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MicroRNA-30e promotes hepatocyte proliferation and inhibits apoptosis in cecal ligation and puncture-induced sepsis through the JAK/STAT signaling pathway by binding to FOSL2

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ABSTRACT

Introduction: Hepatocyte proliferation and apoptosis are critical cellular behaviors in rat liver as a result of a liver injury. Herein, we performed this study in order to evaluate the role of miR-30e and its target Fos-Related Antigen-2 (FOSL2) in septic rats through the JAK/STAT signaling pathway.

Methods: Rat models of sepsis were induced by cecal ligation and puncture. Enzyme-linked immunosorbent assay (ELISA) was performed to access serum levels of lipopolysaccharide (LPS), inflammatory factors, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to confirm the successful establishment of the model. The hepatocytes were subject to miR-30e mimics, miR-30e inhibitors or siRNA-FOSL2. The expressions of miR-30e, FOSL2, apoptosis- and, JAK/STAT signaling pathway-related genes in liver tissues and hepatocytes were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. MTT assay and flow cytometry were performed to evaluate hepatocyte viability and apoptosis, respectively.

Results: The results obtained revealed that in the septic rats, serum levels of inflammatory factors, LPS, ALT and AST, as well as the expression of FOSL2 were elevated and the JAK/STAT signaling pathway was activated, while there was a reduction in the expression of miR-30e. An initial bioinformatics prediction followed by a confirmatory dual-luciferase reporter assay determined that miR-30e targeted and negatively regulated FOSL2 expression. MiR-30e inhibited the activation of JSK2/STAT3 signaling pathway by reducing FOSL2 expression, while miR-30e enhanced hepatocyte proliferation and decreased hepatocyte cell apoptosis in septic rats.

Conclusion: These findings indicated that miR-30e may serve as an independent therapeutic target for sepsis, due to its ability to inhibit apoptosis and induce proliferation of hepatocytes by targeted inhibition of FOSL2 through the JAK/STAT signaling pathway.

1. Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS), which occurs as a result of a serious, life threatening infection or organ dysfunction [1,2]. More concretely, sepsis is caused when the host's immune system responds to bacterial, fungal and infections, which derives from urinary tract, abdomen, skin and respiratory tract [3]. It is also correlated with decreased selenium levels, reduced endogenous antioxidative capacity and increased reactive oxygen species [4]. The occurrence of sepsis increases with age, as a result of which it is more prevalent in the elderly and it develops substantially [5]. Each year, approximately 1,000,000 Americans are affected by sepsis, with 4%–10% death rate in pediatric patients and 28%–50% mortality rate in adult patients [6]. Patients suffering from sepsis have increased risks of shock and multisystem organ failure, resulting in death without the assistance of timely resuscitation, antibiotics and life support institution [7]. Therapies for sepsis contain activated protein C, stringent control of blood glucose, and early goal-directed therapy to treat cellular oxygen deficit [8]. Even after the successful treatment of sepsis, patients are still prone to irreversible physical and cognitive impairment [9]. Recently, the involvement of microRNAs (miRNAs) in the treatment of sepsis has been revealed [10,11].

MiRNAs are single stranded noncoding RNAs of 20–22 nucleotides, which regulate gene expression by triggering mRNA cleavage or repressing translation [12]. MiRNAs are also involved in fundamental cellular processes of cancer cells, including proliferation, differentiation

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and survival [13,14]. Based on previous findings, specific miRNAs have been found to play an important role in inflammation. [15]. MicroRNA-30e (miR-30e), as a member of miR-30 family, has also been proved to be involved in cell proliferation and apoptosis [16]. miR-30e is also associate with several types of cancer including hepatocellular carcinoma (serves as a tumor suppressor) and glioma [17,18]. Fos-Related Antigen-2 (FOSL2), which is regulated by miRNAs, has been demonstrated to be of significance in cellular proliferation and apoptosis and it also affects inflammatory bone and skin disease [19–21]. In recent years, miR-30e has been reported to act as a tumor suppressor in hepatocellular carcinoma via the JAK1/STAT3 pathway [18], which is an important pathway for many pivotal cytokines' signal transduction of sepsis pathogenesis [22]. Nevertheless, the roles of miR-30e and FOSL2 in sepsis are yet to be investigated. Therefore, the present study was conducted with the purpose of determining the expression of miR-30e and FOSL2 in sepsis and their effects on hepatocyte proliferation and apoptosis through the JAK/STAT pathway.

2. Materials and methods

2.1. Model establishment

A total of 50 healthy male Wistar rats (weighing 250–300 g) were purchased form Laboratory Animal Center of China-Japan Friendship Hospital. The rats were kept in cages separately with free access to water and food and circadian rhythm at 25 °C with relative humidity of 50%–70%. The cages and beddings were changed every two days. The experiments began following a week of feeding. The rats were then divided into two groups randomly: the sepsis group and the control group. Cecal ligation and puncture (CLP) was performed in rats from the sepsis group to induce sepsis through the following procedure: the rats were first anesthetized with the intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg). After disinfection, a 2–3 cm incision was made along the median line of abdomen. With the caecum exposed, and the mesentery striped, the end of ileocecal valve was ligated to ensure intestinal continuity. NO.22 needle was used to incise caecum to make the flow of intestinal contents into peritoneum easier [23]. The cecum was then placed back in the abdominal cavity, and the incisions were sutured layer by layer. Normal saline (50 ml/kg) was injected immediately to prevent shock. After the operation, all the rats were fed conventionally. The rats in the control group did not undergo any treatment. The experiments conducted in the present study were approved by the Ethics Committee of China-Japan Friendship Hospital.

