Research Article

MOLECULAR PAIN

Molecular Pain
Volume 13: 1–13

DOI: 10.1177/1744806917706582
journals.sagepub.com/home/mpx
SAGE

Effects of microRNA-223 on morphine analgesic tolerance by targeting *NLRP3* in a rat model of neuropathic pain

Xiao-Juan Xie[1], Li-Gang Ma[1], Kai Xi[2], Dong-Mei Fan[3], Jian-Guo Li[4], Quan Zhang[4] and Wei Zhang[5]

Abstract

Objective: To investigate the effects of microRNA-223 on morphine analgesic tolerance by targeting NLRP3 in a rat model of neuropathic pain.

Methods: Our study selected 100 clean grade healthy Sprague-Dawley adult male rats weighing 200 to 250 g. After establishment of a rat model of chronic constriction injury, these rats were divided into 10 groups (10 rats in each group): the normal control, sham operation, chronic constriction injury, normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups. The real-time quantitative polymerase chain reaction assay, Western blotting, and enzyme-linked immunosorbent assay were used for detecting the mRNA and protein expressions of NLRP3, apoptosis-associated speck-like protein, Caspase-1, Interleukin (IL)-1β, and IL-18 in sections of lumbar spinal cord. Immunohistochemistry was applied for detecting the positive rates of NLRP3, apoptosis-associated speck-like protein, Caspase-1, IL-1β, and IL-18.

Results: The paw withdrawal threshold and percentage maximum possible effect (%MPE) were higher in chronic constriction injury group when compared with the normal control and sham operation groups. Behavioral tests showed that compared with the chronic constriction injury and normal saline groups, the morphine and miR-223 + morphine groups showed obvious analgesic effects. Expressions of miR-223 in the miR-223, miR-223 + morphine, and miR-223 + NLRP3 + morphine were significantly higher than those in the chronic constriction injury, normal saline, and morphine groups. Compared with chronic constriction injury, normal saline and morphine groups, the mRNA and protein expressions of NLRP3, apoptosis-associated speck-like protein, Caspase-1, IL-1β, and IL-18 were significantly decreased in the miR-223 and miR-223 + morphine groups, while mRNA and protein expressions of NLRP3, apoptosis-associated speck-like protein, Caspase-1, IL-1β, and IL-18 were significantly increased in the NLRP3 and NLRP3 + morphine group.

Conclusion: Our study provides strong evidence that miR-223 could suppress the activities of NLRP3 inflammasomes (*NLRP3*, apoptosis-associated speck-like protein, and Caspase-1) to relieve morphine analgesic tolerance in rats by down-regulating *NLRP3*.

Keywords

MicroRNA-223, NLRP3, morphine analgesic tolerance, neuropathic pain

Date received: 25 May 2016; revised: 6 December 2016; accepted: 30 January 2017

[1]Department of Anesthesia, First Affiliated Hospital, College of Clinical Medicine of Henan University of science and technology, Luoyang, China
[2]Department of ENT, First Affiliated Hospital, College of Clinical Medicine of Henan University of science and technology, Luoyang, China
[3]Department of Gynaecology and Obstestrics, First Affiliated Hospital, College of Clinical Medicine of Henan University of science and technology, Luoyang, China
[4]Department of Neurology, Linyi People's Hospital, Linyi, China
[5]Department of Anesthesia, the First Affiliated Hospital of Zhengzhou University, Luoyang, China

Corresponding author:
Wei Zhang, Department of Anesthesia, The First Affiliated Hospital of Zhengzhou University, No. 1, Jianshe East Road, Zhengzhou 450000, Henan Province, China.
Email: 623302914@qq.com

Introduction

Neuropathic pain (NP) refers to the pain caused by damages in the somatosensory system or diseases, whose prevalence is estimated to possibly lie between 6.9% and 10% in the general population with millions of patients all over the world given no exact cause and no treatment methods.[1,2] The most common causes include toxicity, surgery, radiation, trauma, and congenital disorders.[3] Currently, medication remains the mainstay for NP treatment with most commonly used opioids treating chronic and intractable pain.[4,5] Morphine, which can be used for intrathecal infusion therapy for NP, prevents pain signals from being transmitted to the center pivot so that pain signals cannot be delivered to the brain cortex, and the pain is controlled.[6] Morphine is widely used in the clinical application for NP treatment with good analgesic effects and can be considered for first-line use in select clinical circumstances.[7] However, the long-term application of morphine can result in morphine analgesic tolerance, and produce a special kind of hyperalgesia, decreasing effectiveness over time and increasing psychological burden of patients.[8] Currently, there are many drugs treating morphine analgesic tolerance, but side effects, safety issues, and other problems brought by those drugs are outstanding.[9] Molecular targeted therapy, which has been well applied in a variety of diseases in recent years, attracts more and more attention in studies on the treatment of morphine analgesic tolerance.

MicroRNA (miR) is a class of short non-coding RNAs with a length of about 22 nucleotides.[10] Currently, the role of miRs in NP has received more attention in the field of medicine, and a relevant study has confirmed changes in miR expression in NP.[11] MiR-223, expressed in myeloid cells, shows a negative expression in many diseases, such as leukemia, hepatitis B, influenza, lymphoma, and inflammation.[12] *NLRP3* is a type of inflammasome (a multi-protein complex produced by tissue cells after receiving endogenous and exogenous stimulation) which can be activated by cathepsins.[13] In recent years, studies have confirmed that miR-223 can inhibit inflammation, thereby avoiding collateral damage.[14,15] Recently, it is found that *NLRP3* is one of the target genes of miR-223.[16] A study has showed that miR-223 negatively targeting *NLRP3*, thereby promoting the secretion of certain macrophages.[17] Currently, it is considered that miR-223 involved in the development of inflammatory response as a regulatory molecule.[18] However, no relevant studies have reported whether miR-223 targeting *NLRP3* participates in NP and morphine analgesic tolerance. Therefore, the aim of our study is to investigate the effects of miR-223 on morphine analgesic tolerance by targeting NLRP3 in a rat model of NP.

Materials and methods

Ethical statement

The experimental procedures in present study were carried out with the approval of the First Affiliated Hospital of Zhengzhou University. Breeding and use of laboratory animals in this experiment strictly adhered to the International Association for the Study of pain guidelines for the care and protection of laboratory animals.

Experimental animals and groups

In this study, clean grade healthy pure breed Sprague-Dawley male rats ($n = 100$) ($200 \sim 250$ g) were obtained from Hayes Lake Animal experiments Ltd. (Shanghai, China). The rats were group-housed (five rats per cage) in a relatively constant feeding environment (a normal circadian rhythm) at $20 \sim 25^{\circ}$C, with free access to food and water. After one week of feeding, the rats were randomly assigned to 10 groups with 10 rats per group, including the normal control, sham operation, chronic constriction injury (CCI), normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups. Different drug interventions were performed on different groups according to following steps (Table 1). Seven days after CCI, the intrathecal administration was conducted in the normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups, and the doses were as follows: the volumes of 10 mg/kg morphine (Shenyang First Pharmaceutical Factory, Shenyang, Liaoning, China), miR-223 lentivirus vectors, and *NLRP3* lentivirus vectors were all 5 μL (intravitreal injection of titer of miR-223 lentivirus vectors was 2×10^6 TU/mL; intravitreal injection of titer of *NLRP3* lentivirus vectors was 2×10^8 TU/mL; lentivirus vectors were constructed by Shanghai GeneChem Co., Ltd., Shanghai, China). Drugs were first injected for intervention, and then catheters were flushed with normal saline, with a total volume of 20 μL per rat. Rats were administered at 8:00 every morning, which lasted for 10 days. Then rats were scarified, and spinal cord tissues were preserved for further studies.

