Alleviation of sepsis-induced cardiac dysfunction by overexpression of Sestrin2 is associated with inhibition of p-S6K and activation of the p-AMPK pathway

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Abstract. Sepsis-induced myocardial injury is one of the manifestations of multiple organ dysfunction in sepsis. The aim of the present study was to determine the mechanism of alleviation of lipopolysaccharide (LPS)-induced injury on cardiomyocytes by Sestrin2. A sepsis model using LPS injection was constructed in Sprague-Dawley (SD) rats, and after 6, 12 and 24 h, rat blood was collected and cardiac troponin T (CTnT) levels were determined using ELISA. Heart specimens were excised, tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) levels were detected by ELISA, malondialdehyde (MDA) levels were estimated using colorimetric analysis, and phosphorylated (p)-S6K and p-AMP-activated protein kinase (AMPK) levels were determined by western blot analysis. In the septic rats, phenomenon of myocardial fiber rupture, interstitial edema and inflammatory cell infiltration were observed under light microscope. Following LPS injection, CTnT in serum and MDA in myocardial homogenate were increased time-dependently. TNF-α and IL-6 levels were significantly increased, with a peak at 6 h, p-S6K levels were adaptively downregulated, and levels of p-AMPK and Sestrin2 were adaptively upregulated by LPS. In LPS-injured H9c2 cells, Sestrin2 overexpression attenuated the LPS-mediated inhibitory effects on cell viability, suppressed LPS-mediated increase in CTnT, TNF-α, IL-6 and MDA levels, as well as attenuated p-S6K levels and elevated p-AMPK and Sestrin2 levels. Sestrin2 interference showed the opposite effect. Sestrin2 promoted cell viability and inhibited the inflammatory responses of LPS-injured myocardial cells. The phenomena may be associated with inhibition of p-S6K and activation of the p-AMPK pathway.

Introduction

Sepsis is a systemic inflammatory response syndrome caused by infection. With its high morbidity and mortality, sepsis poses a serious threat to human health, and has become a global public health challenge (1). Organ damage is caused by organ ischemia and infection in the progression of sepsis, and mortality rises sharply to 56-100% when multiple organ dysfunctions (MODS) occur (2,3). Sepsis can further develop into septic shock and multiple organ failure, and patients with severe sepsis often suffer from cardiac dysfunction (4). Previous studies have found that the heart is one of the most significantly damaged organs in MODS. Approximately 50% of sepsis patients may have cardiac dysfunctions, and the mortality rate can even rise to 70% in the case that myocardial inhibition occurs in sepsis (5,6). Therefore, in the treatment of sepsis, effective prevention and treatment of myocardial damage is of great significance in controlling the disease and preventing the deterioration of the disease.

In essence, sepsis is caused by infection-induced host immunity (7). Many factors may be involved in sepsis-induced heart dysfunction, such as the production of nitric oxide, release of inflammatory factors, adhesion and infiltration of monocytes, activation of the coagulation system, oxidative metabolic disorders and so forth (8-10). However, the specific mechanism of myocardial injury in sepsis is still not fully elucidated (11). When the system is infected by bacteria, factors such as lipopolysaccharide (LPS) in bacteria participate in the process of initial organism damage (12). LPS is the outermost lipopolysaccharide of the Gram-negative bacterial cell wall, which can bind to various protein molecules on the cell surface, initiate intracellular signaling molecules, activate different tissues or cells to produce cytokines, release oxygen free radicals and lipid mediators, induce microvascular obstruction and changes in vascular homeostasis, leading to coagulopathy, fever, vasodilatation, capillary leakage, and ultimately resulting in multiple organ failure of sepsis (13). Among them, LPS can activate macrophages through the TLR4 receptor on macrophages, and initiate gene expression of inflammatory factors, such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6). These inflammatory cytokines can directly lead to...
myocardial dysfunction (14,15). The process of blocking these pathways may be a potential strategy for treating myocardial damage in sepsis.

