and rats. To detect the expression level of TNIP2 in the blood of MODS patients and rats, RT-qPCR and western blot assay were used, respectively. As shown in Fig. 1A and B, compared with the healthy control, the protein and mRNA levels of TNIP2 were significantly decreased in the blood samples of patients with MODS. The data indicated that TNIP2 may be involved in the development of MODS.

Then the levels of TNIP2 were detected in the blood and in the pulmonary, renal, and hepatic tissues of MODS rats. The findings revealed that TNIP2 was significantly downregulated in the blood and the pulmonary, renal and hepatic tissues of the MODS rats (Fig. 1C-J).

Blood levels of ALT, AST, LDH, BUN, Cr and CK. To assess changes in the cellular integrity and functionality in the organs at 6, 12, 24 and 48 h after trauma, blood tests were performed. As shown in Fig. 2, the levels of ALT, AST, LDH, BUN, Cr and CK in the blood of the MODS rats were significantly higher at different time points after injury than those in the rats of the control group. TNIP2-plasmid administration significantly reduced the levels of ALT, AST, LDH, BUN, Cr and CK in the blood of the MODS rats.

Blood levels of TNF- α and HMGB-1. As shown in Fig. 3, the production of TNF- α and HMGB-1 in the blood of the MODS rats was markedly higher at different time points after injury than those in the rats of the control group. TNIP2-plasmid administration significantly reduced the levels of TNF- α and HMGB-1 in the blood of the MODS rats.

Blood levels of MDA and TAC. As shown in Fig. 4, the production of MDA in the blood of the MODS rats was higher, and TAC was lower, at different time points after injury than those in the rats of the control group. However, TNIP2-plasmid administration significantly decreased the MDA level and increased TAC in the blood of the MODS rats.

Effect of TNIP2 on the activation of NF- κ B in MODS rats. To investigate the mechanism of the effect of TNIP2 on MODS rats, the NF- κ B pathway was detected (Fig. 5). Forty-eight hours after treatment, total protein and RNA were extracted from tissues/blood of rats in the different groups and measured by western blot assay and qRT-PCR. Our results showed that compared with the control group, the mRNA and protein level of TNIP2 was significantly decreased in the MODS model group, and compared with the MODS model group, TNIP2-plasmid notably increased TNIP2 expression. Phosphorylation of

Table I. Basic patient characteristics and clinical data 24 h after admission (n=16).

Patient characteristics	Values
Sex (male/female)	10/6
Age range (years)	34-57
ISS	34.9 \pm 6.7
APACHE II score	25.3 \pm 8.1
CRP (mg/l)	104.7 \pm 13.7
WBCs ($\times 10^9$ /l)	13.2 \pm 9.1
Neutrophils (%)	83.9 \pm 5.5

ISS, injury severity score; APACHE II, acute physiology and chronic health evaluation II; CRP, Creactive protein; WBCs, white blood cells.

p65 was significantly enhanced in the MODS rats compared to the control rats, and this enhancement was eliminated by TNIP2-plasmid administration (Fig. 5).

Discussion

In the present study, it was determined that TNIP2 is significantly decreased in MODS following severe trauma. Our findings also showed that TNIP2 plays a protective role in MODS development by the inhibiting inflammation response and oxidative stress through regulation of NF- κ B activation. We revealed that TNIP2 may be a promising therapeutic target for the treatment of MODS.

Severe post-traumatic complications, such as sepsis and systemic inflammatory response syndrome (SIRS), are the leading cause of mortality in hospitals with a mortality rate of 30-50%. SIRS can eventually lead to (26). Multiple organ failure (MOF) is common in trauma patients with the most severe injuries, and 29% of trauma patients present with one or more systemic organ failure (27,28). MOF is the leading cause of morbidity and delayed mortality in survivors immediately after injury. The mortality rate of trauma patients with failure of three organ systems is approximately 67%, and the mortality rate of patients with failure of four organ systems is close to 100%. There is a distinct difference between MODS and MOF. The function of MODS organs is not completely lost and will not fail, while the organs of MOF patients have sequential failure of function. Early detection of MODS and interventions can improve organ function. At present, the therapeutic effect of MODS remains unsatisfactory. Therefore, it is urgent to find new effective diagnostic markers and therapeutic targets for MODS.