Establishment of a rat model of CCI

Surgical procedures were performed under sterile conditions. After anesthetized by the intraperitoneal injection of sodium pentobarbital (40 mg/kg, Sigma, St. Louis, MO, USA), the rats were fixed in a prone position. Hair on the left hind leg was removed, and the skin was disinfected. Then the sciatic nerve was exposed by skin incision and blunt separation of the thigh muscles

Table 1. Experimental animal grouping.

Groups	Treatment regimens
Normal control group	Experimental rats received no treatment.
Sham operation group	Only sciatic nerve trunk was exposed without ligation.
CCI group	After CCI, rats received no intrathecal catheter and no injection of drugs.
Normal saline group	Seven days after CCI, rats received an injection of saline through intrathecal catheter
Morphine group	Seven days after CCI, rats received an injection of morphine through intrathecal catheter
miR-223 group	Seven days after CCI, rats received an injection of miR-223 lentivirus vectors through intrathecal catheter
NLRP3 group	Seven days after CCI, rats received an injection of NLRP3 lentivirus vectors through intrathecal catheter
miR-223+morphine group	Seven days after CCI, rats received an injection of morphine and miR-223 lentivirus vectors
NLRP3+morphine group	Seven days after CCI, rats received an injection of morphine and NLRP3 lentivirus vectors
miR-223+*NLRP3* +morphine group	Seven days after CCI, rats received an injection of morphine, miR-223 lentivirus vectors, and *NLRP3* lentivirus vectors

CCI: chronic constriction injury.

and was suture ligated ($n = 4$, interval 1 mm, appropriate compactness) using non-absorbable sutures (number 4–0 chromic catgut, Johnson & Johnson, Arlington, TX, USA). After surgery, the rats were injected with penicillin (30,000 units, Sigma, St. Louis, MO, USA) to prevent infection. Only sciatic nerve trunk in the sham operation group rats was exposed without ligation. Other surgical procedures were the same with injury groups. After surgery, rats were fed in a quiet and warm environment without strong light and noise stimulation. If hind limb paralysis or death occurred in the model rats after surgery, those rats were excluded. Behavioral tests were performed on rats after surgery to identify whether CCI models were established successfully.

Intrathecal catheterization in rats

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), and fixed in a prone position. After routine disinfection, atlantooccipital membranes were exposed by skin and fascia incision and blunt separation of muscle. Atlantooccipital membranes were gently picked, and then a gush of cerebrospinal fluid could be seen. Polyurethane microspinal catheters (0.12 mm inner diameter, 0.35 mm outer diameter, Lewiston, NY, USA) were slowly implanted into the lumbar interspace through the laceration. Rats with hind limb paralysis, tail paralysis, or impaired motor function after intrathecal catheterization were excluded. Rats without impaired motor function were selected for identification of catheterization success, in which rats received an injection of 2% lidocaine (15 μL, Sigma, St. Louis, MO, USA) in the lumbar catheter. If double-leg paralysis occurred about 20 s later, and rats returned to the normal within 30 min, intrathecal catheterization was considered to be successful.

Behavioral tests

The morphine-produced analgesia was measured with the paw withdrawal threshold (PWT) and percentage maximum possible effect (%MPE) in the tail flick test. After rats were familiar with the testing environment and became quiet, behavioral tests began. The PWT was measured using von Frey filaments (DanMic Global, San Jose, CA, USA). The filament was applied directly to the plantar hind pawn, and the force of each application was limited within 8 s. Withdrawal of paw during the application was considered as a positive response, otherwise it was considered as a negative response. If no withdrawal was observed, another filament was used. The test was continuously performed four times with an interval of 2 min between two stimuli. The mean value was the PWT. Rats were placed on the tail-flick apparatus, and the light radiation was focused on one-third of the distance from the tail distal end to the base. A cut off time of 15 s was used to avoid tissue damage in the tail. The test was continuously performed four times with an interval of 5 min between two light radiations. The threshold of pain was calculated as the average of four consecutive tail flick latencies (the period from the start of light radiation to the withdrawal of the tail). %MPE = (post-drug threshold of pain − pre-drug threshold of pain)/(cut off time − pre-drug threshold of pain) × 100%.

Luciferase reporter gene assay

TargetScan (http://www.targetscan.org/vert_61/), online bioinformatics software, concluded that *NLRP3* is a downstream target gene of miR-223. The luciferase-3′-UTR reporter constructs were generated by introducing the 3′-UTRs of NLRP3 carrying the putative miR-223 binding site into a luciferase reporter vector.

A plasmid vector containing the wild-type loci of miR-223 binding *NLRP3* (*NLRP3* wild) and a plasmid vector containing the mutant loci of miR-223 binding *NLRP3* (*NLRP3* mut) were designed and constructed. *NLRP3*-3′-UTR primer sequences synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) are as follows (Table 2). After the sequence was verified by sequencing, HEK293 cells (ATCC) were co-transfected with 80 ng *NLRP3* wild plasmid or *NLRP3* mut plasmid, along with 40 ng dual fluorescence plasmid carrying firefly and Renilla luciferase reporters, and the miR-223 mimic, miR-223 inhibitor, or the control (Shanghai GenePharma Co., Ltd., Shanghai, China). After HEK293 cells were incubated at 37°C for 24 h, proteins were extracted and analyzed by the dual-Luciferase reporter assay (Promega, Madison, WI, USA).

Tissue sampling

On day 11 after drug intervention, when rats in each group completed behavioral tests, they were sacrificed after receiving an intraperitoneal injection of pentobarbital (40 mg/kg). Rats were fixed with pre-cooled normal saline (250 mL) and 4% paraformaldehyde (500 mL) through cardiac perfusion. Complete spinal cord of rats (the CCI, normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups) was taken out and placed in freezing tubes which were pretreated with diethyl pyrocarbonate water and presterilized treatment. Freezing tubes were immediately frozen in liquid nitrogen and then transferred to a −80°C refrigerator. The following operation was performed in a RNA biological purification table using sterile surgical instruments.

Table 2. *NLRP3*-3′-UTR primer sequences.

Primers	Sequences
Forward	5′-ACCTCAACAGTCGCTACACG-3′
Reverse	5′-TAGACTCCTTGGCGTCCTGA-3′

Real-time quantitative polymerase chain reaction

RNA in lumbar spinal cord was extracted according to the strict requirements of RNA extraction from tissues. Reverse transcription was performed using the PrimeScript®RT reagent Kit. The cDNAs of miR-223 and *U6* were synthesized using stem-loop reverse transcription primers (miR-223: 5′-GTCGTATCCAGT GCAGGGTCCGAGGTATTCGCACTGGATACGA-CTGGGGT-3′; U6: 5′-AACGCTTCACGAATTTGC GT-3′). According to the gene sequence published in Genbank database, polymerase chain reaction (PCR) reaction primers were designed using Primer 5.0 as follows (Table 3). Primers were synthesized by Shanghai Sangon Company (Shanghai, China). According to instructions on the kit, reaction liquid was prepared, and reaction conditions were set. ABI PRISM 7500 real-time PCR System (ABI, Carls- bad, CA, USA) and SYBR Green I fluorescence kit (Takara, Tokyo, Japan) were used in the PCR reaction. U6/GAPDH served as an internal control. The melting curve was used to evaluate reliability of the PCR results. Threshold cycle (CT) value (the inflection point of the amplification curve) was taken, and the relative expression of target genes was calculated using $2^{-\triangle\triangle Ct}$.

Western blotting

Tissues in lumbar spinal cord were removed from the −80°C refrigerator, and cell lysis solution containing protease inhibitors was added to the tissues.

