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Original Article

miR-451a targeting IL-6R activates JAK2/STAT3 pathway, thus regulates proliferation and apoptosis of multiple myeloma cells

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Abstract

Objectives: To investigate the effects of miR-451a targeting interleukin-6 (IL-6) on the proliferation and apoptosis of multiple myeloma (MM) cells and its potential mechanism via JAK2/STAT3 pathway. **Methods**: mRNA expression of miR-451a and IL-6R in the plasma of patients with MM and normal controls were determined by RT-qPCR. U266 cells were cultured, transfected with miR-451a mimics, the proliferative ability of U266 cells was determined by CCK-8. Potential targets of miR-451a were predicted with the biological software TargetScan, and the direct relationship between miR-451a and the target IL-6R was analyzed by a dual-luciferase reporter assay. U266 cells were stimulated with IL-6R (100 ng/ml), and the proliferative ability and apoptosis rate were determined by CCK-8 and flow cytometry after 48h. **Results**: In the plasma of patients with MM, miR-451a expression was low and IL-6R expression was high. miR-451a targeted and negatively regulated IL-6R. Overexpressing miR-451a inhibited the proliferation and promoted the apoptosis of U266 cells. IL-6R acting on U266 cells promoted the proliferation and inhibited the apoptosis of U266 cells. Overexpressing miR-451a inhibited the activation of JAK2/STAT3 pathway and down-regulating miR-451a promoted the activation of JAK2/STAT3 pathway. **Conclusions**: miR-451a targeting IL-6R activates JAK2/STAT3 pathway, thus regulates the proliferation and apoptosis in MM cells.

Keywords: Apoptosis, IL-6R, miR-451a, Multiple Myeloma, Proliferation

Introduction

Multiple myeloma (MM) is the second most common hematological malignancy, accounting for 1% of all cases of cancers[1]. Although, MM has been widely studied, it is still an incurable disease[2]. Patients with MM may develop drug resistance, resulting in relapse and progression. Therefore, it is crucial to investigate the pathogenic mechanisms underlying the development and progression of MM, particularly in proliferation and apoptosis, to identify new therapeutic strategies.

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules encoded by endogenous genes with the length about 22 nucleotides, which can be bound to the 3′-untranslated region (3′-UTRs) of the specific target mRNA, thereby inhibiting post-transcriptional expression of the target gene[3,4]. Several studies have demonstrated that microRNAs are involved in the development of various diseases[5]. As reported in the literature, miR-152 is involved in the development of colorectal cancer by targeting PIK3R3[6]. Alternatively, miR-185-5p targeting Bcl2 inhibited the proliferation of breast cancer cells[7]. All of the above studies show that microRNAs are closely related to the development of various cancers. miR-451a act as a tumor suppressor and it has been associated with a variety of cancers, including lung cancer, glioma, and osteosarcoma, and inhibits tumor proliferation, migration, and invasion[8]. For example, Dong Hui et al. found that overexpressing miR-451a can inhibit the proliferation of HepG2 cells by targeting migration repressors

The authors have no conflict of interest.

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of macrophages[9]. In the research by Streleckiene et al., it was found that miR-451a is involved in gastric cancer through PI3K/AKT/mTOR pathway[10]. However, the association of miR-451a with MM remains unclear.

Interleukin-6 (IL-6) is a key growth factor that exerts its bioactive link by binding to its receptor (IL-6R). It was found that IL-6 is closely associated with the occurrence and development of various diseases[11,12]. IL-6 promotes the metastasis in non-small cell lung cancer (NSCLC) through NF-κB[13]. Additionally, it has been reported that the proliferation of MM cells is mediated by autocrine or paracrine mechanism during the release[14]. miR-451a can target IL-6R, to inhibit the proliferation, migration, and angiogenesis of tumors[15]. However, it is unclear whether miR-451a inhibits tumor growth in MM and targets IL-6R. Furthermore, the clinical significance of miR-451a in MM has not yet been determined. It has been found that the network structure composed of molecules of non-coding protein miRNAs is involved in regulating the growth and survival of myeloma cells. We further explored the significance of miR-451a in the development of MM and its mechanism, to lay a theoretical foundation for revealing the prognostic indicators of multiple myeloma and further studying the role of miRNA in the pathogenesis of myeloma.

