Original Article
miR-144 inhibits development of meningitis in rats by down-regulating TLR2

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Abstract: Purpose: This article was to investigate the effect of miR-144 on meningitis in rats and its mechanism. Methods: A rat meningitis model was constructed and divided into the Meningitis group, the Meningitis + agomir group, and the Meningitis + antagomir group. A sham group was served as control. Loeffler scores, white blood cell count as well as IL-1β and TNF-α level in the cerebrospinal fluid were measured. TLR2 protein expression in brain tissues was detected by immunohistochemistry and Western blot. miR-144 and TLR2 mRNA expression in brain tissues was determined by qRT-PCR. Luciferase reporter assay was performed to verify the targeting relationship between miR-144 and TLR2. Results: Compared with the sham group, the meningitis rats had much higher body temperature, lower Loeffler score, higher white blood cell count, IL-1β and TNF-α level, markedly lower miR-144 expression, and much higher TLR2 mRNA and protein expression (all $P < 0.05$). Compared with the Meningitis group, infusion of miR-144 agonists decreased white blood cell count as well as IL-1β and TNF-α level, and down-regulated TLR2 expression ($P < 0.05$ or $P < 0.01$). miR-144 inhibited TLR2 expression. Conclusions: miR-144 inhibited development of meningitis in rats by down-regulating TLR2.

Keywords: Meningitis, miR-144, TLR2, IL-1β, TNF-α

Introduction

Bacterial meningitis is a common infectious disease of the central nervous system in infants and young children. It has a high mortality rate and severely affects the life and health of children. In addition, meningitis also causes severe sequelae, such as paralysis, hydrocephalus, epilepsy, deaf, blindness, and intellectual damage [1, 2]. Therefore, early prevention and diagnosis of meningitis are very important for improving the prognosis of patients.

In recent years, the treatment of diseases at the genetic level has received increasing attention. miRNA are also deeply studied in multiple diseases. As a miRNA, miR-144 has also been reported to be involved in many diseases, especially in tumors, and is considered to be a tumor suppressor in glioblastoma, laryngeal squamous cell carcinoma, as well as gastric cancer [3-5]. Meanwhile, miR-144 has also been found to participate in and regulate inflammatory responses. Dongmin et al. [6] illustrated that in rats with non-alcoholic steatohepatitis, down-

regulation of miR-144 could stimulate the release of pro-inflammatory cytokines. Liu et al. [7] also found that, in monocyte-derived macrophages infected with M. tuberculosis, inhibition of miR-144 could lead to accelerated secretion of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. However, expression of miR-144 in meningitis and its effect on the development of meningitis have not been reported before. Therefore, this article established a model of rat meningitis through S. pneumoniae infection. The effect of miR-144 on development of meningitis in rats was also researched in depth. This study provides an important theoretical basis for the application of miR-144 in the prevention and treatment of meningitis.

Methods

Preparation of streptococcus pneumoniae suspension

Streptococcus pneumoniae strains were purchased at the National Institute for the Control of Pharmaceutical and Biological Products

(Bacterial No. 31003). Streptococcus pneumoniae was inoculated in blood agar medium and cultured in a 37°C, 5% CO_2 incubator for 12 hours. Then they were inoculated into VITAL AER broth medium (Bio Mérieux, France), and were collected in the logarithmic phase for centrifugation. After being rinsed with normal saline, Streptococcus pneumoniae was subjected to centrifugation again. They were diluted in normal saline with a density of 3×10^{10}/L.

Construction of rat meningitis model

Forty male Sprague-Dawley rats (weighing 200-250 g, purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences) were housed in cages at 25°C and 40% relative humidity for one week. Rats were randomly divided into four groups with 10 rats in each group: Sham group, Meningitis group, Meningitis + agomir group, Meningitis + antagomir group. All rats were subjected to meningitis model construction surgery after fasting for 12 hours.