Table 3. Real-time fluorescent quantitative PCR primers.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-223	TGGCTGTCAGTTTGTCAAAT	GTGCAGGGTCCGAGGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
NLRP3	CAGCGATCAACAGGCGAGAC	AGAGATATCCCAGCAAACCTATCCA
ASC	GCTGAGCAGCTGCAAACGA	ACTTCTGTGACCCTGGCAATGA
Caspase-1	ACTCGTACACGTCTTGCCCTCA	CTGGGCAGGCAGCAAATTC
IL-1β	CCCTGAACTCAACTGTGAAATAGCA	CCCAAGTCAAGGGCTTGGAA
IL-18	GACTGGCTGTGACCCTATCTGTGA	TTGTGTCCTGGCACACGTTTC
GAPDH	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA

NLRP3: nucleotide-binding and oligomerization domain-like receptor 3; ASC: apoptosis-associated speck-like protein; IL-1β: interleukin-1β; IL-18: interleukin-18; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Centrifugal tubes containing spinal cord tissues of rats were placed in an ice-water mixture to rewarm tissues. After 30 min of rewarming, tissues were homogenized with an electric homogenizer (Thermo, Holbrook, NY, USA) in ice bath. Refrigerated centrifugation was performed at 15,000 r/min for 10 min after tissues were placed in an ice bath for 30 min, and the supernatant was saved. A small amount of protein samples were used to determined protein concentrations according to instructions on the bicinchoninic acid kit (Wuhan Boster Company, Wuhan, Hubei, China). Loading buffer was added to extracted protein samples and boiled at 95°C for 10 min. Then 10% polyacrylamide gel electrophoresis was performed to extract proteins with 30 μg of samples loaded per well. Wet transfer was performed, protein blotting was transferred onto a polyvinylidene fluoride transfer membrane, and then 5% bovine serum albumin was added for block for 1 h at room temperature. Primary antibodies against *NLRP3* (1: 1000), apoptosis-associated speck-like protein (ASC) (1: 1000), Caspase-1 (1: 1000), Interleukin (IL)-1β (1:1000), IL-18 (1:1000), and β-actin (1: 1000) (Abcam Inc., Cambridge, MA, USA) were added, followed by overnight incubation at 4°C. Samples were rinsed in tris-buffered saline for three times/5 min. The appropriate secondary antibodies were added, and then samples were incubated at room temperature for 1 h. The membrane was washed in tris-buffered saline for threetimes/5 min. Chemiluminescent reagents were then added for color development. A gray value analysis of target bands was conducted using Image J with β-actin as an internal reference.

Enzyme linked immunosorbent assay

Tissues in lumbar spinal cord were removed from the −80°C refrigerator and grinded to extract proteins for further experiments. The following operation was performed in strict accordance with instructions on the enzyme-linked immunosorbent assay (ELISA) kit (eBioscience Inc., San Diego, CA, USA). An ELISA kit was equilibrated at room temperature for 20 min, and washing solution was prepared. After the dissolution of the standard, 100 μL of the solution was added to the reaction plate, and serial dilutions of the solution were made to make a standard curve. According to the protein concentration of samples detected by bicinchoninic acid, protein samples were appropriated diluted to prepare samples to be detected. Samples (100 μL) were added into reaction wells and incubated at 37°C for 90 min. Liquid in the reaction wells was flung off, and the reaction wells were washed three times. Freshly prepared biotinylated antibody working solution (100 μL) was added into reaction wells. After shaken gently, reaction wells were incubated at 37°C for 60 min and washed three times. Freshly prepared enzyme binding reactant (away from light) working solution was added into reaction wells. Reaction wells were incubated at 37°C for 30 min and washed three times. Then 100 μL of substrate was added to each well and shaken gently. Reaction wells were incubated away from light for 15 min at 37°C, and then 100 μL of stop solution was quickly added to each well to stop the reaction. Optical density value (450 nm) of each well was detected using a versatile microplate reader within 3 min. According to optical density values, a standard curve was drawn, and results were analyzed combined with the IL-1β, and IL-18 contents in samples.

Immunohistochemistry

Tissues in lumbar spinal cord were removed from the −80°C refrigerator, embedded in optimal cutting temperature medium, sectioned to 8 μm thicknesses by a freezing microtome (Cryotome SME, Cambridge, MA, USA), and equilibrated at room temperature for 10 min. Then tissues were fixed with pre-cooled acetone, dried, washed in the phosphate-buffered saline, and treated with 0.3% H_2O_2 to inactivate endogenous peroxidases. After block, primary antibodies against *NLRP3* (1: 500), ASC (1: 500), Caspase-1 (1: 500), IL-1β (1:500), and IL-18 (1:500) were added. After 4°C overnight incubation, tissues were washed with phosphate-buffered saline Tween-20, and secondary antibodies were added. After incubation at room temperature, diaminobenzidine (DAB) (Wuhan Boster Company, Wuhan, Hubei, China) was added to develop colors, and sections were counterstained with hematoxylin. Then the sections were washed in water and mounted by neutral resin. The staining was observed through a microscope (Olympus, Tokyo, Japan), and results were recorded in photos. Positive stain of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 were mainly tan, which were located at cytoplasm. Three sections of each rat were randomly selected for immunohistochemically staining, and the results were gained after analysis of the ratio between the number of positive cells and the total number of cells.

Statistical analysis

All data analysis was conducted using SPSS (version 18.0) (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation ($\bar{x} \pm s$). For normally distributed measurement data, comparisons between two groups were examined using the t test, while multiple comparisons among groups were examined using the single-factor analysis of variance (one-way analysis of variance). $P < 0.05$ was considered statistically significant.

Results

Morphine analgesic tolerance in rat model of CCI after drug intervention

On D1, D3, D5, D7, D9, and D11 after CCI surgery, PWT and %MPE were measured. The results are as follows: there were significant difference on PWT and %MPE in all time points (except for D1) in the CCI group ($P < 0.05$), while there were no significant difference between the sham operation and normal control groups at each time point (Figure 1(a) and (b)). These results indicated that the rat model of CCI was established successfully. On D1, D3, D5, D7, D9, and D11 after drug intervention following CCI surgery, behavioral tests were performed on rats. There were no significant differences between the CCI group without intrathecal catheter insertion and the normal saline group in which rats received saline injection through intrathecal catheter in the PMT and %MPE over time (both $P > 0.05$). After drug intervention, compared with the normal saline group, the PMT and %MPE in the morphine and miR-223 + NLRP3 + morphine groups were decreased, and on the D9 after drug intervention, they were closed to the normal saline group (both $P > 0.05$); while the PMT and %MPE in the miR-223 group were enhanced first and decreased on the D5 after drug intervention. Compared with the normal saline group, the NLRP3 group had lower PMT and %MPE (both $P < 0.05$). The miR-223 + morphine group maintained good analgesic effects, and significant differences were found in the PMT and %MPE between the normal saline and miR-223 + morphine groups (both $P < 0.05$). The PMT and %MPE in the NLRP3 + morphine group were decreased, which were closed to the normal saline group on the D5 (all $P > 0.05$). Compared with the morphine group, the miR-223 + morphine group had higher PMT and %MPE in all time points (except for D1), while the NLRP3 + morphine group had lower PMT and %MPE in all time points (except for D1) (all $P < 0.05$). No significant difference was found in comparisons of the PMT and %MPE between the morphine and miR-223 + NLRP3 + morphine groups (both $P > 0.05$). Compared with the miR-223 group, the miR-223 + morphine group had higher PMT and %MPE in all time points (both $P > 0.05$). Besides, the NLRP3 + morphine group had higher PMT and %MPE in all time points compared with the NLRP3 group, and the PMT and %MPE in the NLRP3 + morphine group were lower than those in the miR-223 + NLRP3 + morphine group (all $P < 0.05$) (Figure 1(c) and (d)). These results suggested that miR-223 and *NLRP3* influence the morphine analgesic tolerance.