Severe trauma can lead to the release of various inflammatory mediators and inflammatory cytokines in the body and cause systemic inflammatory response syndrome and oxidative stress (29,30). Inflammation response and oxidative stress promote the occurrence and development of MODS (31,32). NF- κ B is an important transcription factor involved in the regulation of proliferation, survival, apoptosis, immune, oxidative stress, and inflammatory reaction (33-36). In addition, studies have revealed the important role of NF- κ B in the

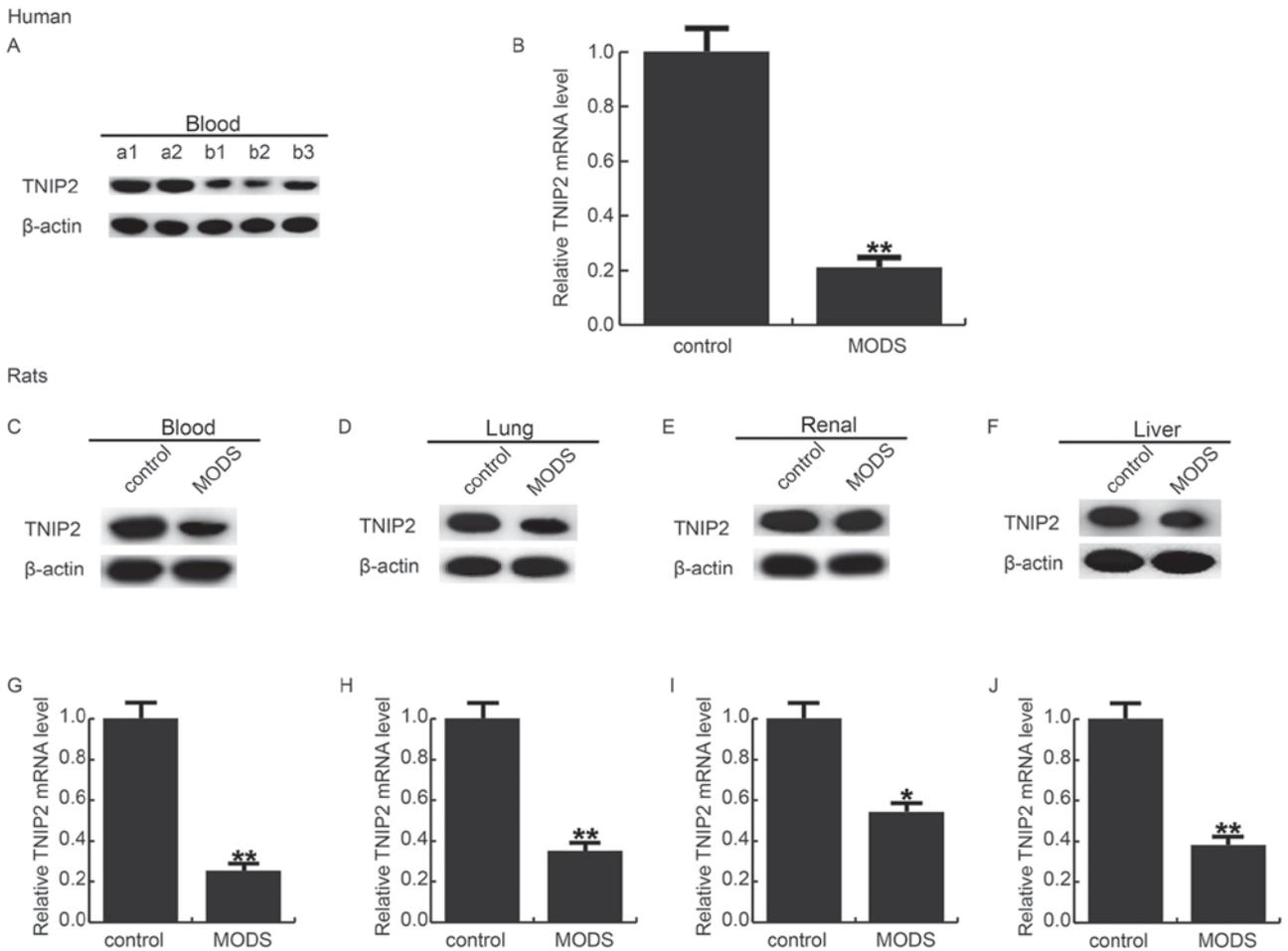


Figure 1. TNIP2 expression in MODS. The level of TNIP2 in MODS was detected using western blot analysis and qRT-PCR, respectively. (A) Protein level of TNIP2 in the blood of MODS patients; a1, a2 indicate the control patients; b1-b3 indicate the MODS patients. (B) Relative mRNA levels of TNIP2 in the blood of MODS patients. Protein levels of TNIP2 in the (C) blood, (D) lung, (E) renal and (F) liver tissues