Sestrin2, an important member of the Sestrin family, is a highly conserved stress response protein that is induced in response to stresses, such as DNA damage, oxidative stress and hypoxia (16,17). Sestrin2 has been reported to prevent oxidative liver damage (18) and age-related pathologies (19). Recent research has discovered that Sestrin2 protects the myocardium from radiation-induced dysfunction (20). There is further evidence that Sestrin2 is involved in ischemic AMP-activated protein kinase (AMPK) signaling and the toxicological actions of ischemic stress to the heart (21,22). The mechanism of Sestrin2 in sepsis-induced cardiac dysfunction remains unclear.

Hence, a model of LPS sepsis-induced myocardial injury was constructed, and the mechanism of Sestrin2 overexpression was investigated using this model. As a result, it was found that Sestrin2 overexpression could relieve cardiac oxidative stress induced by LPS, which is valuable for the treatment of myocardial damage in sepsis.

Materials and methods

Animal models. Female healthy Sprague-Dawley (SD) rats weighing 220-250 g, which were purchased from Beijing Vital River Company (Beijing, China), were used for this study. The animals were maintained in a 25°C atmosphere, with a cycle of 12 h light/12 h dark and ad libitum access to food and water. The animal model of endotoxin shock was established by injecting LPS (10 mg/kg) into the peritoneal cavity of the rats and the control group was injected with normal saline. Blood was collected at 6, 12, and 24 h after LPS injection, rats were anesthetized by injection with 3% (v/v) pentobarbital sodium solution (40 mg/kg) into the abdominal cavity, and blood was collected from the inferior vena cava. The serum was separated by centrifugation at 1,000 x g for 10 min at 4°C and preserved at -80˚C for subsequent experimentation. The cardiac troponin T (CTnT) levels in the serum were assessed by ELISA. In addition, heart specimens were taken 6, 12 and 24 h after LPS injection. After anesthesia, rats were sacrificed using a continuous flow of CO₂ using a flow meter unit for 3-5 min at the flow rate of 2 l/min (displacement rate, 20% chamber volume/min). The right region of the heart was homogenized for ELISA detection of TNF-α and IL-6 levels, as well as colorimetric analysis for malondialdehyde (MDA). The left region of the heart was placed into paraformaldehyde fixative for 24 h, and used for paraffin sections (4-μm thick) for hematoxylin and eosin (H&E) staining. All procedures for animal care were approved by the Animal Management Committee of The First Affiliated Hospital of Soochow University.

Cell treatment. H9c2 rat embryo cardiomyocytes were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and used for cell transfection and LPS injury. Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) were used for cell culture. Sestrin2 siRNA was purchased from MISSION predesigned siRNA (Sigma-Aldrich; Merck KGaA). The sequences of si-Sestrin2 were as follows: Sense, 5'-CAGAGU AUGCACAU-3' and antisense, 5'-AUUGUUAACAA UACUCUG-3'. Sestrin2 siRNA (50 nM, siSestrin2 group) and Sestrin 2 overexpression (Sestrin2 group) recombinant plasmids (Sigma-Aldrich; Merck KGaA), as well as the empty vector (NC group) were respectively transfected into H9c2 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells without any treatment were used for control. Then, 50 μg/ml LPS was applied in all the above groups for 48 h for most experiments of cell functions, namely, LPS, LPS+NC, LPS+Sestrin2, LPS+siSestrin2 groups. After a 48-h incubation, the cells were collected and used for subsequent experiments.

Hematoxylin and eosin (H&E) staining. The paraffin sections of the hearts excised at 6, 12 and 24 h after LPS injection were first dewaxed with xylene I and II for 15 min, and then dewaxed in gradient ethanol at different concentrations (100, 95, 85 and 70%, respectively) for 3 min each, and subsequently rinsed with steaming water for 5 min. Next, the sections were stained with hematoxylin for 10 min and then stained with eosin for 5 min. The sections were then placed into gradient concentrations of ethanol (70, 85, 95 and 100%, respectively) for 3 min each for dehydration. After that, the slices were clarified with xylene I, II for 5 min each time and finally sealed with neutral gum.