All rats were injected intraperitoneally with 10% chloral hydrate (dose: 5 mL/kg) and fixed on a stereotaxic instrument. Totally 50 µL of cerebrospinal fluid was obtained from the cisterna magna by using a microsyringe. At the same injection point, 30 µL of normal saline was injected into rats of the Sham group, while 30 µL of Streptococcus pneumoniae suspension was injected into rats of the Meningitis group, Meningitis + agomir group and Meningitis + antagomir group. One hour later, 20 µL of normal saline was injected into rats of the Sham group again. However, rats of the Meningitis + agomir group were injected with 20 µL of miR-144 agonist, and rats of the Meningitis + antagomir group were injected with 20 µL of miR-144 antagonist. After injection, rats were kept individually in cages with sufficient water and feed. Body temperature was recorded for 5 days. All rats were then anesthetized with 10% chloral hydrate and 50 µL of cerebrospinal fluid was collected from the cisterna magna. Finally, all rats were sacrificed and intact brain tissues were obtained. The use of animals and procedures were approved by Weinan Vocational & Technical College Institutional Animal Care and Use Committee.

Neuroethology assessment by Loeffler's neuroethology score

One day after surgery, neuroethology of rats were assessed using Loeffler's neuroethology score according to the following criteria: 5 points: rats were able to move normally when they were caught on the back, and they were able to turn over within 5 s; 4 points: rats had reduced exercise, but they were able to turn over within 5 s; 3 points: rats were able to turn over more than 5 s; 2 points, rats were unable to turn over; 1 points, rats could not exercise. A higher Loeffler's neuroethology score indicated less neurological impairment in rats.

White blood cell counts and levels of IL-1β and TNF-α in rat cerebrospinal fluid

The number of white blood cells in cerebrospinal fluid collected before and after surgery in rats was measured using the LH750 automatic hematology analyzer (Beckman Coulter, USA). Furthermore, levels of IL-1β and TNF-α in these cerebrospinal fluid were also detected by enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA). All operations are strictly performed according to the instructions.

TLR2 expression detection by immunohistochemistry

Paraffin-embedded rat brain tissues were subjected to continuous coronal slices. Five consecutive sections of brain tissues from each rat were randomly selected for dewaxing, rehydration, and antigen retrieval. After incubation with 3% H_2O_2 for 15 min at room temperature, these brain tissue sections were blocked with goat serum for 15 minutes again. Rabbit anti-Human TLR2 Antibody (1:100, Santa Cruz) was used to incubate these sections for 12 hours at 4°C. Slices were rinsed 3 times by PBS. The secondary antibody was added for 15 minute incubation at 37°C. After rinsed 3 times by PBS, horseradish peroxidase-labeled streptavidin working solution was added to continue incubation for 15 minutes. Brain tissue sections were subjected to DAB chromogenic reaction and were counterstain with hematoxylin for 30 seconds. Neutral gel used to seal them. Five areas of each slice were observed under a microscope and the number of TLR2 positive cells was counted. Brown-yellow particles appearing in the cytoplasm and cell membrane were considered as TLR2-positive cells.

Culture and transfection of 293T Cells

293T cells (purchased from China Type Culture Collection) were cultured in DMEM containing

10% fetal bovine serum (FBS) and were placed in a 5% CO_2, 37°C incubator for subculture. 293T cells in logarithmic growth phase were transfected with miR-144 mimics negative control (NC), miR-144 mimics, miR-144 inhibitors NC and miR-144 inhibitors respectively by using Lipfectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions. These transfected cells were grouped in turn as follows: mimics NC group, miR-144 mimics group, inhibitors NC group and miR-144 inhibitors group. 293T cells without any treatment were served as the control group. Cells of each group were prepared as cell suspensions by DMEM (10% FBS) at a density of 1 × 10^5 cells/Ml, and were inoculated in a 24-well plates with 2 mL of cell suspensions each well. Plates were were placed in a 5% CO_2 37°C incubator for continued culture.

