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ABSTRACT

Sepsis is a kind of systemic inflammatory response syndrome caused by infection, which has high morbidity and mortality. Studies have shown that reducing sepsis-related liver injury and restoring liver function can reduce the morbidity and mortality of it. Current clinical treatment methods for sepsis have many disadvantages. Our study aimed to investigate the mechanism of sepsis-induced liver injury and to find a proper therapeutic target for sepsis. In this paper, we have found that when miR-324-3p was overexpressed, the inflammatory infiltration and ferroptosis in liver injury cells aggravated. Further studies showed that overexpression of miR-324-3p could bind to the 3'-UTR of SNHG11 directly so as to decrease the expression level of SNHG11. Our study indicated that LncRNA SNHG11 can mediate the ferroptosis of liver injury cells induced by sepsis through the miR-324-3p/GPX4 axis. Suggesting that it is a new drug target for clinical treatment of sepsis and sepsis-associated liver injury, then we can improve the survival rate for sepsis patients.

Introduction

Sepsis is a kind of systemic inflammatory response syndrome, it is caused by the dysregulated host response to infection (e.g. bacteria and viruses) and has very high morbidity and mortality (1, 2), tens of millions of people die every year from or related to sepsis, establishing an effective treatment of sepsis has long been a great challenge all over the world (3). The concept of modern sepsis was first proposed in the 19th century by Semmelweis et al. (3) as we know. Since then, scientists have carried out a large number of studies on sepsis. Despite our advanced understanding of sepsis, however, the morbidity and mortality of which have still remained high. It is urgent to find a better way to treat sepsis (5, 6).

The liver is the largest organ in the viscera of the human body which plays a vital role in various physiological processes such as metabolism and immune homeostasis, it is also the organ most susceptible to sepsis (7), liver injury may occur at any stage of sepsis. During the development of sepsis, the liver can be injured by various pathogens (e.g. bacteria or viruses), toxins, or some inflammatory mediators, liver injury generally progresses from active hepatocyte dysfunction to liver injury, and may eventually develop into liver failure (7). Among patients with sepsis, the incidence of sepsis-induced liver dysfunction ranges from 34% to 46%, of which liver failure ranges from 1.3% to 22% (9-12). Liver dysfunction and liver failure are proven to be serious complications of sepsis, and are even directly associated with the development and death of sepsis (13), early detection and intervention can improve the survival rate of patients with sepsis to a certain extent (14, 15). The present clinical treatment of sepsis-induced liver injury is mainly based on traditional methods, such as antibiotics, antibacterial, and anti-platelet therapy (16-18). However, these traditional therapies have disadvantages, such as low specificity, short duration of drug action, and obvious side effects. Therefore, it is necessary to find better drug targets to treat sepsis-induced liver injury with good therapeutic effects and low side effects to treat sepsis and further improve the survival rate of patients who have sepsis.

Ferroptosis is a unique form of programmed cell death, which differs from apoptosis, necrosis and autophagy and has unique morphological, genetic and biochemical characteristics. The abnormal intracellular iron ion concentration and lipid peroxidation are the hallmark symbols of ferroptosis (19), which is actually caused by iron-dependent lipid peroxidation, the lipid peroxidation then produces excessive reactive oxygen species (ROS) in cells (20). The process of ferroptosis can be roughly divided into the following processes: the abnormal increase of intracellular iron ions after the disorder of intracellular iron metabolism, and the iron ions can catalyze Fenton reaction, leading to the production of excess ROS (21, 22), the glutathione (GSH) was depleted in this process, and the activity of glutathione peroxidase 4 (GPX4) was also reduced. GSH depletion and decreased GPX4 activity result in the accumulation of iron ions inside the cell, which then catalyze the production of ROS. The excessive production of ROS causes oxidative stress, damaging the cell membrane and organelle, leading to cell death. This process is called ferroptosis, which is characterized by iron-dependent cell death.

LncRNA SNHG11 induces ferroptosis in liver injury cells through miR-324-3p/GPX4 axis-mediated sepsis

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in the inability to metabolize lipid peroxides, excessive accumulation of lipid peroxides can disrupt the integrity of the cell membrane and eventually induce cell death (23, 24). More and more studies have shown that ferroptosis is involved in a variety of diseases, including cancer (25), heart disease (26) and liver disease, and liver disease is closely related to ferroptosis (26).

GPX4 has been found to be a key factor regulator of ferroptosis since the discovery of ferroptosis (28, 29). GPX4 has excellent antioxidant capacity, and it interrupts lipid peroxidation by using GSH as a cofactor to convert intracellular lipid peroxides to non-toxic lipid alcohols (30, 31). A growing number of studies have proved that GSH biosynthesis and GPX4 function are core regulatory factors of ferroptosis (23, 32). In the proceed of GPX4-regulated ferroptosis, multiple regulators are involved, such as NFE2L2/NRF2, which can indirectly activate GPX4 by activating SLC7A11, leading to the inhibition of ferroptosis (33). Additionally, a recent study showed that NRF1 could suppress ferroptosis by maintaining GPX4 expression in a manner distinct from NRF2 (34, 35).