2.2. Enzyme-linked immunosorbent assay (ELISA)

The death rates of the mice in both groups (the sepsis group and the control group) within 24 h were recorded. The 10 surviving rats were then selected from the sepsis group and the control group, respectively 24 h after model establishment. They were anesthetized through the intraperitoneal injection of 3% pentobarbital sodium (1–2 ml, P3761, Sigma, St. Louis, MO, USA). Following the extraction of blood from the inferior vena cava, the whole blood samples were centrifuged at 4 °C at 2000 r/min for 10 min to separate serum. The expressions of lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-1β (IL-1β) in part of the serum were evaluated using an ELISA kit. The expressions of alanine aminotransferase (ALT) as well as aspartate transaminase (AST) of the rest serum were analyzed using an automatic biochemical analyzer (HITACHI-7170, Hitachi, Tokyo, Japan). The high expression level of endotoxin could be a marker of the successful establishment of septic rat models as it is one of the most sensitive factors that could induce inflammation [24].

All rats were then sacrificed. Some of the liver tissues were fixed in 4% paraformaldehyde (Lot B130053, Biosntech Co., Ltd., Beijing, China) for histopathological and immunological analysis, while the remaining fresh livers were stored in liquid nitrogen at −80 °C for later use.

2.3. Hematoxylin-eosin (HE) staining

After the liver tissues had been fixed in 4% paraformaldehyde for 16 ~ 18 h, they were dehydrated (1 min each time) in graded ethanol (70%, 80%, 90%, 95%, 100%) and cleared twice by xylene (X820585-500 ml, Hongzhe Biotech Co., Ltd., Hefei, Anhui, China). Afterwards, the samples were embedded in paraffin and were sliced into 4 μm sections. Some of the paraffin-embedded sections were then dewaxed and rehydrated for immunohistochemistry. Subsequently, the sections were stained by hematoxylin (Baomanbio Technology Limited Company, Shanghai, China) for 10 min at room temperature and then they were washed with running water for 30–60 s. After that, the sections were dissimilated by 1% ethanol-hydrochloric acid and washed by running water for 1 min each. The sections were then stained by eosin for 5 ~ 10 min at room temperature, and dehydrated by graded ethanol, and then cleared twice by xylene, followed by mounting in neutral balsam. Morphological changes of sections were observed under an Axio Observer fluorescence microscope (Axio Observer A1/D1/Z1, Zeiss, Göttingen, Germany) and photographed. The degree of liver injury was graded and scored according to the score system "Hepatic Injury Severity Scoring" (HISS) [25].

2.4. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

A total of frozen liver tissues were collected from the right lobe was 100 mg, in order to extract total RNA based on the instructions stated on the miRNeasy Mini Kit (217004, Qiagen, Hilden, Germany). The concentration as well as purity of RNA was determined by NanoDrop 2000 micro-ultraviolet spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was reversely transcribed into cDNA based on the instruction of TaqMan MicroRNA Assays Reverse Transcription Primer (4427975, Applied Biosystems Inc., Foster City, CA, USA). The cDNA was diluted to 50 ng/μl, and was added with 2 μl cDNA each time until the amplification system was up to 25 μl. The reaction conditions were reverse transcription at 37 °C for 30 min and inactivation of reverse transcriptase at 85 °C for 5 s. Primer 3.0 online database was used to design miR-30e, FOSL2, JAK2, STAT3, Bcl-2, Bax, TLR4 and HMGB1 primers. After the specificity of the primer was matched using BLAST, and primers were synthesized by TaKaRa Company (Tokyo, Japan) (Table 1). RT-qPCR was conducted by using ABI7500 PCR instrument (7500, Applied Biosystems Inc., Carlsbad, CA, USA). The reaction conditions were as follows: 40 cycles of pre-denaturation at 95 °C for 10 min; denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 70 °C. The fluorescent qPCR reaction system was 20 μl, including 10 μl of SYBR Premix Ex TaqTM II, 0.8 μl of PCR forward primer (10 μM), 0.8 μl of PCR Reverse Primer (10 μM), 0.4 μl of ROX Reference Dye, 2.0 μl of cDNA template and 6.0 μl of sterile distilled water. The calibration curve method was adopted to determine the primer amplification efficiency of qPCR. U6 was adopted as the internal reference of miR-30e and β-actin was adopted as the internal reference of FOSL2, JAK2, STAT3, Bcl-2, Bax, TLR4 and HMGB1. The $2^{-\Delta\Delta Ct}$ method was used to work out the gene expression ratio between the two groups. The following formula was used in the present experiment: $\Delta Ct = Ct_{miRNA/gene} - Ct_{\beta\text{-actin}}$. Ct value referred to the number of amplification cycles until the fluorescence value reached the setting threshold. Each experiment was repeated three times (6 repeated wells were set for each qPCR). After transcription for 48 h, the mRNA expression of cells was determined using the aforementioned method.

2.5. Western blot analysis

A total of 100 mg frozen liver tissues from the right lobe were