

Dysregulation of miR-301a-3p serves as a non-invasive biomarker and is associated with inflammatory responses in traumatic spinal cord injury

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Background: Spinal cord injury (SCI) led by trauma is a serious damage in central nervous system. This study aimed at confirming the expression levels and clinical significance of miRNA-301a-3p (miR-301a-3p) in SCI patients, and exploring the potential mechanisms of miR-301a-3p participating in SCI progression. **Methods:** One hundred and thirty nine acute spinal trauma patients, included neurologically normal, incomplete and complete SCI cases, were analyzed in this study. Quantitative real-time PCR was used to measure the expression of miR-301a-3p. Receiver operating characteristic (ROC) was used to evaluate the diagnostic accuracy of serum miR-301a-3p in SCI. LPS-treated SH-SY5Y cells were used to mimic SCI inflammatory injury. The levels of IL-1 β , IL-6, TNF- α were detected using enzyme-linked immunosorbent assay. **Results:** Expression of serum miR-301a-3p was significantly higher in both incomplete and complete SCI patients than that in neurologically normal controls, and had a significant ability to distinguish SCI patients from controls. Additionally, complete SCI cases had markedly increased miR-301a-3p compared to incomplete cases, and the two groups of patients could be distinguished based on serum deregulated miR-301a-3p. In the SCI cell model, miR-301a-3p overexpression led to decreased cell viability. The inflammatory responses of the cell model were enhanced by miR-301a-3p, which was consistent with the findings in SCI patients that serum miR-301a-3p was positively correlated with pro-inflammatory cytokines. **Conclusion:** The expression of miR-301a-3p is increased in SCI patients, and serves as a candidate biomarker for SCI diagnosis. MiR-301a-3p may be involved in SCI progression by affecting neuronal viability and inflammation.

Keywords: Spinal cord injury, miR-301a-3p, neuroinflammation, viability, IL-1 β , IL-6, TNF- α

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Abbreviations: AUC, area under the curve; miRNAs, microRNAs; ROC, Receiver operating characteristic; SCI, spinal cord injury

INTRODUCTION

Spinal cord injury (SCI) refers to an injury resulting from the direct or indirect effects of external forces on spinal cord, and is a traumatic injury disease that seriously damages central nervous system (Fehlings *et al.*, 2017; Fan *et al.*, 2018). SCI can be divided into complete SCI and incomplete SCI according to the American Spinal Injury Association (ASIA) Impairment Scale in In-

ternational Standards for Neurological Classification of Spinal Cord Injury (Vasquez *et al.*, 2013), and a variable damage degree of the two SCI types depends mainly on the primary and secondary mechanisms of injury (Rouanet *et al.*, 2017). Although the clinical repair strategies are different between complete and incomplete SCI (O'Shea *et al.*, 2017), both have devastating consequences for the physical abilities of patients (Ahuja *et al.*, 2017). Studies confirmed that the pathogenesis after the initial occurrence of SCI involved oxidative stress, posttraumatic inflammation, motor neuron apoptosis and necrosis, leading to a secondary neurological damage to the spinal cord and the peripheral nervous system (Rouanet *et al.*, 2017; Wilson *et al.*, 2013; Ahuja *et al.*, 2016). What's more, SCI leads to spinal inflammatory responses, which is also an important mechanism for aggravating neuronal damage (Sun *et al.*, 2021; Tian *et al.*, 2007). Therefore, the exploration of early diagnostic biomarkers and methods to alleviate neuroinflammation will be beneficial for understanding SCI disease progression and promoting recovery after injury.

MicroRNAs (miRNAs) are small, noncoding RNA particles, and they participate in many biological processes, including inflammation (Xia *et al.*, 2017; Neudecker *et al.*, 2017). miRNA-301a-3p (miR-301a-3p) is an inflammation-related miRNA that promotes inflammatory responses in the development and progression of diseases, such as intestinal mucosal inflammation (He *et al.*, 2016), osteoarthritis (Chen *et al.*, 2017), pulmonary fibrosis (Wang, Li, *et al.*, 2020), or ankylosing spondylitis (Dong *et al.*, 2020). In addition, several studies have demonstrated that miR-301a-3p has the potential to promote the inflammation induced by Japanese encephalitis virus infection in central nervous system (Hazra *et al.*, 2019; Hazra *et al.*, 2017). Kumar and coworkers (Kumar *et al.*, 2013) have demonstrated that aberrantly expressed miR-301a-3p has a potential diagnostic value for distinguishing patients with the neurodegenerative disease, such as Alzheimer's disease. It is worth mentioning that a recent SCI-related study has explored aberrantly expressed miRNAs in SCI animal models, and the research found that miR-301a-3p was significantly upregulated in SCI mice (Wan *et al.*, 2020). Therefore, it is necessary to analyze the expression levels and clinical significance of miR-301a-3p in SCI patients, to further explore new biomarkers and therapeutic targets for SCI.