MicroRNAs (miRNAs) usually refer to short non-coding RNAs, the sequences of which were between 19 to 25 nucleotides, miRNAs mainly regulate gene expression in a manner of post-transcriptionally (36). miR-324-3p is a kind of miRNA, belonging to the miR-324 family. It has been proved to be closely related to the pathogenesis of a variety of cancers, such as affecting the development of gastric cancer in miR-324-3p/Smad4/Wnt pathway (37), suppressing the nasopharyngeal carcinoma via targeting WNT2B (38) and promoting the growth of liver cancer by targeting DACT1 and activating Wnt/β-catenin pathway (39). In addition, miR-324-3p was proved to target GPX4 directly, which was regulated by binding to the 3'-UTR of the gene, decreasing the expression of GPX4 and further promoting ferroptosis (40, 41). In this regard, miR-324-3p is a good target to treat ferroptosis, and it also suggests that miR324-3p inhibitor drugs may have unexpected effects on inhibiting ferroptosis.

Long non-coding RNAs (LncRNAs) are a class of RNA with sequence length greater than 200 nt and no function of protein-coding (42). Transcriptome analysis has identified a growing number of tissue-specific LncRNAs, while the biological functions of many LncRNAs in diseases remain unknown (43-45). A unique ability of LncRNAs is that they can interact with various molecules like RNA, DNA or protein through specific RNA functional domains. Thus they can confer different functions after forming the complexes with different molecules (46). Small nuclear host gene 11 (SNHG11), a newly discovered lncRNA, has been considered a prognostic marker for some cancers (47, 48). Recent studies have found that it is also related to the development of gastric cancer and pancreatic cancer (49, 50). In this paper, we demonstrated that LncRNA SNHG11 expression was regulated by miR-324-3p, and SNHG11 could directly bind to miR-324-3p, which is closely related to the development of ferroptosis. Our study demonstrates that LncRNA SNHG11 induces ferroptosis in sepsis-associated liver injury through miR-324-3p/GPX4 axis, which could provide a new drug target for the treatment of sepsis and sepsis-associated liver injury.

Materials and Methods

Animals

The male C57BL/6 mice used in the experiment were 6 weeks old, weighing 18–22 g, and were provided by Changzhou Cavings Laboratory Animal Co., LTD., animal certificate No.: SCXK (Su) 2021-0013. The animals were kept in an environment of 22 ± 2°C, with food and water. This study was approved by the Animal Ethics Committee of Kunming Medical University Animal Center.

Establishment of animal models

LPS was prepared at a concentration of 1.0 mg/ml in advance. Thirty-six male C57BL/6 mice were randomly divided into (i) Sham operation group (Sham group); (ii) sepsis-induced acute liver injury Model group (Model group); (iii) miR-324-3p overexpression group (miR-324-3p agomir group); (iv) miR-324-3p inhibition group (miR-324-3p antagomir group); (v) antagomir-NC group; (vi) agomir-NC group. Each group has 6 mice. Treatment for different groups is as follows: Group (iii)-(vi) were intravenously injected with different types of RNA fragments, such as miR-324-3p agomir in group (3), miR-324-3p in group (4), the Model and Sham groups were intravenously injected with the same volume of normal saline. After that groups (ii)-(vi) were intraperitoneally injected with 15 mg/kg LPS to establish sepsis mice models, the Sham group was intraperitoneally injected with the same volume of normal saline. 24 h later, the mice were sacrificed for liver tissue extraction and blood sample collection.

Western blot assay

Protein lysate (1 ml RIPA, 1% phenylmethylsulfonyl fluoride (PMSF), 0.1% aprotinin) was used to extract the total protein from liver tissue of each group. Total protein was extracted by lysis on ice for 30 min, protein was quantified by BCA method. 20 μg of protein was subjected to sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis (PAGE) reaction. The protein gel was transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 5% skim milk powder in room temperature, incubated for 1 h, then the membranes were incubated with the primary antibodies (GAPDH, 1:1000 dilution; GPX4, 2 μg/ml) at 4°C overnight. The next day, the membranes were incubated with a secondary antibody (1:10000 dilution) at room temperature after washing with TBST three times. After incubation for 1 h, the membranes were washed three times with TBST, and the electrochemiluminescence (ECL) agent was added to show the target band. The protein bands were digitalized with Image Pro Plus 6.0 software and further statistically analyzed with GraphPad Prism 9.0.