Cell viability assay. The cell viability of H9c2 cells treated with LPS (0, 5, 10, 50 μg/ml) for 0, 24 and 48 h was measured using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Nantong, China) first. In addition, cell viability of the cells in the different groups (control, NC, Sestrin2, siSestrin2, LPS, LPS+NC, LPS+Sestrin2, LPS+siSestrin2 groups) was also determined. The processes were mainly performed as follows: cells were cultured in 96-well plates (5x10³ cells/well) for 0, 24 and 48 h and stained with 20 μl CCK-8 stain for 1 h. The optical density values (OD) at 450 nm as detected by a microplate reader (BioTek, Winooski, VT, USA) were used for cell viability analysis.

Enzyme linked immunosorbent assay (ELISA). The quantities of CTnT in rat blood, and TNF-α and IL-6 in rat hearts collected at 6, 12, and 24 h after LPS injection, as well as CTnT, TNF-α and IL-6 in the different cell groups (control, NC, Sestrin2, siSestrin2, LPS, LPS+NC, LPS+Sestrin2, LPS+siSestrin2 groups) were determined by ELISA kits (Cusabio, Wuhan, China), following the manufacturer's instructions. The samples and standard substances were added into 96-well plate and incubated for 90 min at 37°C, cultured with biotinylated antibodies for 60 min, and later seeded with avidin peroxidase complex for 30 min before tetramethylbenzidine (TMB) coloration. Finally, OD values at 450 nm as read by a microplate reader (BioTek) were used for quantity calculation.

Colorimetric detection. The levels of lipid peroxidation product MDA in rat heart collected at 6, 12, and 24 h after...
LPS injection, as well as the levels in the different cell groups (control, NC, Sestrin2, siSestrin2, LPS, LPS+NC, LPS+Sestrin2, LPS+siSestrin2 groups) were determined with the Lipid Peroxidation MDA Assay kit (Beyotime Institute of Biotechnology). According to the manufacturer’s instructions, MDA reacts with thiobarbituric acid (TBA) and the red MDA-TBA products were observed at 535 nm using a colorimetric method.

Western blot analysis. The protein levels of ribosomal protein S6 kinase (p-S6K), phosphorylated AMP-activated protein kinase (p-AMPK) and Sestrin2 in the heart tissues of rats treated with LPS (10 mg/kg) for 6, 12 and 24 h, as well as these proteins in the different cell groups (control, NC, Sestrin2, siSestrin2, LPS, LPS+NC, LPS+Sestrin2, LPS+siSestrin2 groups) were detected by western blot analysis. First, the total proteins were extracted using RIPA lysis buffer (Boster Biological Technology, China), and quantified by BCA protein assay reagent kit (Beyotime Institute of Biotechnology). Then, 10 µg protein was loaded in each lane and were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Amersham; GE Healthcare, UK). The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% skim milk with 0.1% Tween 20 at 37˚C for 1 h, and then the membranes were incubated overnight at 4˚C with the following primary antibodies: p-AMPK antibody (cat. no. 4186; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), AMPK antibody (cat. no. 12063; dilution, 1:1,000; Cell Signaling Technology, Inc.), p-S6K antibody (cat. no. 9209; dilution, 1:1,000; Cell Signaling Technology, Inc.), S6K antibody (cat. no. 9202; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-Sestrin2 (ab178518; dilution, 1:1,000; Abcam, Cambridge, UK) and anti-GAPDH (cat. no. ab8245; dilution, 1:1,000; Abcam). GAPDH was used as internal control. Subsequently, the proteins were probed with goat anti-rabbit secondary antibody (HRP) (cat. no. Ab205718, 1:5,000; Abcam) for 1 h at 37˚C. The proteins were detected by enhanced chemiluminescence (ECL) reagents (Amersham; GE Healthcare) and X-ray exposure. Finally, the proteins were quantified using Bio-Rad ChemiDoc system with Image Lab software (version 6.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as mean ± standard deviations, and were statistical analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA), one-way analysis of variance (ANOVA), and Dunnett’s test. P<0.05 was considered as indicative of statistical significance.