This study aimed at confirming the expression levels and clinical significance of miR-301a-3p in SCI progression. Considering a close relationship between miR-301a-3p and inflammation, this study further explores the regulatory effects of miR-301a-3p on the inflammatory responses of a SCI inflammatory injury cell model,

which was expected to preliminarily uncover the mechanisms of miR-301a-3p playing in SCI progression. The results of this study may allow researchers to gain new understanding of the function of miR-301a-3p, and provide novel biomarkers and targets for the diagnosis and therapy of SCI.

MATERIALS AND METHODS

Patients and tissue collection

Blood samples were collected from 139 acute spinal trauma patients treated at Weifang People's Hospital between September 2017 and September 2020. According to the American Spinal Injury Association (ASIA) Impairment Scale in International Standards for Neurological Classification of Spinal Cord Injury (Vasquez *et al.*, 2013), 139 acute spinal trauma patients were divided into three groups: neurologically normal controls (controls, $n=33$); incomplete SCI group ($n=67$); complete SCI group ($n=39$). Inclusion criteria for acute SCI patients in this study: (1) the onset time of acute SCI was within 24 h before admission in all patients; (2) the age of patient ≥ 18 years old. Patients with any of the following characteristics were excluded from this study: (1) patients with a history of spinal and craniocerebral related trauma and surgery within a half year period; (2) patients with a severe cardiopulmonary disease including large area myocardial infarction or acute coronary syndrome, severe arrhythmia, and acute respiratory distress syndrome; (3) patients with acute severe head injury (severe brain trauma, massive cerebral hemorrhage, large cerebral infarction and severe brain stem injury, etc.); (4) pregnant women; (5) patients with a previous history of acute SCI or head injury; (6) patients with malignancies, acute and chronic infections, severe hepatic and renal dysfunction, cerebrovascular disease, autoimmune disease and hematological disease. Venous blood samples were collected from the included patients after admission, and clinicopathological characteristics of the patients were recorded. Serum was separated by centrifugation at low temperature and stored at -80°C for subsequent analysis. The protocols for venous blood sample collection and analysis were all in accordance with the guideline of the Ethics Committee, and were approved by the Ethics Committee of Weifang People's Hospital. A signed informed consent was obtained from each patient before sampling.

Cell culture

Human neuroblastoma SH-SY5Y cells obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM; Solarbio, Beijing, China), and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was added to the medium. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 .

Cell treatment

To construct a cell model of SCI inflammatory injury, SH-SY5Y cells were treated with 100 ng/mL of LPS (Sigma-Aldrich, Louis, MO, USA) for 24 h at 37°C . To explore the regulatory effects of miR-301a-3p on model cell viability and inflammation, after SH-SY5Y cell reached 80% confluence, the cells were transfected with miR-301a-3p mimic, miR-301a-3p inhibitor, mimic NC or inhibitor NC (GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at

6 h before LPS treatment. Following were the sequences used for transfection: miR-301a-3p mimic: 5'-CAGUGCAAUGUAAAAAGGGCAU-3'; miR-301a-3p inhibitor: 5'-AUGAAAUUUUUAAACAUUGCACUG-3'; mimic NC: 5'-UUCUCCGAACGUGUCACGU-3'; inhibitor NC: 5'-CAGUACUUUUUGUGUAGUACAA-3'.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

TRIzol reagent (Invitrogen) was used to extract total RNA from serum samples, and the purity of RNA was detected by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse-transcribed into cDNA using PrimeScript RT reagent kit (TaKaRa, Shiga, Japan) following the manufacturer's protocols.

Quantitative real-time PCR (qRT-PCR) was performed using SYBR green I Master Mix kit (Invitrogen, Carlsbad, CA, USA) on a 7500 Real-Time PCE System (Applied Biosystems, USA), and U6 was used as an endogenous control for the reaction. All manipulations were performed following the manufacturer's guidelines. The relative expression results were quantified using the $2^{-\Delta\Delta\text{Ct}}$ method.