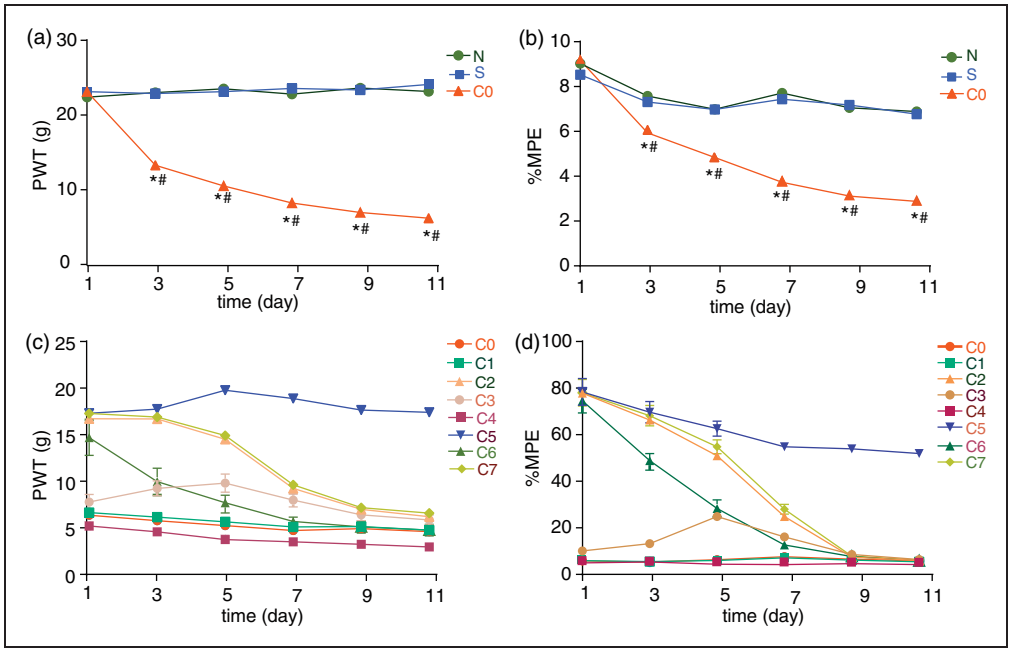

Figure 1. Comparisons of paw withdrawal threshold (PWT) and percentage maximum possible effect (%MPE) of rats in each group at different time points. (a) Comparisons of PWT of rats in each group after CCI. (b) Comparisons of %MPE of rats in each group after CCI. (c) Comparisons of PWT of rats in each group after drug intervention. (d) Comparisons of %MPE of rats in each group after drug intervention. N: the normal control group, $n = 10$; S: the sham operation group, $n = 10$; C0: the CCI group, $n = 10$; C1: the normal saline group, $n = 10$; C2: the morphine group, $n = 10$; C3: the miR-223 group, $n = 10$; C4: the NLRP3 group, $n = 10$; C5: the miR-223 + morphine group, $n = 10$; C6: the NLRP3 + morphine group, $n = 10$; and C7: the miR-223 + NLRP3 + morphine group, $n = 10$. $^{*}P < 0.05$, compared with the normal control group; $^{\#}P < 0.05$, compared with the sham operation group.

NLRP3 is a target gene of miR-223

It was concluded that *NLRP3* is a downstream target genes of miR-223 by TargetScan. Plasmid vectors containing the wild-type loci of miR-223 binding *NLRP3* (*NLRP3* wild) and plasmid vectors containing the mutant loci of miR-223 binding *NLRP3* (*NLRP3* mut) were designed and expressed (Figure 2(a)). Luciferase results showed that the transcriptional activity of *NLRP3* decreased significantly ($P < 0.05$) after instantaneous transfer miR-223 in the *NLRP3* wild group in comparison with the control group ($P < 0.05$), that the transcriptional activity of *NLRP3* increased significantly after instantaneous transfer miR-223 in the miR-223 inhibitor group in comparison with the miR-223 group ($P < 0.05$), and that no factors influenced transcriptional activities in the *NLRP3* mutant group and normal control group, indicating that miR-223 targeted and regulated the *NLRP3* expression negatively (Figure 2(b)). RNA and protein were extracted from HEK293 cells in the transfection groups. The results of real-time quantitative