Luciferase reporter assay

Target scan online prediction software was used to predict the targeted relationship between miR-144 and TLR2. Mutant-type (MT) and wild-type (WT) sequences of TLR2 mRNA containing the binding site were designed according to the predicted results. These sequences were constructed on vectors to transfect cells of mimics NC group and miR-144 mimics group respectively. After 48 hours, residual liquid in each well was discarded and cells were rinsed twice with PBS. Then 100 μL of Passive Lysis Buffer was added into each well for 15 minutes slightly shaking at room temperature. Cell lysates in each well were collected for luciferase activity detection.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Takara, Shiga, Japan) was used to extract total RNA in tissues and cells. A total of 500 ng RNA was underwent reverse transcription to obtain cDNA template by using PrimeScript RT Master Mix (Takara, Dalian, China). qRT-PCR containing three procedures (95°C for 20 seconds, 58°C for 30 seconds, 74°C for 30 seconds) was carried out to detect miR-144 and TLR2 mRNA expression levels. miR-144 expression level was normalized to U6, while TLR2 mRNA was normalized to GAPDH. Primers and their sequences involved in this study were as follows: miR-144-F, CA-CAAGCTTCCACAGGATCAGGGAGA, miR-144-R, ACACTCGAGTTGGCACAGTGAAGAGG; U6-F, CT-

CGCTTCGGCAGCACATATACT, U6-R, ACGCTTCA-CGAATTTGCGTGTC; TLR2-F, CCAAAGAGCTCG-TAGCATCC, TLR2-R, AGGGGCTTCACTTCTCTG-CT; GAPDH-F, TGGCAAAGTGGAGATTGTTGCC, GAPDH-R, AAGATGGTGATGGGCTTCCCG. In this research, $2^{-\Delta\Delta Ct}$ method was selected to calculate the fold change of genes to be detected.

Western blot analysis

Total proteins in tissues and cells were collected after they were lysed by lysis buffer (Cell Signaling Technology). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes after they were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Skim milk (5%) was added to block these membranes for 1 hour at room temperature. Membranes were sequentially subjected to primary antibody (mouse anti-human TLR2 antibody, 1:1000, Abcam, Cambridge, UK) incubation for 12 hours at 4°C and secondary antibody (horse radish peroxidase conjugated secondary antibody, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) incubation for 1 hour at room temperature.
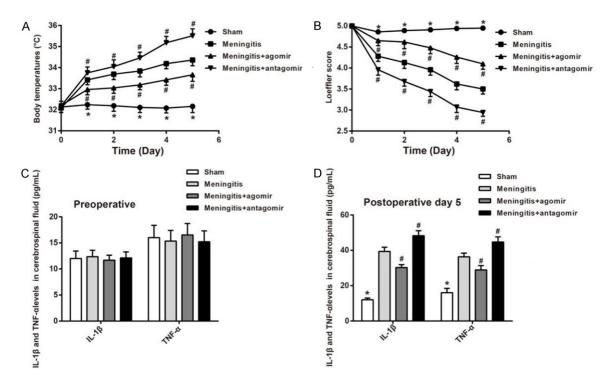
Statistical analysis

All data were processed by SPSS 19.0 and were expressed as mean ± SD. Comparison between two groups was conducted by t-test. One-way ANOVA was selected to perform comparison among multiple groups. $P < 0.05$ was considered statistically significant. In this research, all experiments were repeated 3 times.

Results

Clinical features of rats

Before surgery, the difference in body temperature, Loeffler scores, white blood cell count, IL-1β, and TNF-α level was not obvious among groups. After surgery, compared to the Sham group, rats of the Meningitis group, the Meningitis + agomir group and the Meningitis + antagomir group were with much higher body temperature ($P < 0.05$), obviously lower Loeffler's scores ($P < 0.05$), and markedly higher white blood cell count, IL-1β and TNF-α level ($P < 0.05$). Additionally, when compared to the Meningitis group, rats of the Meningitis + agomir group had dramatically lower body temperature ($P < 0.05$), significant-

Figure 1. Changes in clinical features of rats in each group. A. Changes in body temperature. B. Changes in Loeffler score. C. Levels of IL-1β and TNF-α in the cerebrospinal fluid before surgery. D. Levels of IL-1β and TNF-α in the cerebrospinal fluid after surgery. *P < 0.05 when compared with the other three groups. #P < 0.05 when compared with the Meningitis group.

