



Effect of miR-223-3p on cell pyroptosis in myelodysplastic syndrome and its mechanism via regulating the expression of NLRP3

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ABSTRACT

This study aimed to investigate the regulatory mechanism of the miR-223-3p/NLRP3 signaling axis in the progression of myelodysplastic syndrome (MDS). For this purpose, SKM-1 cells were transfected and three groups were set up according to different transfection protocols: si-NC group (NLRP3 silencing negative control plasmid), si-NLRP3 group (NLRP3 silencing plasmid), miR-223-3p mimic-NC group (miR-223-3p overexpressing negative control plasmid), miR-223-3p mimic group (miR-223-3p overexpressing plasmid), miR-223-3p mimic+oe-NLRP3 group and miR-223-3p mimic+oe-NLRP3 group (NLRP3 silencing combined with miR-223-3p overexpressing plasmid). Normal bone marrow cells were used as the control. qRT-PCR was used to detect relative expressions of NLRP3 and miR-223-3p; and Western blot to detect Ki67, Caspase-1, Gasdermin D, IL-1 β , IL-18 and MMP-9 expressions. Cell proliferation detection used CCK-8 assay, cell cycle distribution detection adopted flow cytometry, and cell migration and invasion analyses relied on Transwell assay. Dual-luciferase reporter assay verified the relationship between NLRP3 and miR-223-3p. An animal experiment was finally conducted to confirm the results obtained in cells. Results showed that compared with normal bone marrow cells and K562 cells, there were significantly upregulated NLRP3 expression and upregulated expression of miR-223-3p in SKM-1 cells (all $P < 0.05$). Compared with the si-NC group and mimic-NC group respectively, the si-NLRP3 group and miR-223-3p mimic group showed inhibited proliferation, blocked cells in the G0/M phase, reduced cells in S phase, inhibited cell invasion and migration, decreased expressions of Ki67, Caspase-1, Gasdermin D, IL-1 β and IL-18, and MMP-9 (all $P < 0.05$). NLRP3 was the direct target of miR-223-3p. Moreover, compared with the miR-223-3p mimic group, the miR-223-3p mimic+oe-NLRP3 group showed increased expressions of NLRP3, Ki67, Caspase-1, Gasdermin D, IL-1 β , IL-18 and MMP-9, promoted proliferation, invasion and migration, and increased cells in S phase (all $P < 0.05$). The animal test revealed that compared with the mimic-NC+ oe-NC group, miR-223-3p mimic+ oe-NC group showed reduced tumor volume and decreased Ki67 expression (both $P < 0.05$); while compared with miR-223-3p mimic+ oe-NC group, miR-223-3p mimic+ oe-NLRP3 group had increased tumor volume and increased Ki67 expression (both $P < 0.05$). It was concluded that overexpression of miR-223-3p can effectively inhibit the expression of NLRP3 and cell pyroptosis in MDS.

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Introduction

Myelodysplastic syndrome (MDS) is a group of heterogeneous clonal diseases originating from myeloid hematopoietic stem cells/hematopoietic progenitor cells (HSCs/HPCs) (1). It is characterized by the abnormal development of erythroid, granulocyte or (and) megakaryocyte at the same time or successively (2). It can be manifested as refractory infection, anemia or (and) bleeding, and high-risk transformation to acute myeloid leukemia (3). At present, the disease can be treated by hematopoietic stem cell transplantation, etc. (4). While owing to its high incidence and mortality, it is urgent to understand the pathogenesis of MDS and find new

therapeutic targets from many angles.

Cell pyroptosis is a newly discovered and confirmed mode of programmed cell death in recent years (5). It is characterized by dependence on Caspase-1 and the release of a large number of pro-inflammatory factors (6). Its morphological characteristics, occurrence and regulation mechanism are different from other programmed cell death modes such as apoptosis and autophagy (7). Recent studies have found that in MDS bone marrow HSC/HPC, there is a Caspase-1-dependent programmed cell death mediated by NLRP3 inflammasome, that is, cell pyroptosis (8,9). This important discovery not only provides a new idea for studying the relationship

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between genetic heterogeneity and abnormal phenotype of MDS but also a new opportunity for the discovery and research of novel targeted therapeutic drugs.

Pyroptosis is a programmed cell death process strictly regulated by inflammasomes (10). It can be divided into typical inflammasome activation pathways and atypical inflammasome activation pathways (11). Inflammasomes are cytoplasmic protein complexes assembled after pattern recognition receptors (PRRs) of innate immune cells recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (12). At present, there are mainly 5 inflammasomes, namely NLRP1, NLRP3, NLRC4, IPAF and AIM2, of which NLRP3 inflammasome is the most widely and comprehensively studied (13,14). It is composed of the leucine-rich-repeat domain at the C-terminal, nucleotide-binding oligomerization domain in the central region and effector domain at the N-terminal (15). Although it can be activated by various DAMPs (16., the relevant activation mechanism is still unclear. Cell pyroptosis is mainly mediated by the activation of inflammasomes such as NLRP3 and the activation of its downstream effector (Caspase-1) (17.. Therefore, the occurrence process of pyroptosis can be directly reflected by detecting the related substances that initiate the activation of the NLRP3 inflammasome and the expression level of downstream effector molecules induced by pyroptosis.