Enzyme-linked immunosorbent assay (ELISA)

To evaluate inflammatory responses in SCI patients and cell model, the levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α (TNF- α) in serum samples and cell supernatants were measured using ELISA kits (R&D Systems, Shanghai, China) following the manufacturer's protocols.

Cell counting kit (CCK)-8 assay

CCK-8 assay was used to evaluate the proliferation abilities of SH-SY5Y cells. Cells were seeded into a 96-well plate (1×10^4 cells/well). Then, the 96-well plate was incubated in a humidified incubator at 37°C with 5% CO_2 . 10 μL portions of CCK-8 reagent were added to the wells, respectively. After 1 h of incubation, a microplate reader was used to read OD (optical density) values at 450 nm.

Statistical analysis

SPSS 22.0 (IBM Corp.) and GraphPad Prism 7.0 software (GraphPad Software, Inc.) were used to perform the statistical analyses. Differences between multiple groups were compared using one-way ANOVA followed by the Tukey's test. The chi-square test was used for comparison between categorical variables. Receiver operating characteristic (ROC) was used to evaluate the diagnostic accuracy and discrimination ability of serum miR-301a-3p in SCI patients. The Pearson correlation test was used to evaluate the correlation between serum miR-301a-3p and inflammatory factors (IL-1 β , IL-6, TNF- α). All data in the present study were presented as the mean \pm standard deviation (S.D.). Each experiment was repeated at least 3 times. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics of the patients

The baseline characteristics of all participants were included in Table 1, which revealed that there were no significant differences in age, body mass index (BMI), body

Table 1. Baseline characteristics of the included patients

Variables	Control (n=33)	Incomplete SCI (n=67)	Complete SCI (n=39)	P-value
Age (years)	43.818±8.869	44.910±10.790	45.692±9.652	0.733
BMI	23.549±1.815	23.164±2.147	23.994±2.165	0.142
Body temperature	37.276±0.734	37.491±0.559	37.661±0.794	0.057
Gender				0.923
Female	15	30	19	
Male	18	37	20	
Site of spinal cord affected				0.001
Cervical cord	1	7	13	
Thoracic Lumbar cord	32	60	26	
ASIA grade				< 0.001
A	0	0	39	
B	0	25	0	
C	0	23	0	
D	0	19	0	
E	33	0	0	

BMI, Body mass index; ASIA, American Spinal Injury Association.

temperature and gender between neurologically normal controls and SCI patients (all $P>0.05$), and SCI patients had significant differences in the site of spinal cord affected ($P=0.001$) and ASIA grade ($P<0.001$) compared with neurologically normal controls.

Overexpression of miR-301a-3p in the serum of SCI patients

The expression levels of miR-301a-3p in the collected serum samples were detected by qRT-PCR. The expression levels of miR-301a-3p were significantly higher in the serum of both incomplete and complete SCI patients than these in neurologically normal controls (Fig. 1, all $P<0.001$). In addition, the relative expression of miR-301a-3p was significantly higher in the serum of complete SCI patients compared with that in incomplete SCI patients. ($P<0.001$).

Diagnostic performance of serum miR-301a-3p in patients with SCI

The ROC curves were generated according to the expression levels of serum miR-301a-3p. Serum expression levels of miR-301a-3p have high diagnostic performance

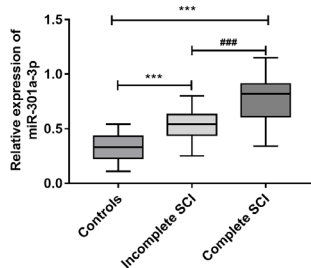


Figure 1. Expression of miR-301a-3p in the serum samples of the study population. The expression levels of miR-301a-3p in the serum of incomplete SCI patients and complete SCI patients were significantly higher than these in neurologically normal controls, and the highest serum miR-301a-3p was observed in complete SCI patients. (***) $P<0.001$, ### $P<0.001$).

in distinguishing between SCI patients and neurologically normal controls, with the area under the curve (AUC) of 0.903. At the cut-off value of 0.465, the sensitivity was 80.19% and the specificity was 87.88%, indicating that this was the optimal value of miR-301a-3p to distinguish SCI patients from neurologically normal controls (Fig. 2A).