Table 1. Changes in white blood cell count of rats (Unit: × 10^6/L)

Group	Before surgery	The 5th day after surgery
Sham group	84 ± 7	88 ± 6*
Meningitis group	82 ± 9	3365 ± 681
Meningitis + agomir group	85 ± 6	2872 ± 574#
Meningitis + antagomir group	83 ± 5	3834 ± 862#

*P < 0.05 when compared with the other three groups. #P < 0.05 when compared with the Meningitis group.

ly higher Loeffler's scores (P < 0.05), and obviously lower white blood cell count, IL-1β and TNF-α level (P < 0.05). However, markedly higher body temperature (P < 0.05), much lower Loeffler's scores (P < 0.05), as well as dramatically higher white blood cell count, IL-1β and TNF-α level (P < 0.05) were found in rats of the Meningitis + antagomir group when compared with the Meningitis group (**Figure 1A-D**, **Table 1**).
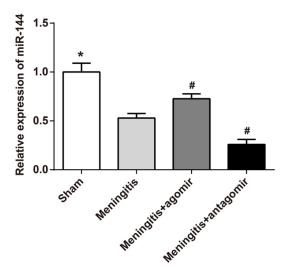
miR-144 expression in rat brain tissues

miR-144 relative expression in rat brain tissues of the Meningitis group, the Meningitis + agomir group and the Meningitis + antagomir group was all significantly lower than that of the Sham group (P < 0.01). In addition, when compared with the Meningitis group, miR-144 relative expression was dramatically increased in the Meningitis + agomir group (P < 0.01), while that was markedly decreased in the Meningitis + antagomir group (P < 0.01) (**Figure 2**).

TLR2 expression in rat brain tissues

Significantly higher TLR2 mRNA and protein relative expression was found in rat brain tissues of the Meningitis group, the Meningitis + agomir group, and the Meningitis + antagomir group when compared with the Sham group (P < 0.01). Furthermore, when compared with TLR2 mRNA and protein relative expression in rat brain tissues of the Meningitis group, they were significantly down-regulated in the Meningitis + agomir group (P < 0.01) and dramatically up-regulated in the Meningitis + antagomir group (P < 0.01) (**Figure 3A**, **3B**). These results of TLR2 protein expression had also been demonstrated by immunohistochemistry (**Figure 3C**).

Figure 2. miR-144 relative expression in rat brain tissues of each group. *$P < 0.01$ when compared with the other three groups. #$P < 0.01$ when compared with the Meningitis group.
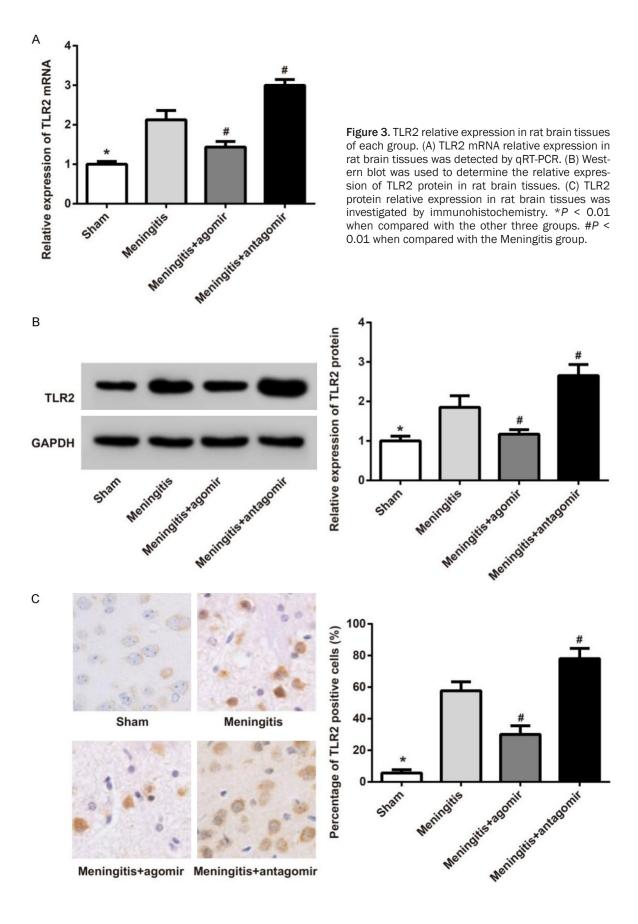
TLR2 was the target gene of miR-144