At present, the pathogenesis of MDS is not completely clear. Relevant researches on MDS involve molecular genetics and cytogenetics, which confirmed that MDS is a clonal disease originating from HSC/HPC (18-20.. Ineffective hematopoiesis in bone marrow is an important link in the pathogenesis of MDS (21., which seriously threatens the life safety of MDS patients. Therefore, it is urgent to clarify the pathogenesis of ineffective hematopoiesis in MDS. Recent studies have found that in addition to apoptosis and autophagy involved in the pathogenesis of MDS, NLRP3 inflammasome mediated HSC/HPC pyroptosis can induce ineffective hematopoiesis in bone marrow (8,22,23., which plays an important role in the pathogenesis of MDS.

MicroRNA (miRNA) is a single-stranded RNA composed of 18-25 nucleotides and does not have a coding function (24,25). It mainly binds to the 3'-

untranslated region (3'-UTR) of the target gene mRNA to control the expression of the target gene at the transcriptional level by degrading the target gene mRNA or inhibiting its translation (26). Over 50% of human miRNAs genes are located in tumor-related genomic regions, and miRNAs can not only play the role as an oncogene but also downregulate the activity of proto-oncogene as a tumor suppressor (27,28). In recent years, increasingly more studies have found that miRNA is involved in normal and abnormal hematopoietic processes, and abnormal expression profiles of miRNAs have also been found in patients with MDS (29-31). More importantly, abnormally expressed miRNAs play an important role in the pathogenesis of MDS. For example, the expression of miR-146a and miR-145 is decreased in patients with 5q-syndrome (32,33). Meanwhile, the expression level of miR-21 was significantly increased in patients with MDS (34), and downregulating the expression of miR-204 can inhibit the proliferation of AML cell lines through the axis of LINC01128/miR-4260/NR3C2 (35).

Considering the clinical emergency of MDS, and the important roles of NLRP3, miRNA, and cell pyroptosis, and limited experimental data related to these parameters in MDS, this study was conducted to investigate the roles and regulatory mechanism of miR-223-3p/NLRP3 signaling axis in the progression of MDS.

Materials and methods

Cell line

MDS cell line SKM-1 was grown in RPMI1640 medium containing 10% fetal bovine serum in a cell incubator at 37°C and 5% CO₂. The culture medium was changed and cells were subcultured every 2 days. Meanwhile, normal bone marrow cells were used as the negative control, and leukemia cell line K562 as the positive control.

Plasmid constructs and cell transfection

Plasmids were purchased from GenePharma (Shanghai, China). The cell transfection was completed according to the instruction by using Lipofectamine 2000 (Invitrogen, USA). SKM-1 cells at logarithmic growth were collected and inoculated in 6-well plates at the concentration of 1×10^5 cells. After 24h of culture, SKM-1 cells were transfected

and three groups were set up according to different transfection protocols: si-NC group (NLRP3 silencing negative control plasmid), si-NLRP3 group (NLRP3 silencing plasmid), miR-223-3p mimic-NC group (miR-223-3p overexpressing negative control plasmid), miR-223-3p mimic group (miR-223-3p overexpressing plasmid), miR-223-3p mimic+oe-NLRP3 group and miR-223-3p mimic+oe-NLRP3 group (NLRP3 silencing combined with miR-223-3p overexpressing plasmid).

qRT-PCR

The cells cultured on 10cm plate were collected, and the total RNA in the cells was extracted by TRIzol kit (Invitrogen, USA). Then, the concentration and extraction quality of the extracted total RNA was measured with a spectrophotometer, and then the total RNA was frozen in a refrigerator at -80°C . An amount of 5 μg of RNA was collected and reverse transcribed to synthesize cDNA according to the

instructions of the cDNA kit (Fermentas Inc., Ontario, CA, USA). NLRP3 and miR-223-3p were analyzed quantitatively according to the instructions of the kit. The reverse transcription reaction procedure included 20 μL reaction system, which was processed at 25°C for 30 min, at 42°C for 30 min, and at 85°C for 5 min, and the obtained cDNA was stored at 4°C . The quantitative PCR reaction procedure also consisted of 20 μL reaction system, PCR reaction solution was prepared on ice in advance, pre-denatured at 95°C for 3 min (1 cycle), followed by PCR reaction at 95°C for 12 s, at 62°C for 40 s (40 cycles). GAPDH and U6 were used as standardized internal parameters, and the relative expressions of NLRP3 and miR-223-3p were analyzed by the $2^{-\Delta\Delta\text{Ct}}$ cycle threshold method. Ct referred to the number of amplification cycles required when the real-time fluorescence intensity of the reaction reached the set threshold. The experiment was repeated three times.