Results

LPS induces changes in myocardial morphology and inflammatory responses in rats. The morphological structures of the myocardial tissues of rats injected with LPS (10 mg/kg) for 6, 12 and 24 h were determined by H&E staining. The images showed that the fiber bundles in the control group were normal, neatly arranged, without fractures and dissolution, and no edema was observed in the interstitial. The sepsis rat tissues at 6 h after injection were observed as normal and neatly arranged myocardial fiber bundles, with moderate inflammatory cell infiltration. At 12 h, the myocardial fiber bundles were loosely arranged; some myocardial fibers were broken and dissolved, accompanied by mild interstitial edema and moderate inflammatory cell infiltration. At 24 h, myocardial fiber bundles were loosely arranged, some myocardial fibers were broken and dissolved, partial myocardial cells were degenerated and dissolved, accompanied by necrosis, interstitial edema, and moderate inflammatory cell infiltration (Fig. 1A). Then, the levels of inflammatory factors including CtnT, TNF-α and IL-6 in rat blood collected at 6, 12 and 24 h after LPS injection were measured by ELISA. The results showed that serum CtnT levels were increased time-dependently. Levels of TNF-α and IL-6 in the heart were increased by LPS, reached a peak at 6 h after LPS injection.
and slightly decreased subsequently (P<0.05; Fig. 1B-D). In addition, the lipid peroxidation product MDA levels in the heart were found to be increased time-dependently, as determined by the colorimetric method (P<0.05; Fig. 1E).

**LPS regulates the expression levels of p-S6K, p-AMPK and Sestrin2 in the rats.** The protein levels of S6K, p-S6K, p-AMPK, AMPK and Sestrin2 were determined by western blot analysis in the rats injected with LPS (10 mg/kg) for 6, 12 and 24 h. The results demonstrated that the protein levels of p-S6K were adaptively decreased, and those of p-AMPK and Sestrin2 were adaptively increased by LPS treatment. Moreover, changes at 6 h were the most significant (P<0.01; Fig. 2). Notably, the protein expression levels of total S6K and AMPK were not significantly changed.

**Sestrin2 alleviates LPS-mediated injured cell viability and LPS-enhanced inflammatory response in cardiomyocytes.** The injury effect of LPS on cardiomyocytes was evaluated by CCK-8 assay. Cell viability of the cardiomyocytes treated with LPS (5, 10 and 50 µg/ml) for 24 and 48 h was inhibited time-dependently, and the decrease in cell viability of the cardiomyocytes treated with 50 µg/ml LPS at 48 h was most significant (P<0.05; Fig. 3A). Then, the function of Sestrin2 on cell viability of the cardiomyocytes was detected at 24 and 48 h after LPS treatment. The results showed that the cell viability changed significantly after 48 h of LPS injury. Compared with the control group, cell viability in the LPS and LPS+NC groups was significantly suppressed following treatment with LPS. Cell viability in the LPS+Sestrin2 group was significantly increased, and cell viability in the LPS+siSestrin2 group was significantly decreased, compared with the LPS group. Cell viability in the LPS+Sestrin2 group was lower than that in the Sestrin2 group, and a similar tendency between that in the LPS+siSestrin2 group and in the siSestrin2 group was observed (P<0.05; Fig. 3B). Then, levels of inflammatory factors such as CTnT, TNF-α and IL-6 were detected, showing a significant increase in the LPS and LPS+NC groups, a decrease in the LPS+Sestrin2 group by Sestrin2, and an increase in the LPS+siSestrin2 group by siSestrin2 (Fig. 3C-E). In addition, levels of lipid peroxidation product MDA as determined by colorimetric method, indicated that the MDA levels were increased in the LPS group, and decreased in the LPS+Sestrin2 group (Fig. 3F).
Sestrin2 alleviation of LPS‑mediated injured cell viability is related to regulated levels of p‑S6K and p‑AMPK in cardiomyocytes. Levels of p‑S6K, p‑AMPK and Sestrin2 in cardiomyocytes were evaluated by western blot analysis after Sestrin2 overexpression or siSestrin2 transfection or LPS treatment. The results demonstrated that p‑S6K levels were significantly decreased in the LPS, LPS+NC and Sestrin2 groups, and markedly increased in the siSestrin2 group, compared with the control group. Meanwhile, p‑S6K levels were decreased in the LPS+Sestrin2 group, and increased in the LPS+siSestrin2 group, compared with the LPS group (Fig. 4A and B). In addition, levels of p‑AMPK and Sestrin2 were increased in the LPS, LPS+NC and Sestrin2 groups, and decreased in the siSestrin2 group, compared with the control group; while p‑AMPK and Sestrin2 levels were increased in the LPS+Sestrin2 group, and decreased in LPS+siSestrin2 group, compared with the LPS group. The present results suggested that there was no significant difference in S6K and AMPK levels between the LPS+siSestrin2 group and the LPS group (Fig. 4A, C-F).