of MODS rats, respectively. The relative mRNA levels of TNIP2 in the (G) blood, (H) lung, (I) renal and (J) liver tissues of MODS rats, respectively. Data are expressed as mean ± standard deviation. *P<0.05, **P<0.01 vs. the control group. MODS, multiple organ dysfunction syndrome; TNIP2, TNFAIP3-interacting protein 2.

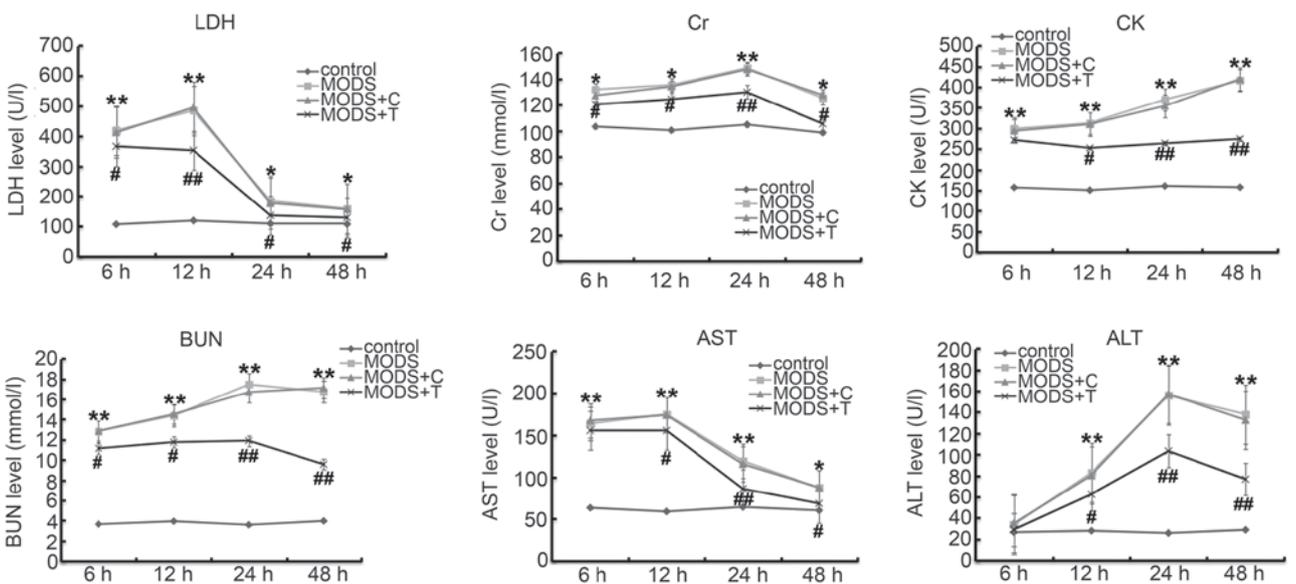


Figure 2. Serum concentrations of major blood biochemical parameters in the control and MODS group at different time points. The levels of LDH, Cr, CK, BUN, AST and ALT in the blood of the MODS rats was measured using ELISA assay. Data are expressed as mean ± standard deviation. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. MODS group. Groups: Control, MODS (MODS model), MODS model + control-plasmid (MODS + C) and MODS model + TNIP2-plasmid (MODS + T). MODS, multiple organ dysfunction syndrome; LDH, lactate dehydrogenase; Cr, creatine; CK, creatine kinase; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