Via Target Scan, we observed that miR-144 was bound to the 3'-UTR region of TLR2 mRNA (**Figure 4A**). Therefore, reporter gene plasmids with TLR2 3'-UTR (MT or WT) were constructed. After 293T cells of mimics NC group and miR-144 mimics group were transfected by these plasmids, we found that there was no statistically significant difference of luciferase activity intensity between MT + NC group and MT + mimics group. However, when compared with the WT+NC group, the luciferase activiyy intensity was significantly decreased in the WT + mimics group ($P < 0.05$) (**Figure 4B**).

The role of miR-144 in the regulation of TLR2 was further studied. Results showed that, compared with the control and mimics NC groups, cells of the miR-144 mimics group were with much higher miR-144 relative expression and markedly lower TLR2 mRNA and protein relative expression ($P < 0.01$). At the same time, significantly increased miR-144 relative expression ($P < 0.01$) and dramatically increased TLR2 mRNA and protein relative expression ($P < 0.01$) were found in the miR-144 inhibitors group when compared with the control and inhibitors NC group (**Figure 4C-E**). The above results further indicated that TLR2 was a target gene of miR-144 and TLR2 was negatively regulated by miR-144.

Discussion

Bacterial meningitis is one of the most common diseases in children, which can cause severe neurological sequelae [8]. Streptococcus pneumoniae meningitis is the most common bacteria in infants with intracranial infections [9]. In this study, the rat meningitis model infected by Streptococcus pneumoniae was successfully constructed. Furthermore, this research also investigated the effect of miR-144 on rats with meningitis. The results demonstrated that miR-144 was down-regulated in brain tissues of meningitis rats. Up-regulation of miR-144 could inhibit the development of meningitis in rats through suppressing expression of TLR2.

miRNAs are a type of noncoding small RNA that is widely spread in eukaryotes [10]. It has been found to play a pivotal role in many major human diseases, especially in multiple cancers [11]. The influence of miRNAs on inflammatory responses has also been studied [12]. For example, it has been reported that miR-221 could regulate endothelial inflammatory response by targeting AdipoR1 [13]. miR-155 was found to stimulate expression of IL-1β and it could participate in regulation of inflammatory responses in ischemic cerebral tissues [14]. miR-138 could decrease the levels of IL-6 and IL-8 in human coronary artery endothelial cell injury. It relieved human coronary artery endothelial cell injury as well as related inflammatory reactions [15]. In this study, white blood cell count as well as IL-1β and TNF-α level were increased significantly in cerebrospinal fluid of meningitis rats. IL-1β and TNF-α were two major pro-inflammatory factors. Elevated levels were indicative of aggravated inflammatory responses [16]. White blood cells are an important part of the human body's defense system. Once the human body is invaded by external bacteria, white blood cells could pass through capillary walls through deformation. Then they are concentrated on the bacteria invading parts in order to surround and devour them [17-20]. Therefore, it is likely that the body develops inflammation if the number of white blood cells exceeds the normal range. We observed from this study that white blood cell count and levels of IL-1β and TNF-α in rats cerebrospinal fluid were dramatically decreased after meningitis rats were injected with miR-144 agonist. While

Figure 3. TLR2 relative expression in rat brain tissues of each group. (A) TLR2 mRNA relative expression in rat brain tissues was detected by qRT-PCR. (B) Western blot was used to determine the relative expression of TLR2 protein in rat brain tissues. (C) TLR2 protein relative expression in rat brain tissues was investigated by immunohistochemistry. *$P < 0.01$ when compared with the other three groups. #$P < 0.01$ when compared with the Meningitis group.