RNA isolation and qRT-PCR

Total RNA was isolated from the liver of mice by TRIZol (9109, TaKaRa, Tokyo, Japan). Synthesis of the first strand cDNA was performed using the cDNA Synthesis Kit (1708891EDU, Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. qRT-PCR was used to detect the expression of miR-324-3p, SNHG11 and GPX4 in liver tissues of various groups. The qRT-PCR reaction system was configured according to the instructions of the qRT-PCR kit (RR420Q, TaKaRa, Tokyo, Japan). The relative expression level of miRNA was calculated by the 2⁻ΔΔCT method (51). The primer sequences used in the study were SNHG11-F,
Detection of the content of ALT and MDA

The blood of each mouse was collected by eyeball blood sampling, serum was separated for detection by centrifugation at 800 × g for 10 min. The expression levels of ALT (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and MDA (Solarbio, Beijing, China) in the serum of each group were detected using the kit according to the manufacturer’s instructions.

Statistical analysis

Statistical analyses were produced using GraphPad Prism 9.0 (La Jolla, CA, USA). Data were presented as the mean ± standard deviation (SD) from three independent experiments. Significant differences were analyzed using Tukey’s test and one-way ANOVA. Statistical significance was determined by a P value <0.05. Densitometry analysis of western blot bands was based on three independent experiments using Image Pro Plus 6.0 software.

Results

miR-324-3p overexpression caused the disorder of mice liver tissue structure and aggravated inflammatory infiltration

Liver injury, which is one of the common accompanying symptoms of sepsis (52), after establishing the sepsis model, we examined the liver tissue injury in the mice. As shown in Figure 1, the liver tissue structure of mice in the Model group was disordered and inflammatory infiltration was obvious compared with the Sham group. While agomir-NC group and antagonim-NC group had no significant changes in liver tissue structure compared with the Model group. The results indicated that the structure of liver tissue in septic mice was significantly disordered; indicating that sepsis caused liver injury in mice. Compared with the agomir-NC group, the liver tissue structure of the miR-324-3p agomir group was disordered and inflammatory infiltration was more severe. While the structure disorder and inflammatory infiltration of liver tissue in the miR-324-3p antagonim group were relieved compared with the control group. The results showed that the overexpression of miR-324-3p could cause the disorder of liver tissue structure and aggravate inflammatory infiltration, and the inhibition of miR-324-3p can alleviate the above phenomenon (Figure 1).

miR-324-3p overexpression increased ROS expression and MDA content

ROS and MDA contents abnormally increase when ferroptosis occurs (53). To investigate the effect of miR-324-3p on the occurrence of ferroptosis in liver cells, we examined the expression of ROS and MDA under the condition of overexpression and inhibition of miR-324-3p, respectively. As is shown in Figure 2, ROS expression (Figure 2A-B) and MDA content (Figure 2C) were increased significantly in septic mice. The overexpression of miR-324-3p can increase ROS expression (Figure 2A-B) in liver tissue and MDA content in serum (Figure 2C) in septic mice. However, when the effect of miR-324-3p was inhibited, that is, the injection of miR-324-3p antagonim compared with the control group could reduce the expression of ROS (Figure 2A-B) in liver tissue and the content of MDA (Figure 2C) in serum. These results indicated that miR-324-3p can increase ROS expression in liver tissue...
and MDA content in serum, which means the occurrence of ferroptosis.

Overexpression of miR-324-3p inhibited the expression levels of GPX4 and SNHG11 in liver tissue

After injection of miR-324-3p agomir and miR-324-3p antagonir, we detected the expression levels of miR-324-3p compared with the control groups, respectively. As shown in Figure 3, miR-324-3p mRNA expression level was significantly increased in the Model group compared with its control group, i.e. Sham group (Figure 3A). After that we detected the RNA expression levels of SNHG11 and GPX4. RNA expression levels of SNHG11 and GPX4 in the Model group were significantly decreased when overexpressed miR-324-3p (Figure 3B and 3C). And the RNA expression levels of SNHG11 and GPX4 in the miR-324-3p agomir group were also significantly decreased compared with the control group (Figure 3B and 3C). The RNA expression levels of SNHG11 and GPX4 in the miR-324-3p antagonir group were significantly increased (Figure 3B and 3C). After that we detected the protein expression level of GPX4, the results were the same as that of RNA expression levels (Figures 3D and 3d). These results indicated that overexpression of miR-324-3p decreased the expression levels of SNHG11 and GPX4.