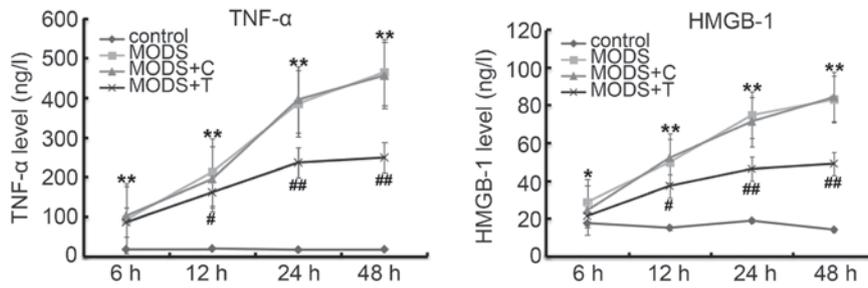


Figure 3. Expression of inflammatory factors in the control and MODS group at different time points. The levels of TNF- α and HMGB-1 in the blood of the MODS rats were determined using ELISA assay. Data are expressed as mean \pm standard deviation. *P<0.05, **P<0.01 vs. the control group. #P<0.05, ##P<0.01 vs. the MODS group. Groups: Control, MODS (MODS model), MODS model + control-plasmid (MODS + C) and MODS model + TNIP2-plasmid (MODS + T). MODS, multiple organ dysfunction syndrome; TNF- α , tumor necrosis factor α ; HMGB-1, high-mobility group box 1.

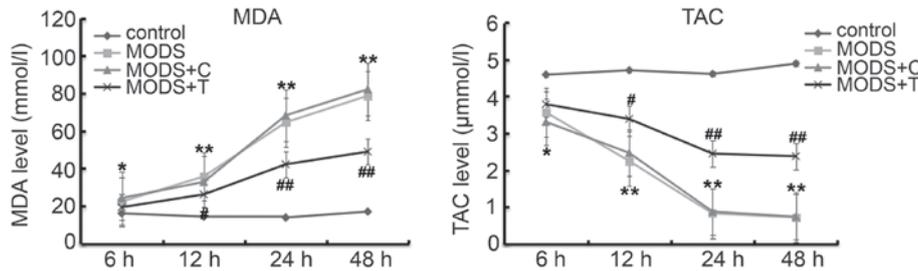


Figure 4. MDA and TAC content in the control and MODS group at different time points. The levels of MDA and TAC in the blood of the MODS rats were determined using ELISA assay. Data are expressed as mean \pm standard deviation. *P<0.05, **P<0.01 vs. the control group. #P<0.05, ##P<0.01 vs. the MODS group. Groups: Control, MODS (MODS model), MODS model + control-plasmid (MODS + C) and MODS model + TNIP2-plasmid (MODS + T). MODS, multiple organ dysfunction syndrome; MDA, malondialdehyde; TAC, total antioxidant capacity.