A

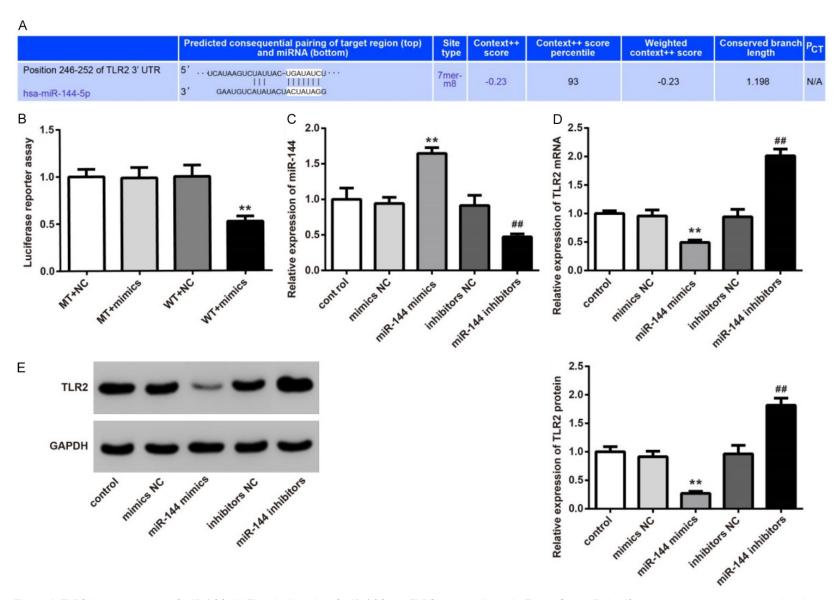|	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_CT
Position 246-252 of TLR2 3' UTR hsa-miR-144-5p	5' · · UCAUAAGUCUAUUAC–UGAUAUCU · · · ||| ||||||| 3' GAAUGUCAUAUACUACUAUAGG	7mer-m8	-0.23	93	-0.23	1.198	N/A

Figure 4. TLR2 is a target gene of miR-144. (A) The binding site of miR-144 and TLR2 was predicted by Target Scan. (B) Luciferase reporter assay was used to detect the targeting relationship between miR-144 and TLR2. **$P < 0.01$ when compared with the WT + NC group. (C) miR-144 relative expression in 293T cells of each group was detected by qRT-PCR. (D) TLR2 mRNA relative expression in 293T cells of each group was determined by qRT-PCR. (E) TLR2 protein relative expression in 293T cells of each group was explored by Western blot. **$P < 0.01$ when compared with the control and mimics NC groups. ##$P < 0.01$ when compared with the control group inhibitors NC groups.

these indicators were all increased after meningitis rats were injected with miR-144 antagonist. It can be concluded from these results that miR-144 might have an inhibitory effect on the inflammatory response in meningitis rats. Liu et al. [7] noticed from their research that, lower miR-144 expression could stimulate the release of inflammatory factors in monocyte-derived macrophages after they were infected by mycobacterium tuberculosis. Their research was consistent with our results. This article first studied the regulation effect of miR-144 on meningitis rats.

TLR2 is an important member of the Toll-like receptor family, which has an irreplaceable role in innate immunity and inflammation [21]. TLR2 could recognize multiple bacterial lipoproteins, which is the initial barrier to bacterial infection [22]. Previous research has suggested that increased TLR2 expression in monocytes could result in systemic inflammatory response syndrome after liver transplantation [23]. Li et al. [24] demonstrated that, in burn-related cellular inflammatory reactions, application of TLR2 agonists could stimulate the expression of inflammatory factors, such as IL-1β and TNF-α. There have been some studies on the role of TLR2 in meningitis. TLR2 has also been found to be regulated by some miRNAs. Philippe et al. [25] revealed that TLR2 was closely related to rheumatoid arthritis and miR-19a/b regulated the release of IL-6 and matrix metalloproteinase 3 through decreasing expression of TLR2 protein. Xu et al. [26] also noted that TLR2 was expressed lower in rats with chronic obstructive pulmonary disease, which was negatively regulated by miR-344b-1-3p. In this article, we found that TLR2 was negatively regulated by miR-144.

In conclusion, the rat meningitis model was successfully constructed through Streptococcus pneumoniae infection. miR-144 agonist significantly inhibited development of meningitis. The relevant mechanism involved in this process was that miR-144 could relieve meningitis development by suppressing expression of TLR2. miR-144 might serve as one of the potential therapeutic targets for the diagnosis and treatment of meningitis caused by Streptococcus pneumoniae.

Disclosure of conflict of interest

None.

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