Table 1. Primer sequences of qRT-PCR

Sequences	Forward (5'→3')	Reverse (5'→3')
miR-223-3p	GTTGCTCCTGTCAGTTTGTCAAA	TATGGTTGTTACGACTCCTTCAC
U6	GCTTCGGCAGCACATATACTAA	AACGCTTCACGAATTTGCGT
NLRP3	GCCACGCTAATGATCGACTT	ACAGTGGGATTGAAACACG
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Western blot

The cells were placed on a 10cm plate. After transfected cells were cultured for 48h, the cells were lysed with RIPA lysate containing PMSF (Sigma, Chemical Co, MO, USA). The protein concentration was quantitatively analyzed by the BCA method. Before loading, the protein was heated at 95°C for 5 min, fully denatured, then added with SDS-PAGE, separated by electrophoresis, and further transferred to polyethylene film. After the film was sealed with 5% BSA for 1 h, primary antibodies of Ki67, Caspase-1, Gasdermin D, NLRP3, IL-1 β , IL-18, MMP-9 and β -actin were added and incubated overnight at 4°C . The film was then washed with TBS buffer added with Tween 3 times, 10 min each time, and incubated with secondary antibody at room temperature for 2 h. The strip was scanned by a Tanon5200 automatic chemiluminescence image analyzer and the data were normalized to β -actin. Image J software analyzed the band gray to obtain the relative expression of the

corresponding proteins. The experiment was repeated three times.

Cell proliferation assay

SKM-1 cells infected with lentivirus were taken and 5×10^3 cells were added to every 96-well plate, the experimental group was the same as before, and 3 replicates were set in each group. CCK-8 reagent was used to detect cell proliferation. An amount of 10 μL CCK-8 reagent was added to each well for reaction at 37°C in a cell incubator for 2h. The optical density value (OD value) was measured at the wavelength of 450nm by Microplate Reader.

Cell cycle distribution detection

Cells were collected and washed with cold PBS 3 times, centrifuged at 1000 rpm for 5 min, washed with PBS twice, centrifuged at 1000 rpm for 5 min, and collected finally, with the supernatant absorbed carefully. The cells were added with pre-cooled 70% ethanol and fixed at 4°C for 2 h. The fixed cells were

centrifuged at 1000 rpm for 5 min, the cells were collected, the supernatant was discarded, and then the cells were washed with PBS twice, centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. Each tube of cells was added with 500 μ L staining buffer for a slow and complete re-suspension. After that, the 25 μ L propidium iodide staining solution was added and mixed well, followed by the addition of 10 μ L RNase A and mixed well. The mixture was incubated in dark at 37°C for 30 min, and stored in an ice bath in dark for subsequent flow cytometry.

Cell migration and invasion analyses

Cells at logarithmic growth were used in all experiments. Matrigel gel was thawed overnight at 4 °C, and the diluted 10 μ L Matrigel was fully mixed with 20 μ L culture medium on ice. The Transwell chamber was taken and placed into a 24-well plate, the cell membrane was wrapped with pre-diluted Matrigel gelatin, and placed in a 37°C incubator for 2 h to solidify the gelatin. The cells were washed with PBS 3 times and digested with 0.25% trypsin to prepare a single-cell suspension (1×10^5 cells/ml). The coated Transwell chamber was placed into a 24 well-plate, and the lower chamber was added with 800 μ L of culture medium containing 20% FBS, 200 μ L cell suspension was added to the upper chamber, and the number of cells was 2×10^4 cells/well. The Transwell chamber was incubated at 37°C, 5% CO₂ and saturated humidity for 24 h. The Transwell cell was taken out and rinsed gently with PBS, wiped off the cells on the upper layer of the microporous membrane with a cotton swab, and then fixed with paraformaldehyde at room temperature for 20 min, stained with hematoxylin dye for 5 min, and rinsed with distilled water finally. The cells that migrated to the lower layer of the microporous membrane were counted under an inverted microscope (200 \times). Five visual fields were selected for each sample to count the number of cells and take the average number. The only difference was that there was no matrix glue on the upper chamber in the cell migration experiment.

Dual-luciferase reporter assay

The binding sites of miR-223-3p and 3'-UTR sequence in human NLRP3 mRNA sequence were

analyzed by TargetScan software, and primers were designed for the binding sites of miR-223-3p. Plasmid and mir-223-3p mimic or negative control agents were transfected into cells at the same time, and the fluorescence value was collected after 48 h. The experimental group included wild-type plasmid miR-223-3p negative control group (WT-NLRP3+NC), wild-type plasmid miR-223-3p mimic group (WT-NLRP3+miR-223-3p), mutant-type plasmid miR-223-3p negative control group (MT-NLRP3+NC) and mutant-type plasmid miR-223-3p mimic group (MT-NLRP3+miR-223-3p). Glomax20/20 luminometer fluorescence detector (Promega) was applied to detect luciferase activity. Each experiment was repeated three times.

Animal experiment

SKM-1 cells were processed according to different transfection protocols (further transfection of NLRP3 overexpression-related plasmids) and inoculated subcutaneously at 1×10^7 cells in the left groin area of mice. Mice were randomly divided into 3 groups: mimic-NC+ oe-NC group (with the transfection of miR-223-3p overexpression and NLRP3 overexpression negative control sequences), miR-223-3p mimic+oe-NC group (with the transfection of miR-223-3p overexpression sequence and NLRP3 overexpression negative control sequence), and miR-223-3p mimic+oe-NLRP3 group (with the transfection of miR-223-3p overexpression and NLRP3 overexpression sequences). On the 28th day after inoculation, mice were killed by cervical dislocation, the tumor tissues were separated and weighed, and the tumor volume was measured with a caliper every other day. The tumor volume was calculated according to the following formula: V (mm^3) = $L \times W^2 / 2$, where L represents the longest diameter of the tumor and W represents the shortest diameter. The tumor tissues were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned. The animal research was approved by the Institutional Animal Care and Use Committee of our hospital.