In addition, the accuracy of serum miR-301a-3p in the differential diagnosis between incomplete SCI and complete SCI patients was analyzed in this study. The ROC result showed that miR-301a-3p had the ability to differentiate complete SCI patients from incomplete SCI patients, and the AUC was 0.836. At a cut-off value of 0.625, the sensitivity was 71.79% and the specificity was 74.63%, which indicated the optimal value of miR-301a-

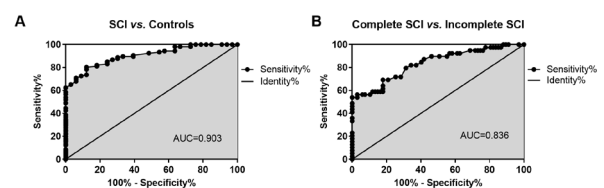


Figure 2. Diagnostic performance of serum miR-301a-3p in patients with SCI.

(A) Serum expression levels of miR-301a-3p have high diagnostic performance between SCI patients and neurologically normal controls, and AUC was 0.903. (B) MiR-301a-3p has the ability to differentiate between incomplete SCI and complete SCI patients, and AUC was 0.836.

3p to distinguish complete SCI from incomplete SCI patients (Fig. 2B).

Correlation of miR-301a-3p with pro-inflammatory cytokines in patients with SCI

The inflammatory responses of SCI patients were evaluated by examining the levels of IL-1 β , IL-6, TNF- α in serum samples, and the correlation between serum miR-301a-3p and inflammation was analyzed. The results revealed that the expression levels of miR-301a-3p were

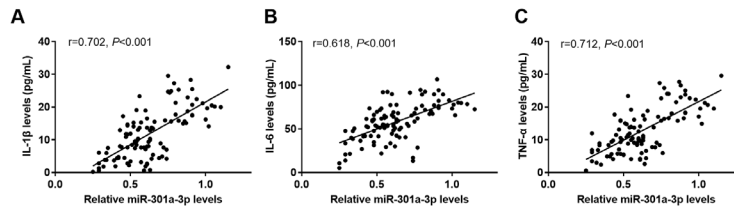


Figure 3. Correlation of miR-301a-3p with pro-inflammatory cytokines in patients with SCI.

(A) The expression levels of miR-301a-3p were positively correlated with the levels of IL-1 β ($r=0.702$) in patients with SCI. (B) The expression levels of miR-301a-3p were positively correlated with the levels of IL-6 ($r=0.618$) in patients with SCI. (C) The expression levels of miR-301a-3p were positively correlated with the levels of TNF- α ($r=0.712$) in patients with SCI. (all $P<0.001$).

positively correlated with the levels of IL-1 β ($r=0.702$), IL-6 ($r=0.618$), TNF- α ($r=0.712$) in patients with SCI (Fig. 3A–C, all $P<0.001$).

Regulatory effects of miR-301a-3p on LPS-induced inflammatory injury in SH-SY5Y cells

By using LPS stimulation, the human neuroblastoma SH-SY5Y cells were induced as a SCI inflammatory injury cell model, and the potential regulatory role of miR-301a-3p in neuroinflammation was investigated. As shown in Fig. 4A, the expression level of miR-301a-3p was significantly increased in SH-SY5Y cells after treating with LPS compared with untreated ($P<0.001$), which was consistent with the expression changes observed in SCI patients. After cell transfection in the LPS-induced cell model, we found that the increased miR-301a-3p by LPS was further enhanced by miR-301a-3p mimic compared with the LPS group, while the promoting effects of LPS on miR-301a-3p expression were significantly inhibited by the inhibitor of miR-301a-3p (Fig. 4A, both $P<0.001$).

In addition, as shown in Fig. 4B, the relative cell proliferation viability was significantly decreased after treating with LPS compared with the untreated cells

($P<0.001$). In the LPS-induced cell injury model, the inhibited cell viability was further decreased by the overexpression of miR-301a-3p, while silencing miR-301a-3p abolished the restriction in cell viability by LPS (Fig. 4B, both $P<0.05$).

For cell inflammatory responses to LPS treatment, we found that LPS significantly increased the release concentration of inflammatory factors (IL-1 β , IL-6, TNF- α) in SH-SY5Y cells, indicating that the inflammatory response was activated in SH-SY5Y cells (Fig. 4C–E, all $P<0.001$). By regulating the expression of miR-301a-3p in the cell model, it was found that miR-301a-3p overexpression led to further increases in IL-1 β , IL-6, TNF- α levels (all $P<0.001$), while the promoting effects of LPS on IL-1 β , IL-6, TNF- α levels were significantly reversed by the reduction of miR-301a-3p (all $P<0.001$). Therefore, the expression level of miR-301a-3p reveals an effect on the release concentration of inflammatory factors in the SCI inflammatory injury cell model *in vitro*.