Experimental methods

Sample source

A total of 58 patients with MM (35 males and 23 females aged 37-88 years old) and 15 healthy controls (7 males and 8 females aged 32-85 years old) were collected in this study from October 2020 to April 2021. The inclusion criteria were: 1) Patients who were clinically diagnosed with MM; 2) Patients without the treatment of surgery, radiotherapy and chemotherapy; Exclusion criteria were: 1) Patients with acute or chronic inflammatory infections; 2) patients with non-MM-related renal injury factors such as severe infection, dehydration, heart failure, and use of nephrotoxic drugs; 3) patients with primary kidney disease and secondary kidney disease. 2 mL of fasting venous blood were extracted from patients and healthy controls in the early morning, which was placed in an anticoagulant tube and centrifuged at 3000 r/min for 10min to isolate plasma. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical College (Qiqihar Ethics Approval No. [2021] 66). This study was conducted in accordance with the principles of the Declaration of Helsinki. Signed written informed consents were obtained from each patient and/or their guardians.

Cell culture

U266 cells in good condition and log growth period from Shanghai Cell Bank of Chinese Academy of Sciences were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were observed under the microscope for their status and density every day, replaced with fresh media in time, added trypsin digestion and subcultured and diluted in the culture plate for subsequent experiments.

Cells transfection

miR-451a mimics and miR-451a inhibitors were synthesized by Shanghai GenePharma Company. When the density reached 70%, the cultured U266 cells were transfected with Lipofectamine 3000 according to the instructions. miR-451a expression was measured by RT-qPCR to confirm the successful transfection. U266 cells in the log growth period were taken and inoculated in the 6-well plate. After 12h of starvation culture, the cells were stimulated with IL-6R at 100 ng/ml and cells were collected after 48h.

ELISA

2 mL of fasting venous blood extracted from patients and healthy controls in the morning was placed in the anticoagulant tube, which was centrifuged at 3000 r/min for 10 min, the supernatant was placed in a new EP tube, and the IL-6R content in the serum was analyzed by the ELISA kit. The collected cell samples were analyzed after centrifugation at 1000 r/min for 10 min for the apoptosis rate according to the apoptosis rate detection kit of ELISA cells.

Dual-luciferase reporter gene detection for the activity

The online software TargetScan (TargetScan Human 7.0, https://www.targetscan.org/vert_70/) was adopted to determine the relationship between miR-451a and IL-6R. We amplified a miR-451a wild sequence vector containing IL-6R 3'UTR and inserted it into the luciferase reporter vector pmiR-RB-REPORT to form a mutant (Mut) vector. U266 cells were transfected with a luciferase reporter vector (30 ng). Renilla luciferase activity levels were continuously measured after 24 h of transfection according to the manufacturer's protocol.

Cell invasion assay

The upper chamber of Transwell was coated with matrix, and 1×10^5 digested U266 cells were inoculated into the upper chamber. The lower chamber was supplemented with 500 μL of medium. After standing for 24h, 4% paraformaldehyde was added for fixation, and 0.5% crystal violet staining was performed to detect the number of cells invaded.

CCK-8 for cell proliferative ability

Cell viability was detected with the CCK-8 kit according to the instructions. Cell proliferation was assessed with BrdU staining at room temperature for approximately 2.5 h. OD was assessed at a 370 nm wavelength with a microplate reader.

RT-qPCR

Total RNA was extracted with the TRIzol reagent according to the manufacturer's protocol, which was