Statistical analysis

SPSS21.0 (IBM SPSS Statistics, Chicago, IL, USA) was used for statistical analysis. The measurement data were expressed by Mean \pm SD, and

the two groups of data were compared by t-test. One-way ANOVA and Tukey's post-test were used for multi-group data comparison and post hoc test, respectively. $P < 0.05$ was used to indicate the existence of a statistically significant difference.

Results and discussion

Inhibited expression of NLRP3 inhibited the proliferation and pyroptosis of MDS cells

The activation of NLRP3 inflammasome has always been considered to play an important role in inflammatory signaling pathways and play an important role in autoimmune diseases, cancer and other diseases, but the role of NLRP3 in MDS needs to be further explored. Therefore, our study first detected the expression of NLRP3 in MDS cell line SKM-1, normal bone marrow cells and K562 cells by qRT-PCR. It was found that compared with normal bone marrow cells and K562 cells, there was a significantly upregulated expression of NLRP3 in SKM-1 cells (both $P < 0.05$, Figure 1A). In order to

further study the role of NLRP3 in the development of MDS, the cells were provided with NLRP3 knockdown treatment. NLRP3 was lowly expressed in a si-NLRP3 group than that in si-NC group ($P < 0.05$, Figure 1B). The proliferation ability of cells was detected with CCK-8. The results showed that compared with the si-NC group, the si-NLRP3 group showed inhibited proliferation of cells ($P < 0.05$, Figure 1C). Further flow cytometry detection of cell cycle distribution revealed that compared with the si-NC group, the si-NLRP3 group had blocked cells in the G0/M phase and significantly reduced cells in the S phase ($P < 0.05$, Figure 1D). Western blot detection of Ki67 revealed decreased expressions in si-NLRP3 than those in the si-NC group ($P < 0.05$, Figure 1E); besides, the si-NLRP3 group also had reduced expressions of Caspase-1, Gasdermin D, IL-1 β and IL-18 (all $P < 0.05$, Figure 1E). These results suggest that inhibited expression of NLRP3 inhibits the proliferation and pyroptosis of MDS cells.