DISCUSSION

Studies have shown that many abnormally regulated miRNAs are involved in the development of SCI (Zhou

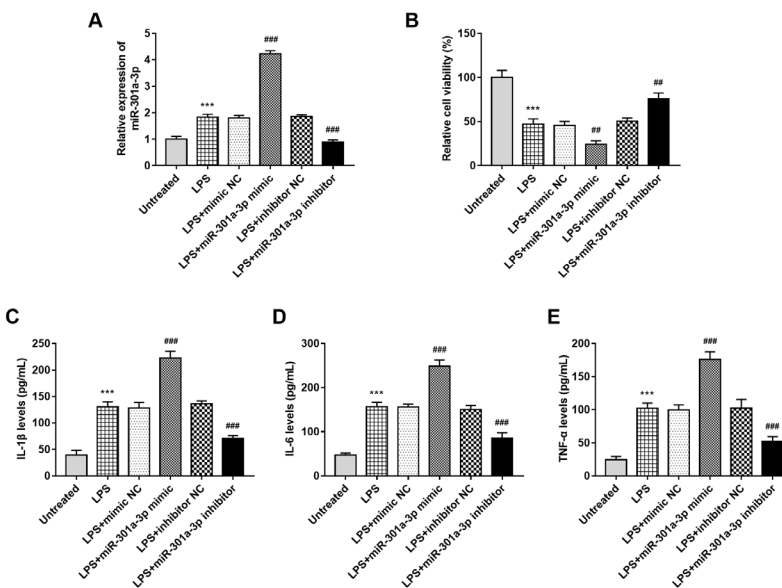


Figure 4. Regulatory effects of miR-301a-3p on cell viability and inflammation in LPS-induced SCI cell model.

(A) The expression level of miR-301a-3p was significantly increased by LPS in SH-SY5Y cells, and the miR-301a-3p mimic enhanced this effect, but the miR-301a-3p inhibitor reversed this effect on miR-301a-3p expression. (B) LPS significantly inhibited cell model viability, and this inhibition was enhanced by miR-301a-3p overexpression, while it was relieved by miR-301a-3p reduction. (C–E) The levels of IL-1 β , IL-6 and TNF- α were increased in SH-SY5Y cells after LPS induction, and these changes were further promoted by miR-301a-3p overexpression, while their levels were decreased under the condition of miR-301a-3p silencing. (***) $P<0.001$ vs. Untreated; (**) $P<0.01$, (###) $P<0.001$ vs. LPS).

et al., 2020; Zhang *et al.*, 2019). For example, Zhang *et al.* indicated that downregulation of miRNA-127-5p could aggravate SCI through activating MAPK1 (Zhang *et al.*, 2019). Zhou and others (Zhou *et al.*, 2020b) revealed that the overexpression of miRNA-433-5p protected SCI-induced motor dysfunction and inflammatory responses. According to the research results of Yang and others (Yang *et al.*, 2018) high expression of miR-544a could promote the recovery of spinal cord. In the present study, we have found that the expression levels of miR-301a-3p were significantly higher in the serum of SCI patients and LPS-treated SH-SY5Y cells. Therefore, we reasonably speculate that miR-301a-3p may be involved in the development of SCI.

The aim in treating acute traumatic spinal trauma was to preserve residual neurologic function and avoid secondary injury (Mourelou Farina *et al.*, 2017). Therefore, in the early occurrence of acute spinal trauma, it is quite important to promptly diagnose the situation of damage in patients and conduct appropriate clinical treatment (Mourelou Farina *et al.*, 2017; Ryken *et al.*, 2013; Jones *et al.*, 2015). MiR-301a-3p has been considered as a diagnostic biomarker for several diseases (Wang, Zhao, *et al.*, 2020; Ramirez-Ardila *et al.*, 2016). In previous studies, Wang and others (Wang *et al.*, 2020b) demonstrated that miR-130 family members (including miR-301a-3p) in serum could effectively distinguish bladder cancer patients from healthy controls. In addition, miR-301a-3p also had the ability to discriminate breast cancer patients from controls (Ramirez-Ardila *et al.*, 2016). In the present study, the results of ROC analysis indicated that miR-301a-3p had the ability to significantly distinguish SCI patients from neurologically normal controls, and complete SCI patients from incomplete SCI patients, with considerable diagnostic accuracy. Therefore, we speculated that miR-301a-3p may have diagnostic implications in SCI patients, and may be a candidate for an early diagnostic biomarker.