Discussion

Lipopolysaccharide (LPS), an important component of the outer membrane of the cell wall of Gram‑negative bacteria, plays an important role in the infection and disease evolution of Gram‑negative bacteria, and is considered to be the main cause of systemic inflammatory syndrome (23). Sepsis‑induced myocardial injury is one of the manifestations of multiple organ dysfunctions in sepsis, which has been confirmed in clinical and sepsis animal experiments (24). LPS is an important substance that causes myocardial damage, by triggering the inflammatory response and seriously affecting cardiac function (25). The sepsis model by injection of LPS can mimic the complex pathophysiological changes of the body caused by bacterial release of LPS during sepsis (26). In the present study, an animal model of sepsis was prepared by intraperitoneal injection of LPS (10 mg/kg) into SD rats. In the septic rats, myocardial fiber rupture, interstitial edema, and inflammatory cell infiltration were observed under light microscope. The above processes were gradually increased as time prolonged, and myocardial tissue necrosis was observed at 24 h after LPS injection. Thus, it is feasible to prepare an animal model of myocardial injury in sepsis by intraperitoneal injection of LPS (10 mg/kg) into SD rats. Serum CTnT is a sensitive indicator of myocardial injury, and elevated serum CTnT is observed in 31‑85% of patients with severe sepsis (27). In patients with septic shock, an increase in serum CTnT predicts a higher mortality rate and a poor prognosis (28). In the present study, it was observed that CTnT was increased in serum 6 h after LPS injection, and gradually increased time‑dependently.

LPS, by initiating transcription and translation of various cytokine genes, causes inflammatory cells to release large amounts of inflammatory factors (29). TNF‑α is recognized as a main mediator of septic shock, and is the first inflammatory factor produced in the early stage of sepsis (30). As a triggering factor of inflammation, TNF‑α is involved in the induction of IL‑1 production, and together with IL‑1 induces the formation of secondary inflammatory factors such as IL‑6, resulting in an inflammatory cascade (31,32). In the serum of patients with sepsis, inflammatory factors, such as TNF‑α and IL‑6 are elevated, and these inflammatory factors have a direct inhibitory effect on myocardial cell contraction (33). IL‑6 causes myocardial damage by activating nitric oxide synthase, and elimination of IL‑6 relieves myocardial damage in sepsis (34). Injecting TNF‑α into animals can replicate the
complex metabolic, hemodynamic, and pathological changes in septic shock syndrome, whereas rats with TNF-α-encoding gene loss do not develop septic shock after LPS injection (35). Therefore, TNF-α and IL-6 are the main inflammatory factors in sepsis-induced multiple organ dysfunctions (MODS). In the present study, TNF-α and IL-6 were significantly increased in the myocardial homogenate, indicating that TNF-α and IL-6 were involved in the inflammatory reaction after LPS injection. In addition, the concentrations of TNF-α and IL-6 at 6 h were higher than those at 12 and 24 h after LPS injection, indicating that concentrations of TNF-α and IL-6 peaked in the myocardial homogenate 6 h after LPS injection, and gradually decreased time-dependently. The cause of its occurrence is unknown and may be related to the metabolism and the adaptive upregulation response. In addition, MDA is a degradation product of lipid peroxide, and its content is positively correlated with the degree of cell damage (36). The changes in MDA content can indirectly reflect the changes in