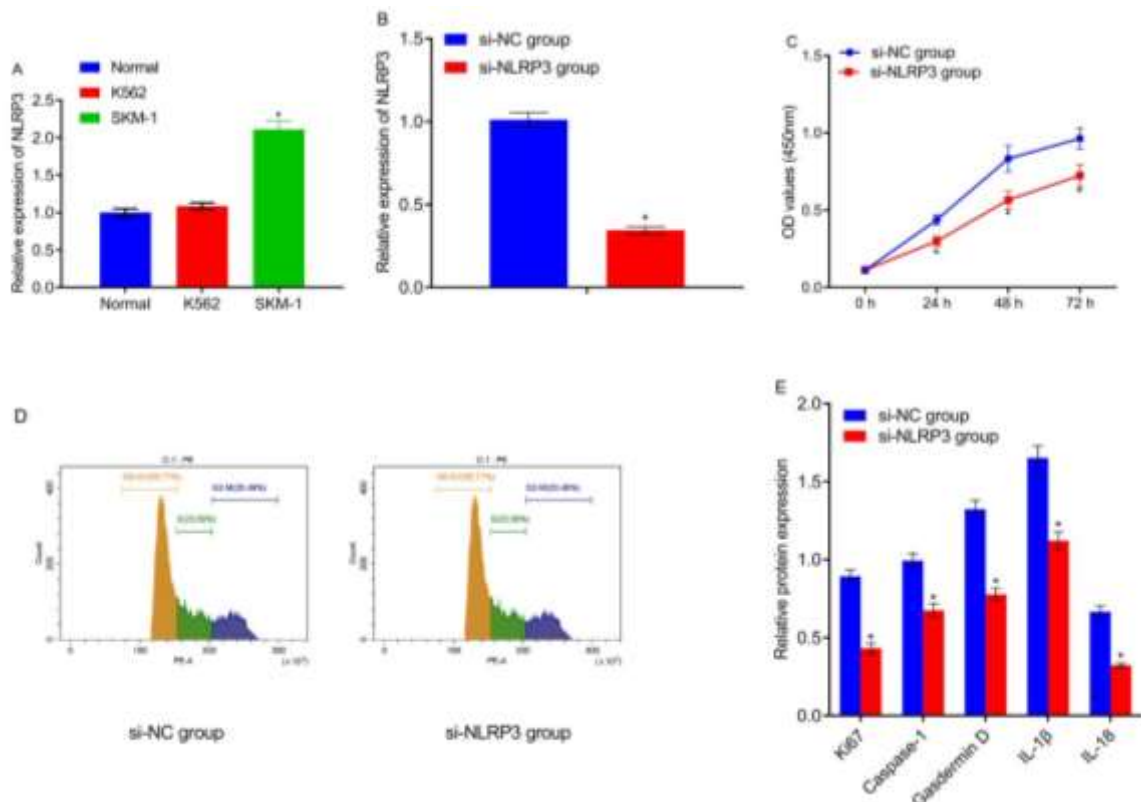


Figure 1. Inhibited expression of NLRP3 inhibited the proliferation and pyroptosis of MDS cells. Note: A: qRT-PCR detection of NLRP3 expression in MDS cell line SKM-1 and normal bone marrow cells (compared with normal bone marrow cells and K562 cells, * $P < 0.05$). B: A: qRT-PCR detection of NLRP3 expression in si-NLRP3 group and si-NC group (compared with the si-NC group, * $P < 0.05$). C: CCK-8 detection of cell proliferation in MDS cells (compared with the si-NC group, * $P < 0.05$). D: Flow cytometry of cell cycle distribution of MDS cells (compared with the si-NC group, * $P < 0.05$). E: Western blot detection of Ki67, Caspase-1, Gasdermin D, IL-1 β and IL-18 in MDS cells (compared with the si-NC group, * $P < 0.05$).

Inhibited expression of NLRP3 inhibited the invasion and migration of MDS cells

Further detection was performed to examine the effect of inhibited expression of NLRP3 on the invasion and migration of MDS cells. Transwell assay revealed that compared with the si-NC group, the si-NLRP3 group showed significantly inhibited SKM-1 cell invasion and migration (both $P < 0.05$, Figure 2AB). Consistent with the above, the expression of MMP-9, an invasion-related protein, decreased significantly in si-NLRP3 group than that in si-NC group ($P < 0.05$, Figure 2C). These results suggest that inhibited expression of NLRP3 inhibits the invasion and migration of MDS cells.

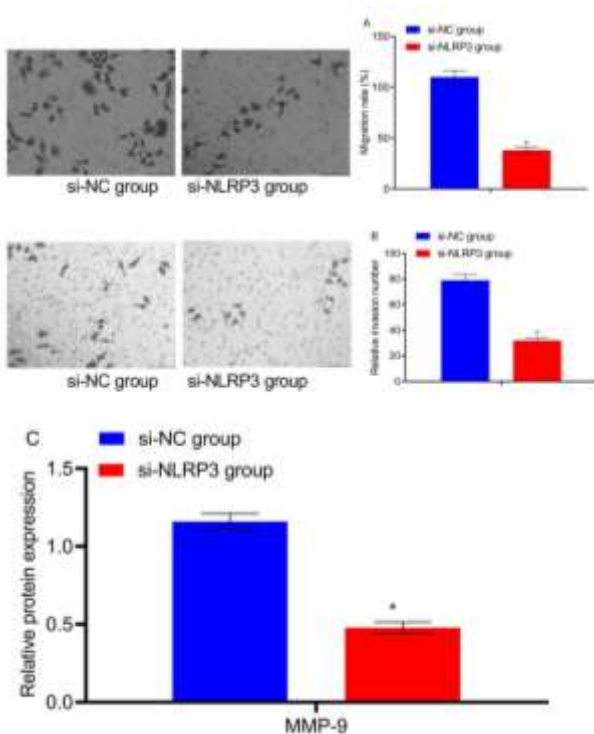


Figure 2. Inhibited expression of NLRP3 inhibited the invasion and migration of MDS cells. Note: A: Transwell assay of SKM-1 cell migration (200X). B: Matrigel invasion assay of SKM-1 cell invasion (200X). C: Western blot detection of MMP-9 in MDS cells (compared with si-NC group, $*P < 0.05$).

miR-223-3p could inhibit NLRP3 targetedly

Our experiment used the most commonly used target gene website Targetscan (<http://www.targetscan.org/>) to predict the target gene of miR-223-3p. The results showed that NLRP3 was one of the targets of miR-223-3p, and there was a potential binding site between miR-223-3p and NLRP3 3'-UTR (Figure 3A). The dual-luciferase

reporter assay was further used to determine whether NLRP3 was the direct target of miR-223-3p. As shown in Figure 3B, overexpression of miR-223-3p resulted in significantly decreased luciferase activity in miR-223-3p mimic+NLRP3 3'UTR-WT when compared with the mimic-NC+NLRP3 3'UTR-WT group ($P < 0.05$). However, there was no apparent change in the luciferase activity after the transfection of the NLRP3 3'UTR-MUT vector ($P > 0.05$). The above results show that NLRP3 was the direct target of miR-223-3p.