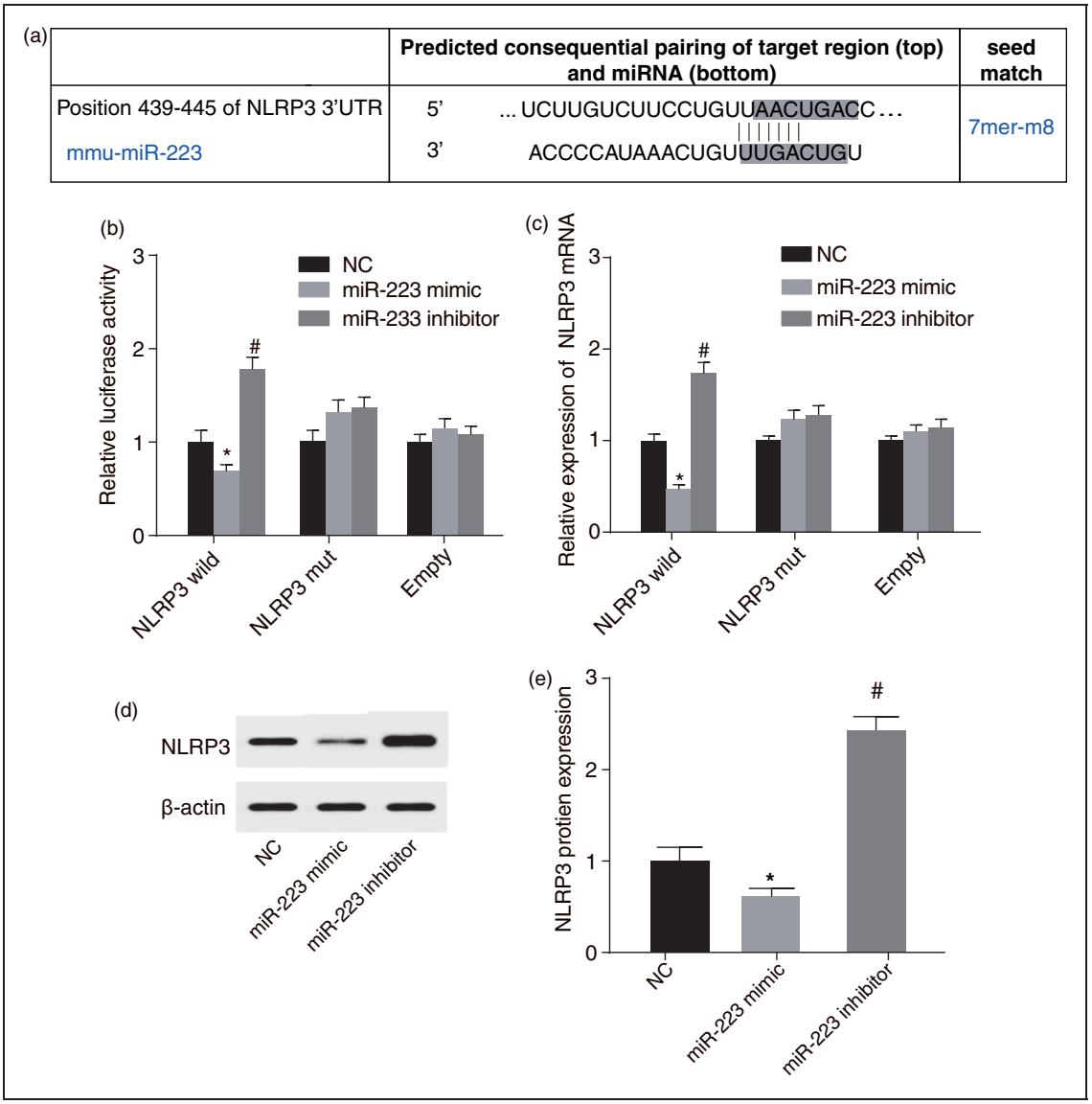

Figure 2. MicroRNA-223 negatively regulates the *NLRP3* expression. (a) MiR-223 binding sites in the *NLRP3*-3′UTR predicted by TargetScan online database. (b) Dual-luciferase reporter gene assay showed that miR-223 negatively regulates the expression of *NLRP3*. (c) qRT-PCR assay confirmed that miR-223 could regulate the mRNA expression of *NLRP3*. (d) Western blotting indicated that miR-223 negatively regulates the protein expression of *NLRP3*. (e) The gray value analysis of Western blotting results. $^{*}P < 0.05$, compared with the normal control (NC) group, $^{\#}P < 0.05$ as compared with the miR-223 mimic group.

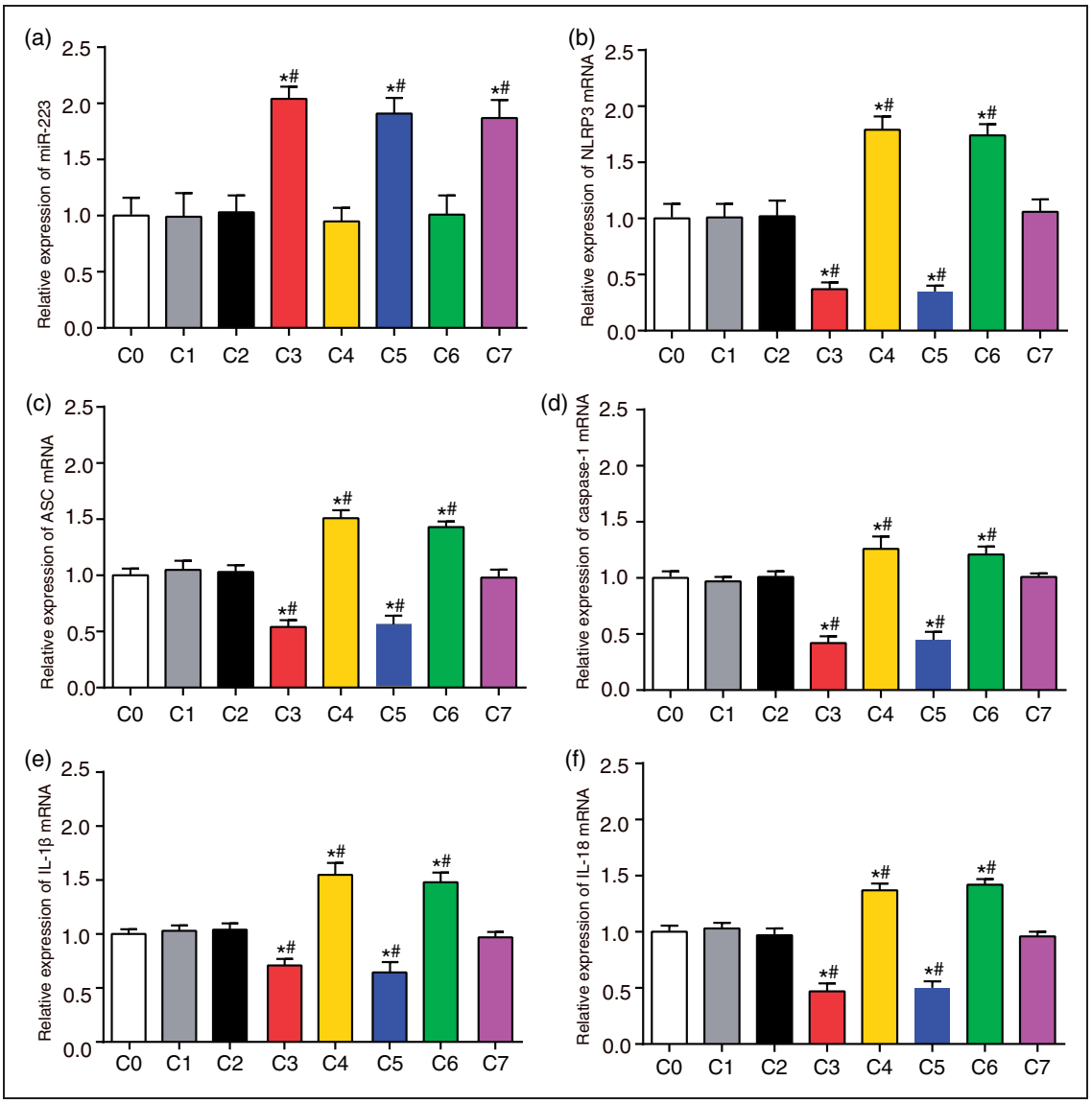

Figure 3. The expressions of miR-223, NLRP3, ASC, Caspase-1, IL-1β, and IL-18 mRNA in the spinal cord tissues of rats in each group after drug intervention. (a) The relative expression of miR-223 in the spinal cord tissues of rats in each group. (b) The mRNA expression of *NLRP3* the spinal cord tissues of rats in each group. (c) The mRNA expression of ASC in rat lumbar spinal cord tissues. (d) The mRNA expression of Caspase-1 in rat lumbar spinal cord tissues. (e) The mRNA expression of IL-1β in rat lumbar spinal cord tissues. (f) The mRNA expression of IL-18 in rat lumbar spinal cord tissues.

Note: NLRP3: nucleotide-binding and oligomerization domain-like receptor 3; ASC: apoptosis-associated speck-like protein; IL-1β: interleukin-1β; IL-18: interleukin-18; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; C0: the CCI group, $n = 10$; C1: the normal saline group, $n = 10$; C2: the morphine group, $n = 10$; C3: the miR-223 group, $n = 10$; C4: the NLRP3 group, $n = 10$; C5: the miR-223 + morphine group, $n = 10$; C6: the NLRP3 + morphine group, $n = 10$; C7: the miR-223 + NLRP3 + morphine group, $n = 10$.

*$P < 0.05$, compared with the CCI and normal saline groups, #$P < 0.05$, compared with the morphine group.

polymerase chain reaction and Western blotting were similar to the results of luciferase reporter gene assay: transfected miR-223 inhibited the *NLRP3* expression, and the *NLRP3* expression increased in the miR-223 inhibitor group after transfection (Figure 2(c) and (d)).