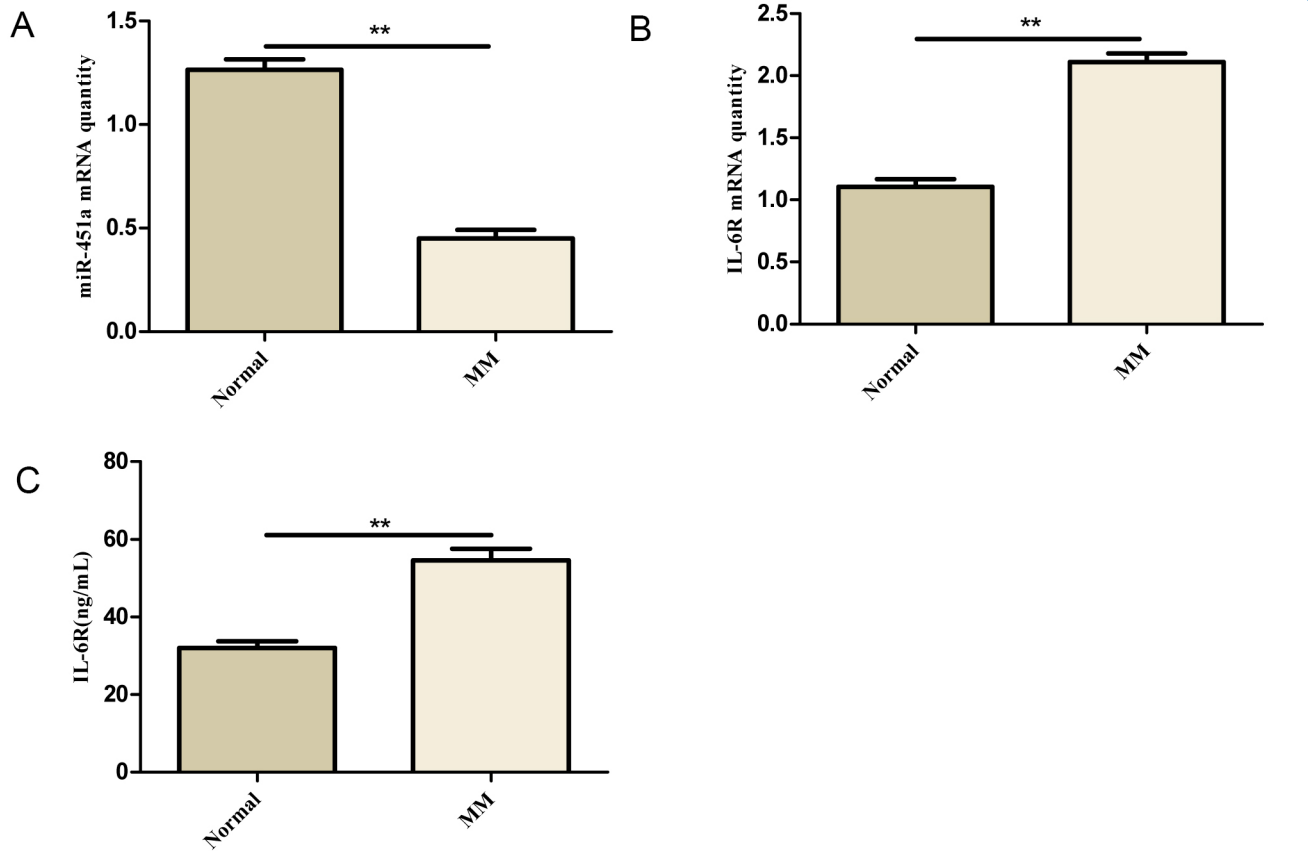

Figure 1. miR-451a expression was low and IL-6R expression was high in the plasma of patients with MM. A): RT-qPCR analysis for mRNA expression of miR-451a in the plasma patients with MM and healthy controls. B): RT-qPCR analysis for mRNA expression of IL-6R in the plasma of patients with MM and healthy controls. C): ELISA analysis for IL-6R content in the serum of patients with MM and healthy controls.

reverse transcribed with a reverse transcription kit. Relative quantitative analysis was performed with the SYBR Green PCR kit. Reaction conditions: the initial denaturation at 95°C for 10 min, followed by 95°C for 2s, 60°C for 20s and 70°C for 10s, for 40 cycles in total. The primer sequences used in the experiment were: miR–451a: forward: 5′-ACCGTTACCATTACT-3'; reverse: 5′-CTCACACGACTCACG-3'. IL-6R: forward: 5′-CCTCTGCATTCATTGTTC-3'; reverse: 5′-GAGATGAGAGGAACAAGCAC-3'. p-JAK2: forward: 5′-TTTGGATCCCTGGATACATACCTGA-3'; reverse: 5′-TGGCACACACATTCCCATGA-3'. p-STAT3: forward: 5′-TGGGTCTGGCTAGACAAT-3'; reverse: 5′-CGTTGGTGTCACACAGAT-3'. Data analysis was performed with $2^{-\Delta\Delta Ct}$, with GAPDH expression as an internal control. Each sample was run in triplicate.

Western blot

To assess the changes in cellular protein levels, transfected U266 cells were collected after 48h, washed with PBS, resuspended with 100 µl lysis buffer, and placed on ice for 2 h, centrifuged for 20 min. And then, the supernatant was stored at -80°C. The proteins (20 µg) were subjected to 10% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk powder in Tris buffered saline containing 0.05% Tween 20 and incubated overnight with primary antibodies (p-JAK2, JAK2, p-STAT3 and STAT3) at 4 °C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for protein exposure with an enhanced chemiluminescence reagent.

Statistical analysis

Statistical analysis was performed with Graphpad prism 5.0 software. Data were expressed as mean ± standard deviation. One-way ANOVA was conducted for comparisons between groups, the LSD-t test for pairwise comparisons. $P<0.05$ indicates statistical significance.