qRT-PCR was performed to detect the expression of miR-223-3p in MDS cell line SKM-1, normal bone marrow cells and K562 cells. Compared with normal bone marrow cells and K562 cells, MDS cell line SKM-1 showed significantly downregulated expression of miR-223-3p (both $P < 0.05$, Figure 3C). After that, our experiment overexpressed miR-223-3p in SKM-1 cells. It was found that compared with the mimic-NC group, the miR-223-3p mimic group had evidently upregulated expression of miR-223-3p ($P < 0.05$, Figure 3D), downregulated expression of NLRP3 ($P < 0.05$); as well as obviously reduced expressions of Ki67, Caspase-1, Gasdermin D, IL-1 β , IL-18 and MMP-9 (all $P < 0.05$, Figure 3E-H). Moreover, compared with the miR-223-3p mimic group, the miR-223-3p mimic+oe-NLRP3 group showed obviously increased NLRP3 expression; as well as notably increased expressions of Ki67, Caspase-1, Gasdermin D, IL-1 β , IL-18 and MMP-9 (all $P < 0.05$, Figure 3EF). In addition, when compared with the mimic-NC group, the miR-223-3p mimic group had remarkably inhibited cell proliferation, migration, invasion, blocked cell distribution in the G0/M phase and decreased cell distribution in the S phase (all $P < 0.05$). While the miR-223-3p mimic+oe-NLRP3 group showed reversed trends when compared with the miR-223-3p mimic group (all $P < 0.05$, Figure 3G-J).

miR-223-3p/NLRP3 signaling axis regulated the progression of MDS

In order to further illustrate the regulatory role of miR-223-3p/NLRP3 signaling axis in the progression of MDS, we screened stably transfected SKM-1 cells for in vivo experiments. According to the in vivo test, compared with mimic-NC+ oe-NC group, miR-223-3p mimic+ oe-NC group showed reduced tumor

volume; while compared with miR-223-3p mimic+ oe-NC group, miR-223-3p mimic+ oe-NLRP3 group had increased tumor volume (both $P < 0.05$, Figure 4A). The expression of cell proliferation marker Ki67 was further detected by immunohistochemistry. Compared with mimic-NC+ oe-NC group, miR-223-

3p mimic+ oe-NC group showed significantly decreased Ki67 expression in tumor tissue; while compared with miR-223-3p mimic+ oe-NC group, miR-223-3p mimic+ oe-NLRP3 group had increased Ki67 expression (both $P < 0.05$, Figure 4B).