The mRNA expressions of miR-223, NLRP3 inflammasomes, and inflammatory cytokines in the spinal cord tissues of rats

Real-time quantitative polymerase chain reaction results of all groups (as shown in Figure 3) showed that there

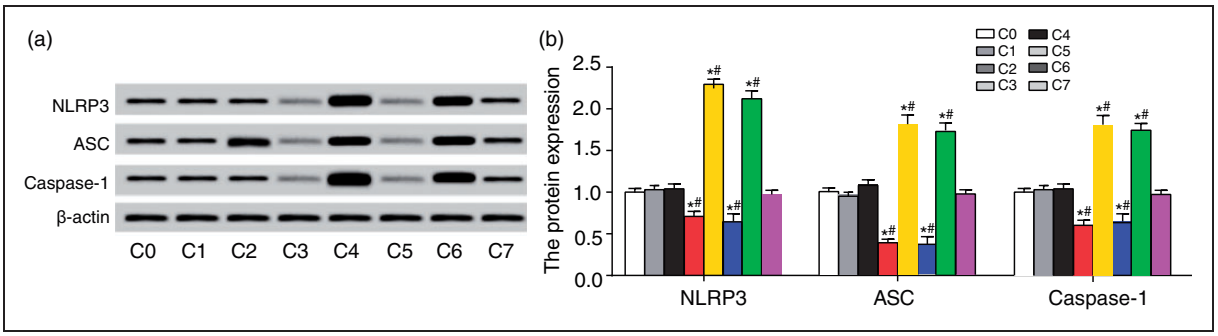

Figure 4. The protein expressions of NLRP3, ASC, and Caspase-1 in the spinal cord tissues of rats in each group. (a) Protein expressions of *NLRP3*, ASC, and Caspase-1 in rat lumbar spinal cord tissues were detected by Western blotting. (b) The gray value analysis of Western blotting results.

Note: NLRP3: nucleotide-binding and oligomerization domain-like receptor 3; ASC: apoptosis-associated speck-like protein; C0: the CCI group, $n = 10$; C1: the normal saline group, $n = 10$; C2: the morphine group, $n = 10$; C3: the miR-223 group, $n = 10$; C4: the NLRP3 group, $n = 10$; C5: the miR-223 + morphine group, $n = 10$; C6: the NLRP3 + morphine group, $n = 10$; C7: the miR-223 + NLRP3 + morphine group, $n = 10$.

$^{*}P < 0.05$, compared with the CCI and normal saline groups, $^{\#}P < 0.05$, compared with the morphine group.

were no significant differences among the CCI, normal saline, morphine, NLRP3, and NLRP3 + morphine groups in the miR-223 expression, and no significant differences among the miR-223, miR-223 + morphine, and miR-223 + NLRP3 + morphine groups in the miR-223 expression (all $P > 0.05$). The miR-223, miR-223 + morphine, and miR-223 + NLRP3 + morphine groups had higher miR-223 expression compared with that in the CCI, normal saline, and morphine groups (all $P < 0.05$). Besides, no significant difference was found among the CCI, normal saline, morphine, and miR-223 + NLRP3 + morphine groups in the expressions of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 (all $P > 0.05$). Compared with the CCI, normal saline and morphine groups, the miR-223, and miR-223 + morphine groups had decreased mRNA expressions of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 (all $P < 0.05$), while the NLRP3 and NLRP3 + morphine groups had increased mRNA expressions of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 (all $P < 0.05$). Compared with the CCI, normal saline and morphine groups, no significant differences were found among those mRNA expressions in the miR-223 + NLRP3 + morphine group (all $P > 0.05$). These results suggested that miR-223 could negatively regulate the expressions of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18.

Protein expressions of NLRP3 inflammasomes in the spinal cord tissues of rats

Proteins were extracted from the spinal cord tissues of rats. Western blotting was adopted to detect protein expressions of *NLRP3* inflammasomes, and the results showed that there were no significant differences among the CCI, normal saline, and morphine groups in protein expressions of *NLRP3*, ASC, and Caspase-1 (all $P > 0.05$). Compared with the CCI, normal saline and morphine groups, protein expressions of *NLRP3*, ASC, and Caspase-1 in the miR-223, and miR-223 + morphine groups significantly reduced, while protein expressions of *NLRP3*, ASC, and Caspase-1 in the NLRP3 and miR-223 + morphine groups significantly increased (all $P < 0.05$). No significant differences were found among those protein expressions in the miR-223 + NLRP3 + morphine group compared with the CCI, normal saline and morphine groups (all $P > 0.05$) (Figure 4(a) and (b)). These results indicated that miR-223 could negatively regulate the expression of *NLRP3* and inhibit the activities of inflammasomes (*NLRP3*, ASC, and Caspase-1).

Protein expressions of IL-1β, and IL-18 in the spinal cord tissues of rats

Western blotting and ELISA were adopted to detect protein expressions of IL-1β and IL-18. Results in Figure 5 showed that there were no significant differences among the CCI, normal saline morphine, and miR-223 + NLRP3 + morphine groups in protein expressions of IL-1β and IL-18 (all $P > 0.05$). Compared with the normal saline group, protein expressions of IL-1β and IL-18 in the miR-223 and miR-223 + morphine groups significantly reduced, while protein expressions of IL-1β and IL-18 in the NLRP3 and NLRP3 + morphine groups significantly increased. Our results indicated that miR-223 affects the processing and secretion of IL-1β and IL-18 through negatively regulating the expression of *NLRP3*.

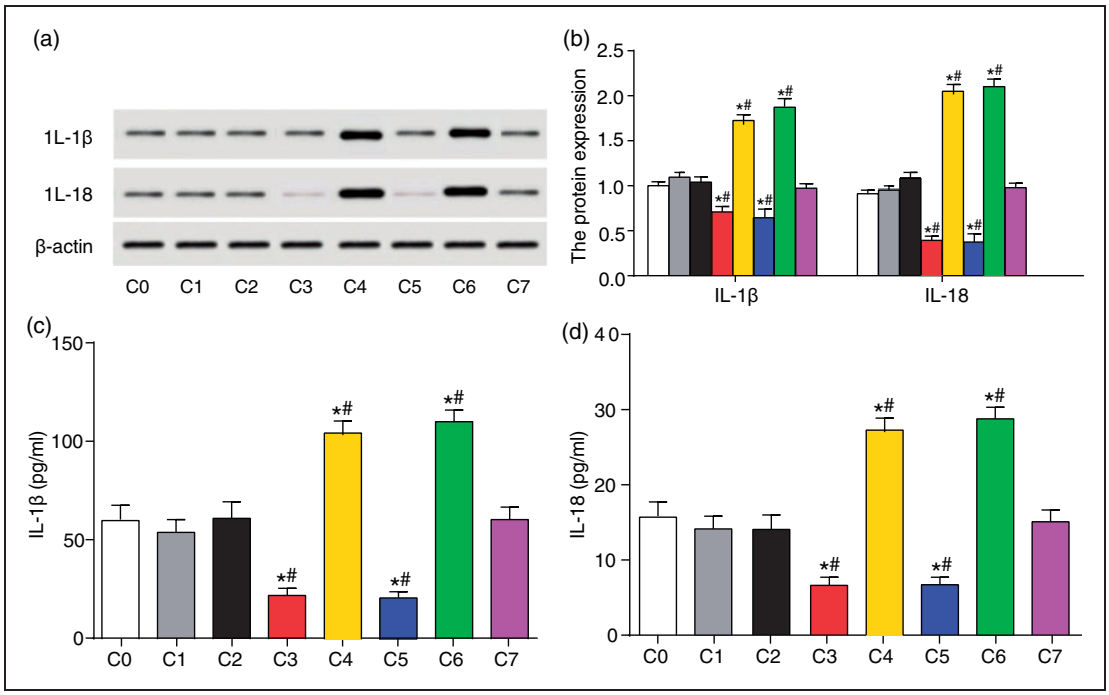

Figure 5. The protein expressions of IL-1β and IL-18 in the spinal cord tissues of rats in each group. (a) The protein expressions of IL-1β and IL-18 in rat lumbar spinal cord tissues detected by Western blotting. (b) The gray value analysis of Western blotting results. (c) The protein expressions of IL-1β in rat lumbar spinal cord tissue homogenate detected by ELISA assay. (d) The protein expressions of IL-18 in rat lumbar spinal cord tissue homogenate detected by ELISA assay.
Note: IL-1β: interleukin-1β; IL-18: interleukin-18; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; C0: the CCI group, $n = 10$; C1: the normal saline group, $n = 10$; C2: the morphine group, $n = 10$; C3: the miR-223 group, $n = 10$; C4: the NLRP3 group, $n = 10$; C5: the miR-223 + morphine group, $n = 10$; C6: the NLRP3 + morphine group, $n = 10$; C7: the miR-223 + NLRP3 + morphine group, $n = 10$. $^{*}P < 0.05$, compared with the CCI and normal saline groups, $^{\#}P < 0.05$, compared with the morphine group.