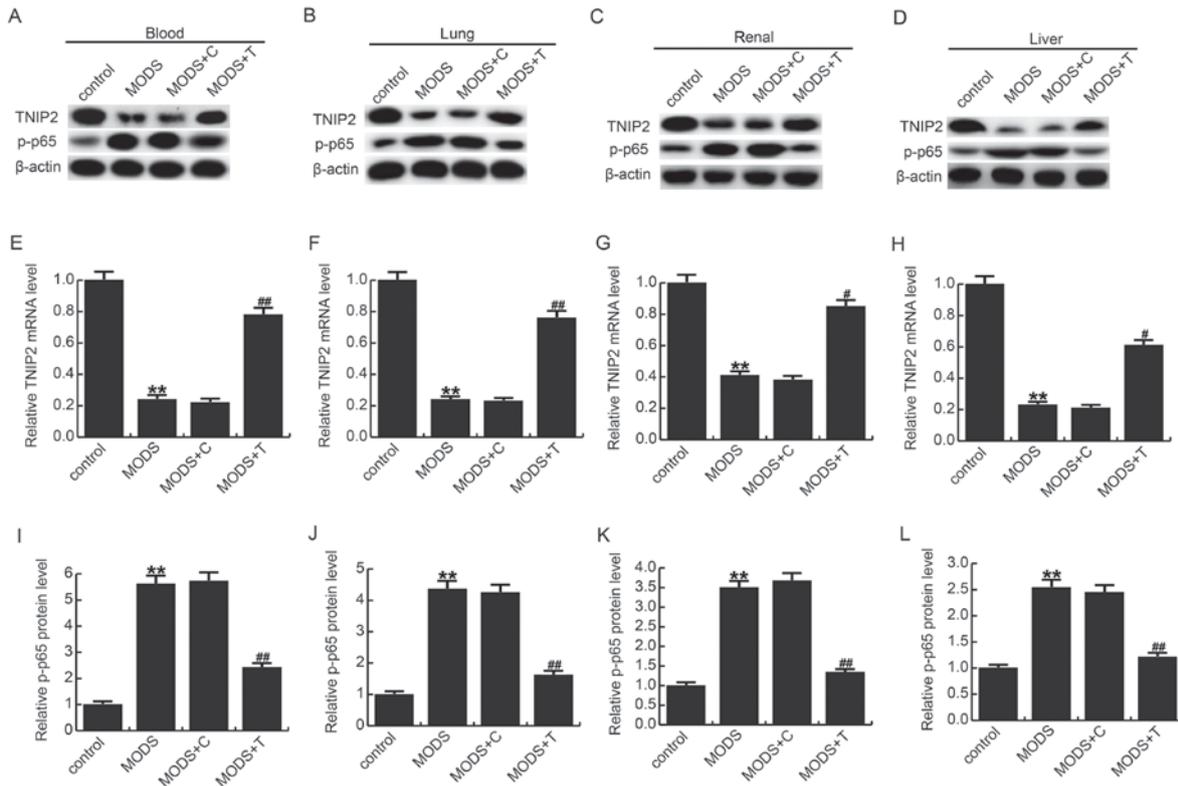


Figure 5. Effect of TNIP2 on p-NF- κ B (p-p65) expression. Protein levels of TNIP2 and p-p65 in the (A) blood, (B) lung, (C) renal and (D) liver tissues of MODS rats were detected using western blot assay. mRNA levels of TNIP2 in the (E) blood, (F) lung, (G) renal and (H) liver tissues of MODS rats was detected using reverse transcription-quantitative polymerase chain reaction. Relative protein levels of p-p65 were analyzed in the (I) blood, (J) lung, (K) renal and (L) liver tissues of MODS rats. Data are expressed as mean \pm SD. **P<0.01 vs. the control group. #P<0.05, ##P<0.01 vs. the MODS group. Groups: Control, MODS (MODS model), MODS model + control-plasmid (MODS + C) and MODS model + TNIP2-plasmid (MODS + T). MODS, multiple organ dysfunction syndrome; TNIP2, TNFAIP3-interacting protein 2; p-, phospho-; NF- κ B, nuclear factor- κ B.

progression of MODS (15,37). Over-activated NF- κ B may significantly enhance the inflammatory response and oxidative stress in MODS patient and cause tissue injury, thus leading to patient death. TNIP2 gene encodes a protein that can repress the activation of NF- κ B, thus we hypothesized that it may be involved in the development of MODS.

We firstly investigated the expression level of TNIP2 in MODS patients and MODS rats, and the MODS rat model was conducted. The findings indicated that TNIP2 was significantly decreased in the blood of MODS patients, and significant downregulation of TNIP2 was also observed in the blood, pulmonary, renal, and hepatic tissues of MODS rats, indicating the downregulation of TNIP2 in MODS. Then, to study the effect of TNIP2 on MODS rats, the rats were treated with or without the TNIP2-plasmid. It was found that the increased expression levels of markers of cellular integrity and organ function including ALT, AST, LDH, BUN, Cr and CK in the blood of rats induced by MODS were inhibited by TNIP2-plasmid administration. Moreover, it was found that blood levels of TNF- α , HMGB-1 and MDA were significantly enhanced in MODS rats, while TAC was notably decreased, and these changes were notably reversed by TNIP2-plasmid treatment, indicating that TNIP2-plasmid administration prevented inflammation response and oxidative stress in MODS rats. Finally, to investigate the mechanism of the effect of TNIP2 on MODS rats, the NF- κ B pathway was detected. The results revealed that the phosphorylation of p65 was significantly enhanced in MODS rats compared to the control rats, and these enhancements were eliminated by TNIP2-plasmid administration.

Taken together, it was found for the first time that TNIP2 is decreased in MODS, and it plays a protective role in MODS development by inhibiting inflammation response and oxidative stress via repressing the activation of the NF- κ B pathway. TNIP2 may be a potential diagnostic marker and therapeutic target for MODS treatment.

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Availability of data and materials

The data sets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HG contributed to study design; HG, XS and JX contributed to data collection, statistical analysis and data interpretation; DZ contributed to data collection, manuscript preparation and literature searching; All authors contributed to the development of the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from every patient enrolled, and the present study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University. The experimental protocol was approved by the Animal Care Committee of Nantong University, and experiments were performed following with the guidance of guide for the care and use of laboratory animals (21).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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