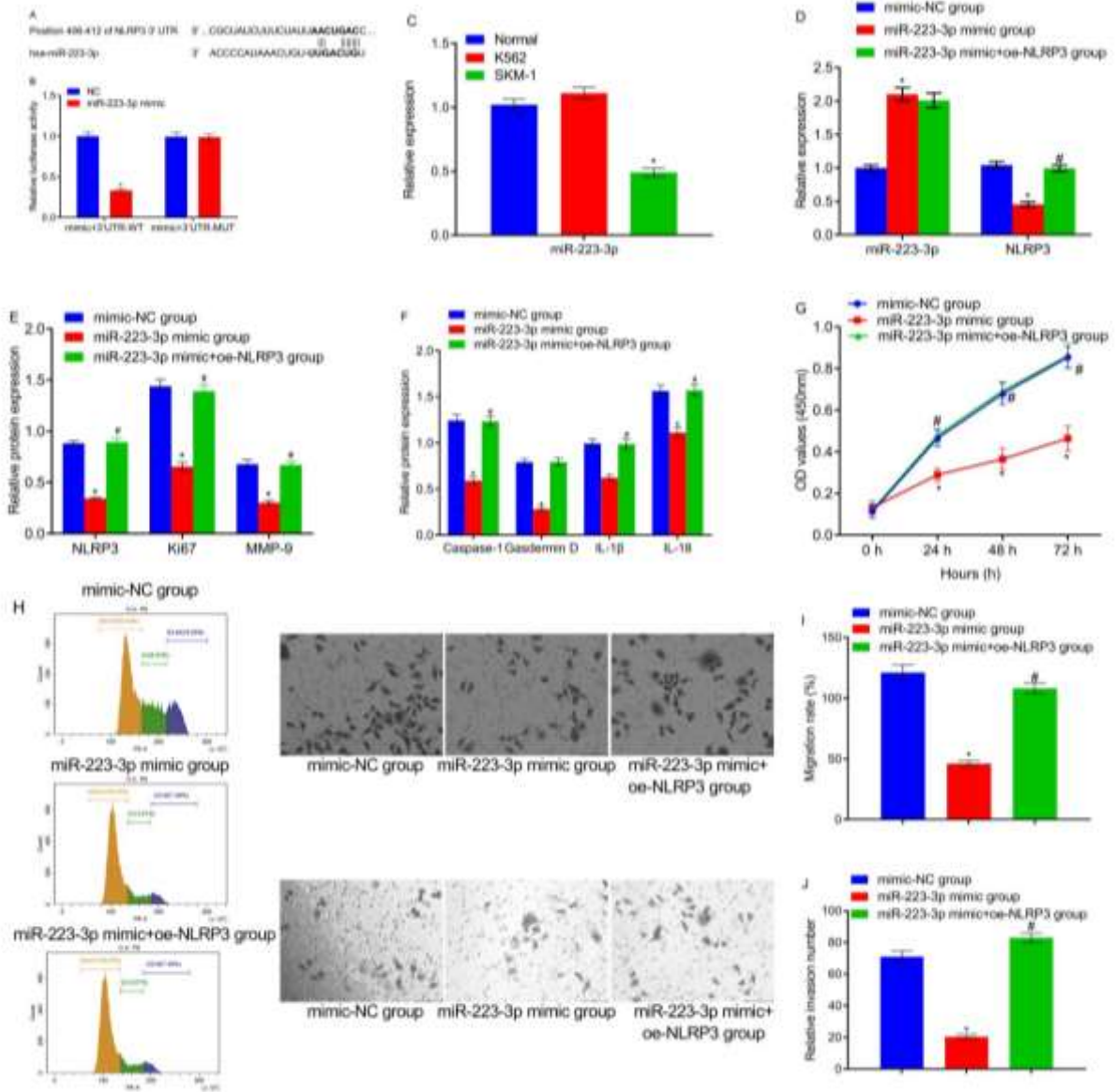


Figure 3. miR-223-3p could inhibit NLRP3 targetedly. Note: A: Prediction of the target relationship between miR-223-3p and NLRP3 by TargetsScan. B: The dual-luciferase reporter assay to determine whether NLRP3 was the direct target of miR-223-3p (compared with the mimic-NC+NLRP3 3'UTR-WT group, $*P < 0.05$). C: qRT-PCR detection of the expression of miR-223-3p in MDS cell line SKM-1, normal bone marrow cells and K562 cells (compared with normal bone marrow cells and K562 cells, $*P < 0.05$). D: qRT-PCR detection of miR-223-3p and NLRP3 expressions in SKM-1 cells after cell transfection (compared with mimic-NC group, $*P < 0.05$; compared with miR-223-3p mimic group, $\#P < 0.05$). E: Western blot detection of NLRP3, Ki67 and MMP-9 in MDS cells (compared with si-NC group, $*P < 0.05$). F: Western blot detection of Caspase-1, Gasdermin D, IL-1 β and IL-18 in MDS cells (compared with si-NC group, $*P < 0.05$). G: CCK-8 detection of SKM-1 cell proliferation (compared with mimic-NC group, $*P < 0.05$; compared with miR-223-3p mimic group, $\#P < 0.05$). H: Flow cytometry of cell cycle distribution of MDS cells (compared with si-NC group, $*P < 0.05$). I: Transwell assay of SKM-1 cell migration (200X, compared with mimic-NC group, $*P < 0.05$; compared with miR-223-3p mimic group, $\#P < 0.05$). J: Matrigel invasion assay of SKM-1 cell invasion (200X, compared with mimic-NC group, $*P < 0.05$; compared with miR-223-3p mimic group, $\#P < 0.05$).

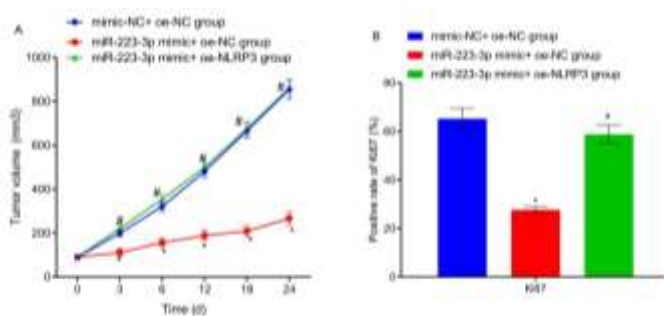


Figure 4. miR-223-3p/NLRP3 signaling axis regulated the progression of MDS. Note: A: Tumor volume of mice in each group. B: The expression of cell proliferation marker Ki67 by immunohistochemistry. Compared with mimic-NC+ oe-NC group, * $P < 0.05$; compared with miR-223-3p mimic+ oe-NC group, # $P < 0.05$.

MDS is a group of hematological malignancies originating from HSCs (1). MDS patients often show anemia and/or other hemocytopenia, and some patients will develop acute myeloid leukemia (3,36). Known risk factors for MDS include aging, chemotherapy or radiotherapy, occupational exposure and some genetic syndromes such as Fanconi anemia (2). Various risk factors lead to gene mutation, epigenetic abnormalities, changes in the hematopoietic microenvironment and immunity (37), all of which may lead to defects of hematopoietic stem cells and further promote the occurrence of MDS. However, the mechanism of the occurrence and development of MDS has not been fully clarified, not to mention the current diagnosis and treatment methods for MDS.

Cell pyroptosis is a kind of programmed cell death phenomenon found in the pathological process of various diseases in recent years (5). At present, the known classical cell pyroptosis pathway involves the synthesis and activation of a variety of key proteins, in which Caspase-1 plays a major mediating role (6). Caspase-1 generally exists in the cytoplasm of cells in the form of zymogen before activation, and activated Caspase-1 can promote the maturation of IL-1 β precursor and IL-18 precursor, enabling them to be activated and secreted extracellularly (38). At the same time, Caspases-1 can shear the common substrate of inflammatory Caspases, Gasdermin D (GSDMD), release the active N-terminal, and induce cell membrane perforation, which is the key to cell osmotic dead and mature IL-1 β and IL-18 secretion outside the cell (39). The activation of Caspase-1 plays a decisive role in the initiation of programmed