Immunohistochemical staining results of spinal cord tissues of rats

The results of positive expression rates of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 were in Figure 6. There were no significant differences among the CCI, normal saline, morphine, and miR-223 + NLRP3 + morphine groups in *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 expressions (all $P > 0.05$). Compared with the normal saline group, positive expression rates of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 in the miR-223 and miR-223 + morphine groups significantly reduced, while positive expression rates of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 in the NLRP3 and NLRP3 + morphine groups significantly increased (Figure 6). These results indicated that miR-223 could negatively regulate the expression of *NLRP3* and inhibit the activities of inflammasomes and affect the expressions of IL-1β and IL-18.

Discussion

The current situation of NP treatment is not ideal with some NP patients unable to adequately relieve pain.[19] Morphine is considered as first-line drugs in NP treatment, but it was easy to develop morphine tolerance during the use of morphine.[20] The mechanisms underlying morphine analgesic tolerance are not fully understood.[21] If an effective therapeutic target which prevents morphine tolerance can be found, it is of great significance for the treatment of NP.

Experimental results have shown that miR-223 and *NLRP3* have an influence on morphine analgesic tolerance. Many relevant studies have shown that miRs have a regulatory role in NP.[22–24] And several miRs have been proven to alleviate the effect of morphine tolerance.[25] Because chronic pain and morphine tolerance have similar signaling pathways, miRs are possible to participate in the formation of morphine analgesic tolerance.[26] Morphine tolerance results from changes in gene transcription levels of the second messenger and neurotransmitter levels because of a series of compensatory reactions caused by prolonged use of morphine.[27] Previous evidence showed that the functions of NLRP3 inflammasome were crucial for the regulation of neuroinflammation mediated by microglia, thus, compounds that inhibited the activation of NLRP3 inflammasome may contribute to the improvement of morphine

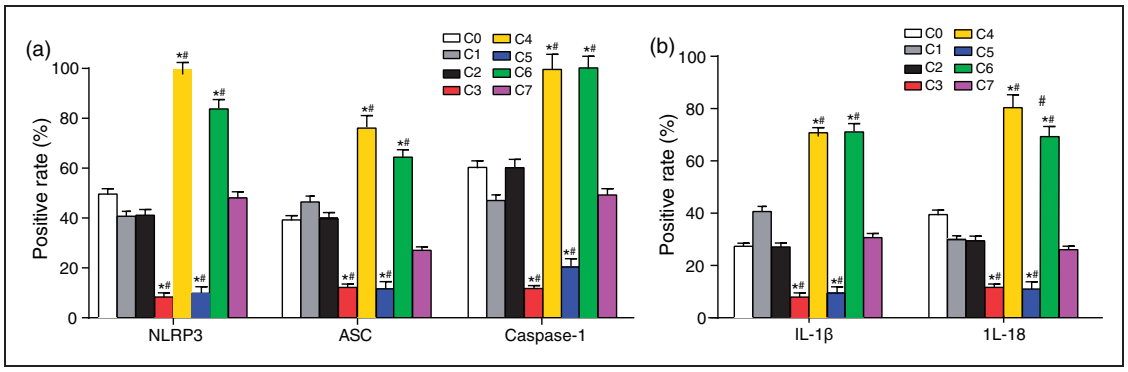

Figure 6. The positive expression rates of *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 in the lumbar spinal cord tissues of rats in each group. (a) Comparisons of the positive expression rates of *NLRP3*, ASC, and Caspase-1 in the spinal cord tissues of rats in each group. (b) Comparisons of the positive expression rates of IL-1β, and IL-18 in the spinal cord tissues of rats in each group.
Note: IL-1β: interleukin-1β; IL-18: interleukin-18; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; C0: the CCI group, $n = 10$; C1: the normal saline group, $n = 10$; C2: the morphine group, $n = 10$; C3: the miR-223 group, $n = 10$; C4: the NLRP3 group, $n = 10$; C5: the miR-223 + morphine group, $n = 10$; C6: the NLRP3 + morphine group, $n = 10$; C7: the miR-223 + NLRP3 + morphine group, $n = 10$. $^{*}P < 0.05$, compared with the CCI and normal saline groups, $^{\#}P < 0.05$, compared with the morphine group.

tolerance.[28,29] Besides, Bauernfeind et al.[30] found that miR-223 could target the 3′UTR area of NLRP3 mRNA to inhibit NLRP3 expression.

It is found that miR-223 could negatively regulate *NLRP3*, ASC, Caspase-1, IL-1β, and IL-18 expressions. Inflammasomes contain high-molecular weight signaling platforms that resulted in the activation of inflammatory caspases.[31] Activated Caspase-1 plays an important role in the proteolytic processing and, thus, maturation of the cytokine precursors of IL-1β and IL-18.[32] Besides, morphine also can modulate immune responses, including the inflammatory response, by affecting the pro-inflammatory cytokines production such as IL-1β.[33] Furthermore, morphine-tolerance suppresses immune activation, thereby decreasing levels of proinflammatory cytokines, and studies have even reported that IL-1β treatment reduces morphine-induced analgesia, thereby contributing to the development of tolerance.[34,35] A study has showed that overexpression of miR-223 inhibits NLRP3 protein expressions and inhibits IL-1β production from the inflammasome.[17] Yang et al.[18] found that miR-223 could downregulate NLRP3 to inhibit inflammation through Caspase-1 and IL-1β after intracerebral hemorrhage. *NLRP* family proteins, proteins scattered in the cell, were able to quickly identify inflammation induced by a pathogen and involved in the processing of IL-1β and IL-18. In addition, they can secrete IL-1β which may lead to apoptosis.[36] Precise and ordered protein oligomers of *NLRP3* are formed by polymerizing ATP, and the oligomers through combining with ASC containing a caspase recruitment domain and cysteine-aspartic proteases (caspase) form a multi-protein complex called the inflammasome, which controls Caspase-1 activation.[37,38] Caspase-1 is a converting enzyme of IL-1β. Activated Caspase-1 can form a mature and activated IL-1β through reactions, which is then secreted outside the cell.[39,40] Apart from participation in inflammasome-related signaling pathways, NLPR3 can also activate NF-κB, starting the transcription of IL-1β precursor.[41] Lamkanfi and coworkers[42] found that Caspase-1 can activate NF-κB, and that NF-κB activation and IL-1β maturation occur simultaneously. The latest study in 2015 found that miR-223 can negatively regulate the activity of *NLRP3* inflammasomes, and that activated inflammasomes promote the body to produce IL-1β and IL-18 and have an automatic negative feedback regulation on IL-1β and IL-18, inhibiting specific pathological damage and maintaining normal physiological activities.[43]

In summary, the experimental results in this study showed that miR-223 could suppress the activities of *NLRP3* inflammasome (*NLRP3*, ASC, and Caspase-1) to relieve morphine analgesic tolerance in rats by down-regulating *NLRP3*, which is expected to become a new target for the treatment of morphine analgesic tolerance. However, the mechanism of miR-223 and *NLRP3* in the occurrence, development, and prognosis of NP needs further confirmation, there is no clinical validation of effects of targeted regulation of *NLRP3* by miR-223 on morphine analgesic tolerance, and there is no in situ hybridization for detecting the miR-223 expression and the expression position in cells due to limitation in funding. Continuous improvement and relevant research are still needed.