Figure 4. Alleviation of LPS-injured cell viability by Sestrin2 is associated with regulation of p-S6K and p-AMPK in cardiomyocytes. (A) p-S6K, p-AMPK and Sestrin2 protein levels were detected by western blot analysis. GAPDH acted as an internal control. (B) Sestrin2 decreased p-S6K levels. (C) Sestrin2 increased p-AMPK levels. (D) Sestrin2 increased the Sestrin2 protein levels in LPS-induced H9c2 cells. (E) Sestrin2 decreased the ratio of p-S6K/S6K, and siSestrin2 had opposite effect. (F) Sestrin2 increased the ratio of p-AMPK/AMPK, and siSestrin2 had opposite effect. *P<0.05 and **P<0.01 vs. the control group, #P<0.05 and ##P<0.01 vs. the Sestrin2 group, ^P<0.05 and ^^P<0.01 vs. the siSestrin2 group, &P<0.05 and &&P<0.01 vs. the LPS group. LPS, lipopolysaccharide; p-, phosphorylated; AMPK, AMP-activated protein kinase.
oxygen free radical content in tissues (37). In the present study, after the establishment of the sepsis model, MDA content in the myocardial homogenate increased significantly, and the concentration gradually increased with time, indicating that the oxidative damage gradually increased time-dependently after LPS injection.

The signaling pathways related to inflammatory reactions should play important roles in sepsis-induced cardiac dysfunction. mTOR is a type of conserved kinase, which stimulates cell anabolism and growth by increasing the synthesis of proteins and lipids through p-S6K (19). p-S6K was found to be increased in tubulointerstitial inflammation and fibrosis (38). In addition, the important regulatory role of the AMPK pathway in the inflammatory response has also drawn much attention. In vitro studies have found that AMPK activation can also significantly reduce the expression of inflammatory mediators and tissue inflammatory damage, and it is an important new target for inflammation regulation (39-41). In this study, after the establishment of the sepsis model, the expression levels of p-S6K were decreased at 6 h after LPS injection, and increased again at 12 and 24 h. The expression levels of p-AMPK and Sestrin2 were increased at 6 h after LPS injection, and decreased at 12 and 24 h. The cause of its occurrence is uncertain and may be related to the adaptive upregulation response.

Sestrin2, as an important oxidative stress response protein, was reported to protect the myocardium from radiation-induced dysfunction (20). We overexpressed Sestrin2 and silenced Sestrin2 in LPS-injured H9c2 cells. The results indicated that the cell viability of the LPS-injured H9c2 cells was enhanced by Sestrin2 over-expression, whereas the cell viability was inhibited by Sestrin2 interference. Decreased levels of TNF-α, IL-6 and MDA in the LPS-injured H9c2 cells by Sestrin2 overexpression were observed. The results suggested that Sestrin2 overexpression inhibited the inflammatory response and the lipid peroxidation chain reaction of the cell membrane, leading to a reduction in myocardial tissue damage. In regards to the inflammation-related signaling, Sestrin2 overexpression promoted levels of Sestrin2 and p-AMPK, and inhibited p-S6K levels, while Sestrin2 interference inhibited levels of Sestrin2 and p-AMPK, and promoted p-S6K levels in H9c2 cells with or without LPS injury. Although LPS showed the same tendency as Sestrin2 in H9c2 cells, this may depend on the adaptive upregulation response.

In conclusion, Sestrin2 promoted cell viability and inhibited inflammatory responses in LPS-injured myocardial cells. The phenomena may be associated with inhibition of p-S6K and activation of the p-AMPK pathway.

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Availability of data and materials

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

ZW wrote the manuscript. LB and PY performed the experiments. ZW designed the study. SF and FX analyzed the data. ZW revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures for animal care were approved by the Animal Management Committee of The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