pyroptosis (8,9). Moreover, NLRP3 inflammasomes belong to the Nod-like receptor protein (NLR) family (13,39). Under the action of stimulation signals, some NLRs can form macromolecular complexes, i.e., inflammasomes. The activation of inflammasomes may result in the shearing of pro-Caspase-1 to form mature caspase-1, and then the inactive IL-1 and IL-18 precursors are cleaved into active IL-1 β and IL-18 and mediated their secretion, resulting in related inflammatory reactions (40). In eukaryotic genomes, the RNA sequences that can encode proteins are less than 2%, and those in most of the remaining genomes can not encode proteins, which are called non-coding RNA. miRNA is the most widely studied non-coding RNA, which is closely related to the pathogenesis of a variety of diseases (26-28,41). Significantly, it has been commonly revealed that noncoding RNA plays an important regulatory role in various diseases (including MDS) involving NLRP3 inflammasome (42,43).

Each miRNA can regulate hundreds of target genes encoding proteins, so as to participate in the biological processes of cell proliferation, differentiation and apoptosis. In addition, miRNA can regulate the differentiation and development of hematopoietic stem cells (44,45). The current evidence shows that the expression level of miR-223-3p is significantly downregulated in multiple malignant tumors, and plays an inhibitory role by inhibiting cell proliferation, invasion and migration or promoting apoptosis (46). Moreover, miR-223-3p has been reported in MDS (47), which was hence selected as the object of study.

Many target gene prediction software predict the target genes of miRNA based on the interaction principle of miRNA and mRNA 3'-UTR. In this study, we used the commonly used target gene prediction software Targetscan to predict the target gene of miR-223-3p. The results of dual-luciferase reporter assay confirmed that miR-223-3p had a direct effect on the 3'-UTR of NLRP3. Meanwhile, the expression of NLRP3 decreased after the overexpression of miR-223-3p, which further proved that NLRP3 was a target of miR-223-3p. At the same time, our study identified the role of the inhibited expression of NLRP3 and overexpression of miR-223-3p on the proliferation and pyroptosis of MDS cells. It was found that compared with normal bone marrow cells, there was a significantly upregulated expression of NLRP3 and

downregulated miR-223-3p expression in SKM-1 cells. These results support a low expression of miR-223-3p and a high expression of NLRP3 in MDS, proposing a hypothesis that inhibited expression of NLRP3 and overexpression of miR-223-3p might exert beneficial effects on the development of MDS, and there might exist an association between miR-223-3p and NLRP3 based on the above target relationship prediction.

Furthermore, in order to further study the role of NLRP3 and miR-223-3p in the development of MDS, the cells were provided with NLRP3 knockdown and miR-223-3p overexpression treatment. Corresponding results showed that silencing the expression of NLRP3 and overexpressing miR-223-3p could inhibit cell proliferation, pyroptosis, migration and invasion, with downregulated expressions of Ki67, Caspase-1, Gasdermin D, IL-1 β , IL-18 and MMP-9. More importantly, since miR-223-3p could inhibit NLRP3 targetedly. Our experiment used the most commonly used target gene website Targetscan (<http://www.targetscan.org/>) to predict the target gene of miR-223-3p. The results showed that NLRP3 was one of the targets of miR-223-3p, and there was a potential binding site between miR-223-3p and NLRP3 3'-UTR. The dual luciferase reporter assay was further used to determine whether NLRP3 was the direct target of miR-223-3p. As shown in Figure 3B, overexpression of miR-223-3p resulted in significantly decreased luciferase activity in miR-223-3p mimic+NLRP3 3'UTR-WT when compared with mimic-NC+NLRP3 3'UTR-WT group. But there was no apparent change in the luciferase activity after the transfection of NLRP3 3'UTR-MUT vector. The above results show that NLRP3 was the direct target of miR-223-3p, a simultaneous treatment of silencing the expression of NLRP3 and overexpressing miR-223-3p was performed to confirm the mechanism of overexpressing miR-223-3p via silencing NLRP3 expression in MDS. It was found that simultaneous treatment resulted in obviously increased NLRP3 expression, increased expressions of Ki67, Caspase-1, Gasdermin D, IL-1 β , IL-18 and MMP-9, remarkably promoted cell proliferation, migration, invasion, and increased cell count in S phase. These results support that miR-223-3p/NLRP3 signaling axis regulated the progression of MDS. Subsequently, we conducted an in vivo animal test to verify this finding.

Overexpressing miR-223-3p and NLRP3 resulted in enlarged tumor volume and increased expression of Ki67 as compared with miR-223-3p overexpression alone, which in turn support the role of miR-223-3p overexpression suppressing NLRP3 expression in alleviating the progression of MDS.

To sum up, our study suggests that overexpression of miR-223-3p can effectively inhibit the expression of NLRP3 and cell pyroptosis in MDS to suppress the development of MDS. Findings in our study provide evidence for molecular therapy of MDS on the basis of the role of miR-223-3p/NLRP3 signaling axis. Anyway, the complex regulatory network between miRNA and its target genes in MDS needs to be further explored by supplementing in vivo and in vitro experiments comprehensively. It is hoped that miRNA-oriented targeted therapy may become a reality in MDS in the near future.

Acknowledgments

None.

Conflict interest

None.

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