Acknowledgments

The authors would like to acknowledge the helpful comments on this paper received from our reviewers.

Author Contributions

Xiao-Juan Xie and Li-Gang Ma are regarded as co-first author.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Cohen SP and Mao J. Neuropathic pain: mechanisms and their clinical implications. *BMJ* 2014; 348: f7656.
2. van Hecke O, Austin SK, Khan RA, et al. Neuropathic pain in the general population: a systematic review of epidemiological studies. *Pain* 2014; 155: 654–662.
3. Lema MJ, Foley KM and Hausheer FH. Types and epidemiology of cancer-related neuropathic pain: the intersection of cancer pain and neuropathic pain. *Oncologist* 2010; 15: 3–8.
4. Pasternak GW. Multiple opiate receptors: deja vu all over again. *Neuropharmacology* 2004; 47: 312–323.
5. Ananthan S. Opioid ligands with mixed mu/delta opioid receptor interactions: an emerging approach to novel analgesics. *AAPS J* 2006; 8: E118–E125.
6. Zorn KE and Fudin J. Treatment of neuropathic pain: the role of unique opioid agents. Agents that work against central nervous system receptors may be the key to successful pain management in patients with nerve damage or injury. *Pract Pain Manag* 2011; 11: 26–33.
7. Dworkin RH, O'Connor AB, Backonja M, et al. Pharmacologic management of neuropathic pain: evidence-based recommendations. *Pain* 2007; 132: 237–251.
8. Benyamin R, Trescot AM, Datta S, et al. Opioid complications and side effects. *Pain Physician* 2008; 11: S105–S120.
9. Huxtable CA, Roberts LJ, Somogyi AA, et al. Acute pain management in opioid-tolerant patients: a growing challenge. *Anaesth Intensive Care* 2011; 39: 804–823.
10. Acunzo M, Romano G, Wernicke D, et al. MicroRNA and cancer–a brief overview. *Adv Biol Regul* 2015; 57: 1–9.
11. von Schack D, Agostino MJ, Murray BS, et al. Dynamic changes in the microRNA expression profile reveal multiple regulatory mechanisms in the spinal nerve ligation model of neuropathic pain. *PLoS One* 2011; 6: e17670.
12. Gilicze AB, Wiener Z, Toth S, et al. Myeloid-derived microRNAs, miR-223, miR27a, and miR-652, are dominant players in myeloid regulation. *Biomed Res Int* 2014; 2014: 870267.
13. Jin C and Flavell RA. Molecular mechanism of NLRP3 inflammasome activation. *J Clin Immunol* 2010; 30: 628–631.
14. Wang J, Bai X, Song Q, et al. miR-223 inhibits lipid deposition and inflammation by suppressing toll-like receptor 4 signaling in macrophages. *Int J Mol Sci* 2015; 16: 24965–24982.
15. Cardoso AL, Guedes JR and de Lima MC. Role of microRNAs in the regulation of innate immune cells under neuroinflammatory conditions. *Curr Opin Pharmacol* 2016; 26: 1–9.
16. Bauernfeind F, Rieger A, Schildberg FA, et al. NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol* 2012; 189: 4175–4181.
17. Haneklaus M, Gerlic M, Kurowska-Stolarska M, et al. Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1beta production. *J Immunol* 2012; 189: 3795–3799.
18. Yang Z, Zhong L, Xian R, et al. MicroRNA-223 regulates inflammation and brain injury via feedback to NLRP3 inflammasome after intracerebral hemorrhage. *Mol Immunol* 2015; 65: 267–276.
19. Tan T, Barry P, Reken S, et al. Pharmacological management of neuropathic pain in non-specialist settings: summary of NICE guidance. *BMJ* 2010; 340: c1079.
20. Dumas EO and Pollack GM. Opioid tolerance development: a pharmacokinetic/pharmacodynamic perspective. *AAPS J* 2008; 10: 537–551.
21. Ueda H and Ueda M. Mechanisms underlying morphine analgesic tolerance and dependence. *Front Biosci (Landmark Ed)* 2009; 14: 5260–5272.
22. Tam Tam S, Bastian I, Zhou XF, et al. MicroRNA-143 expression in dorsal root ganglion neurons. *Cell Tissue Res* 2011; 346: 163–173.
23. Shi G, Shi J, Liu K, et al. Increased miR-195 aggravates neuropathic pain by inhibiting autophagy following peripheral nerve injury. *Glia* 2013; 61: 504–512.
24. Kusuda R, Cadetti F, Ravanelli MI, et al. Differential expression of microRNAs in mouse pain models. *Mol Pain* 2011; 7: 17.
25. Wu Q, Hwang CK, Zheng H, et al. MicroRNA 339 down-regulates mu-opioid receptor at the post-transcriptional level in response to opioid treatment. *FASEB J* 2013; 27: 522–535.
26. McAdams RM, McPherson RJ, Beyer RP, et al. Dose-dependent effects of morphine exposure on mRNA and microRNA (miR) expression in hippocampus of stressed neonatal mice. *PLoS One* 2015; 10: e0123047.
27. Enquist J, Kim JA, Bartlett S, et al. A novel knock-in mouse reveals mechanistically distinct forms of morphine tolerance. *J Pharmacol Exp Ther* 2011; 338: 633–640.
28. Gustin A, Kirchmeyer M, Koncina E, et al. NLRP3 inflammasome is expressed and functional in mouse brain microglia but not in astrocytes. *PLoS One* 2015; 10: e0130624.
29. Cai Y, Kong H, Pan YB, et al. Procyanidins alleviates morphine tolerance by inhibiting activation of NLRP3 inflammasome in microglia. *J Neuroinflammation* 2016; 13: 53.
30. Bauernfeind F, Rieger A, Schildberg FA, et al. NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol* 2012; 189: 4175–4181.
31. Schroder K and Tschopp J. The inflammasomes. *Cell* 2010; 140: 821–832.

32. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 2009; 27: 519–550.
33. Watanabe H, Gaide O, Petrilli V, et al. Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 2007; 127: 1956–1963.
34. Shavit Y, Wolf G, Goshen I, et al. Interleukin-1 antagonizes morphine analgesia and underlies morphine tolerance. *Pain* 2005; 115: 50–59.
35. Mao X, Sarkar S and Chang SL. Involvement of the NLRP3 inflammasome in the modulation of an LPS-induced inflammatory response during morphine tolerance. *Drug Alcohol Depend* 2013; 132: 38–46.
36. van de Veerdonk FL, Netea MG, Dinarello CA, et al. Inflammasome activation and IL-1beta and IL-18 processing during infection. *Trends Immunol* 2011; 32: 110–116.
37. Willingham SB, Allen IC, Bergstralh DT, et al. NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J Immunol* 2009; 183: 2008–2015.
38. Franchi L, Eigenbrod T, Munoz-Planillo R, et al. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* 2009; 10: 241–247.
39. Ferrero-Miliani L, Nielsen OH, Andersen PS, et al. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin Exp Immunol* 2007; 147: 227–235.
40. Ting JP and Davis BK. CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu Rev Immunol* 2005; 23: 387–414.
41. Kinoshita T, Imamura R, Kushiyama H, et al. NLRP3 mediates NF-kappaB activation and cytokine induction in microbially induced and sterile inflammation. *PLoS One* 2015; 10: e0119179.
42. Kanneganti TD, Lamkanfi M and Nunez G. Intracellular NOD-like receptors in host defense and disease. *Immunity* 2007; 27: 549–559.
43. Yang CA and Chiang BL. Inflammasomes and human autoimmunity: a comprehensive review. *J Autoimmun* 2015; 61: 1–8.