Overexpression of miR-324-3p increased the levels of TNF-α, IL-1β and ALT in serum

As shown in Figure 4, compared with the Sham group, the contents of TNF-α, IL-1β and ALT in serum were all increased significantly in the Model group. The contents of TNF-α, IL-1β and ALT in serum of the agomir-NC group and antagonir-NC group had no significant changes compared with the Model group, respectively. Compared with the Agomir-NC group, the contents of TNF-α, IL-1β and ALT in serum of miR-324-3p agomir group were increased. Compared with the Antagomir-NC group, the contents of TNF-α, IL-1β and ALT in the miR-324-3p antagonir group were decreased in serum. The results proved that the miR-324-3p could increase the TNF-α, IL-1β and ALT contents in serum, that is, the overexpression of miR-324-3p aggravated the inflammatory response of liver-injured cells in septic mice.

miR-324-3p can bind to the 3’-UTR of LncRNA SNHG11

In a previous experiment, we detected that overexpression of miR-324-3p inhibits SNHG11 expression. To explore the mechanism, we performed a dual luciferase reporter assay. As shown in Figure 5, through sequence alignment, the binding sites between mmu-miR-324-3p and SNHG11 were predicted. Luciferase reporter assay result further indicated that miR-324-3p mimic significantly
SNHG11 is successfully verified that miR-324-3p can directly bind to SNHG11, we have predicted miR-324-3p binding sites and decreased the luciferase activity of the wide-type (WT) of SNHG11 3'-UTR but there is no obvious changes in the SNHG11-MUT 3'-UTR sequence through dual-luciferase reporter assay. Sepsis is the systemic inflammatory syndrome which is caused by infection, with high morbidity and mortality. Our study found that in sepsis-induced liver injury mice, LncRNA SNHG11 can mediate ferroptosis in liver-injured cells through the miR-324-3p/GPX4 axis, which provided a new target for the treatment of sepsis and sepsis-induced liver injury. Long non-coding RNAs (LncRNAs) are RNA sequences of more than 200 nt in length that do not encode proteins, which have been found to be important regulators of cellular physiology and pathology (54, 55). In the past decade, an increasing number of LncRNAs have been identified by RNA deep sequencing (56, 57). Increasing evidence suggested that LncRNAs were dysregulated in different types of cancer and play important roles in the progression of various cancers (50, 58, 59). SNHG11 is a newly discovered LncRNA, there is currently little information for the function of SNHG11 in cancer. It has been reported that the increased expression of SNHG11 may represent a poor prognosis in hepatocellular carcinoma (HCC). SNHG11 can promote the proliferation, migration, apoptosis and autophagy of HCC by regulating AGO2 through miR-184 (60). It has also been proved that SNHG11 has a diagnostic ability to identify early tumor formation and can promote the proliferation of colorectal cancer through mutual regulation with c-Myc (59).

In our study, SNHG11 expression was found to be related to sepsis-induced liver injury mice, LncRNA SNHG11 can mediate ferroptosis for the function of SNHG11 in cancer. It has been reported that the increased expression of SNHG11 may represent a poor prognosis in hepatocellular carcinoma (HCC). SNHG11 can promote the proliferation, migration, apoptosis and autophagy of HCC by regulating AGO2 through miR-184 (60). It has also been proved that SNHG11 has a diagnostic ability to identify early tumor formation and can promote the proliferation of colorectal cancer through mutual regulation with c-Myc (59).

miRNA is a kind of small non-coding RNA encoded by endogenous genes, about 19-25 bp in length. miRNAs usually are able to negatively regulate the expression of its target genes by recognizing specific target mRNA at the post-transcriptional level, such as promoting mRNA degradation and/or inhibiting the translation process (61). Studies have shown that miRNAs also play an important role in the apoptosis, proliferation and other processes of certain tumor cells as oncogene or tumor suppressor genes (62-64). miR-324-3p was first discovered in mammalian neurons (65), later studies showed that it was abnormally expressed in a variety of tumors. Previous studies have shown that the 3'-UTR of GPX4 can target and bind to miR-324-3p, thereby down-regulating its expression and promoting ferroptosis (40, 41). Our study found that in septic mice, overexpression of miR-324-3p aggravated liver injury and verified infiltration in mice, while inhibition of miR-324-3p alleviated this phenomenon (Figure 1). Meanwhile, overexpression of miR-324-3p was found to increase the levels of ROS and MDA in serum (Figure 2), indicating that miR-324-3p aggravated the ferroptosis of liver-damaged cells in sepsis-induced mice.

In summary, we found that the expression of miR-324-3p could affect the inflammatory response of liver-injured cells in septic mice, inhibiting the expression of miR-324-3p could alleviate the inflammatory infiltration phenomenon in liver-injured cells. In addition, LncRNA SNHG11 was involved in the process of miR-324-3p/GPX4 axis-mediated ferroptosis in liver-injured cells. Studies have shown that liver injury is one of the direct causes of death in patients with sepsis (13), and early intervention against sepsis-induced liver injury can greatly improve the survi-
val rate of patients. Our findings provide new drug targets for the treatment of sepsis and new possibilities for reducing mortality in patients with sepsis.

**Conflict of Interests**
The authors declared no conflict of interest.

**References**

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