Evidences showed that inflammatory response aggravates neuronal injury, and neuronal damage, in turn, promotes neuroinflammation in the pathogenesis of SCI. Studies have indicated that various miRNAs were associated with inflammatory responses in SCI (Sun *et al.*, 2020; Yang *et al.*, 2018). For example, miRNA-411 has been found to attenuate inflammatory damages and apoptosis following SCI (Sun *et al.*, 2020). Additionally, miRNA-544a regulated the inflammation of SCI by inhibiting the expression of NEUROD4 (Yang *et al.*, 2018). The analysis of serum samples collected from SCI patients showed that the expression levels of miR-301a-3p were positively correlated with serum concentration of inflammatory factors (IL-1 β , IL-6 and TNF- α) in SCI patients, demonstrating a possible correlation of serum miR-301a-3p with SCI neuroinflammation. However, the effects of miR-301a-3p on SCI neuroinflammation *in vitro* is unknown. Human neuroblastoma SH-SY5Y cells are recognized as a well-established *in vitro* model to evaluate neural function (Li *et al.*, 2020; Jia *et al.*, 2021). After cell transfection in the LPS-induced cell model, we found that the miR-301a-3p expression was significantly upregulated by LPS, which was consistent with the expression changes observed in SCI patients. Previous studies concluded that miRNA-301a-3p regulated the inflammation and process of diseases (Jiang & Lv, 2021; Martinez-Gutierrez *et al.*, 2019). For instance, miRNA-301a-3p increased inflammation and apoptosis in ox-LDL-induced HUVECs by targeting KLF7 (Jiang & Lv, 2021). The downregulation of miR-301a-3p was associated with a higher overall survival in metastatic breast cancer (Mar-

tinéz-Gutierrez *et al.*, 2019). The inflammatory responses were enhanced by miR-301a-3p in the development of ankylosing spondylitis (Dong *et al.*, 2020). In the SCI inflammatory injury cell model of our study, analysis of inflammatory factors levels indicated that miR-301a-3p overexpression led to further increases in IL-1 β , IL-6, TNF- α levels, while the promoting effects of LPS on IL-1 β , IL-6, and TNF- α levels were significantly reversed by the reduction of miR-301a-3p. Regarding cell viability of the cell model, miR-301a-3p overexpression inhibited cell viability, while miR-301a-3p reduction enhanced this process. Therefore, combining the experimental results and previous studies, we speculate that the dysregulation of miR-301a-3p may be involved in SCI progression by regulating inflammatory responses. After the occurrence of SCI, miR-301a-3p overexpression may promote the progress of neuroinflammation, miR-301a-3p reduction may slow down inflammatory responses, and miR-301a-3p may be regarded as an indicator of inflammatory responses in SCI.

The limitation of this study is that the specific regulatory mechanism of miR-301a-3p in the inflammatory response of SCI was not further explored, thus we give a further perspective on the mechanism of action. Previous studies indicated that miRNA-301a-3p promoted inflammation and apoptosis by targeting KLF7 in ox-LDL-induced HUVECs (Jiang & Lv, 2021). In addition, Guo *et al.* revealed that miR-301a-3p induced by endoplasmic reticulum stress mediates the occurrence and transmission of trastuzumab resistance in HER2-positive gastric cancer (Guo *et al.*, 2021). The above studies provided us with the ideas that some other inflammation-related pathways or target genes might mediate the regulatory role of miR-301a-3p in the inflammation of SCI pathogenesis. These will be exploratory directions of our future studies, and we will continue to explore the mechanisms of miR-301a-3p acting in the progression of SCI.

In conclusion, the expression of miR-301a-3p was increased in SCI patients, and had the ability to discriminate SCI patients from those neurologically normal patients who also suffered from spinal trauma. The inhibition of miR-301a-3p alleviated inflammatory responses and promoted cell viability in the SCI cell model. All the findings of this research suggest that miR-301a-3p may be a novel biomarker and therapeutic target for traumatic SCI.

Declaration

Ethics approval and consent to participate. The experimental procedures were all in accordance with the guideline of the Ethics Committee of Weifang People's Hospital and has approved by the Ethics Committee of Weifang People's Hospital. This study complies with the Declaration of Helsinki. A signed written informed consent was obtained from each patient.

Consent for publication. Written informed consent for publication was obtained from each participant.

Availability of data and materials. The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests. The authors declare that they have no competing interests.

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Authors' contributions. LM carried out the research design and conception; MX analyzed and interpreted the data regarding; MX and EL performed the examination of cells; EL and LM delivered essential reagents or

tools; all authors participated in writing and revision of the manuscript. All authors read and approved the final manuscript.

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