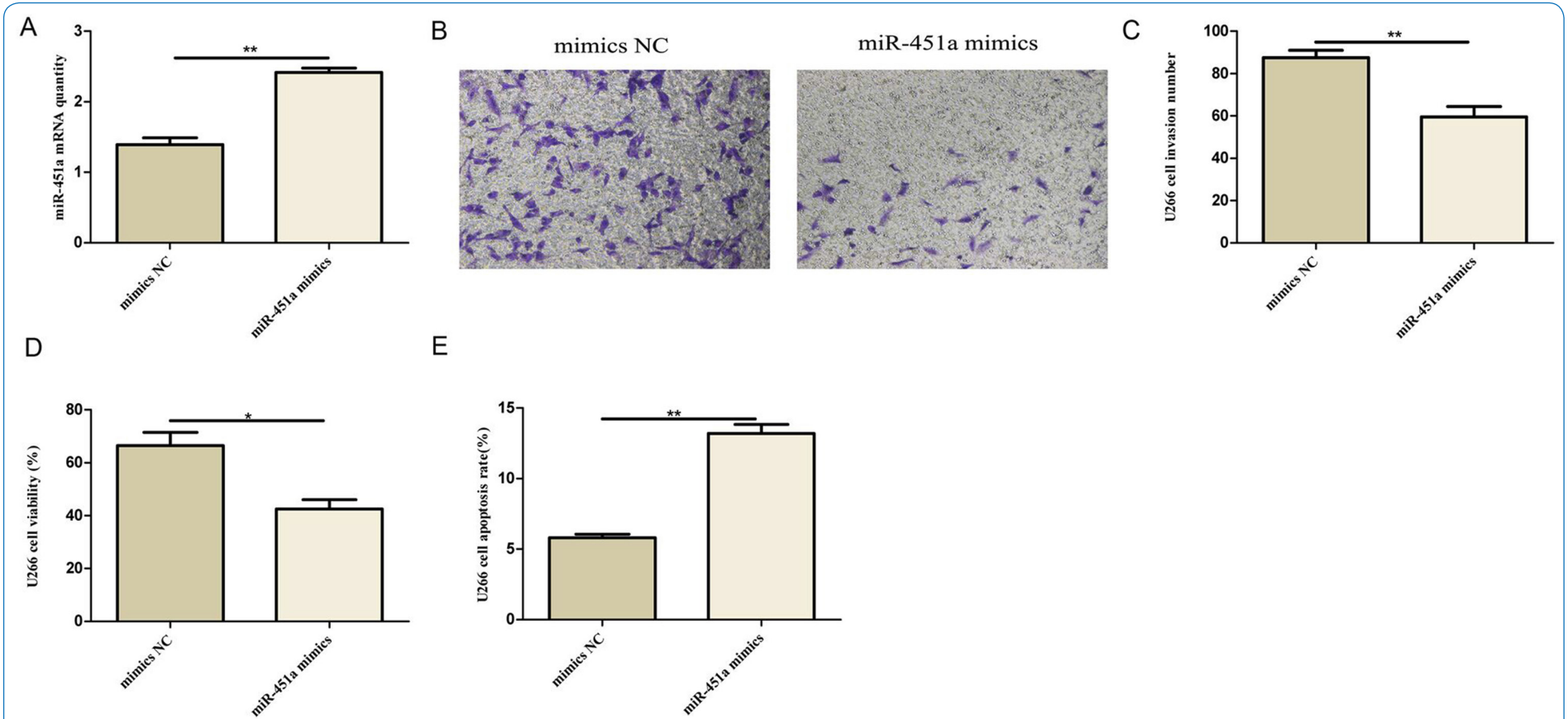

Figure 2. Overexpressing miR-451a inhibited the proliferation and promoted the apoptosis of U266 cells. U266 cells were transfected with miR-451a mimics and mimics control groups, miR-451a expression was further detected by RT-qPCR (A). After 48h, the invasion of cells was detected with Transwell (B-C). The proliferation vitality of cells was detected with MTT (D). The apoptosis rate of the cells was determined by ELISA (E).

Results

miR-451a expression was low and IL-6R expression was high in the plasma of patients with MM

To investigate the association of miR-451a and IL-6R expression with patients with MM, we examined the mRNA expression of miR-451a and IL-6R in the plasma of patients with MM and healthy controls with RT-qPCR. The results showed that compared with those of healthy controls, mRNA expression of miR-415a significantly decreased in the plasma in patients with MM ($P<0.01$) (Figure 1A), and mRNA expression of IL-6R significantly increased in the plasma in patients with MM ($P<0.01$) (Figure 1B). IL-6R content in the serum significantly increased in patients with MM ($P<0.01$) (Figure 1C). These results suggested that miR-451a expression is low in the plasma of patients

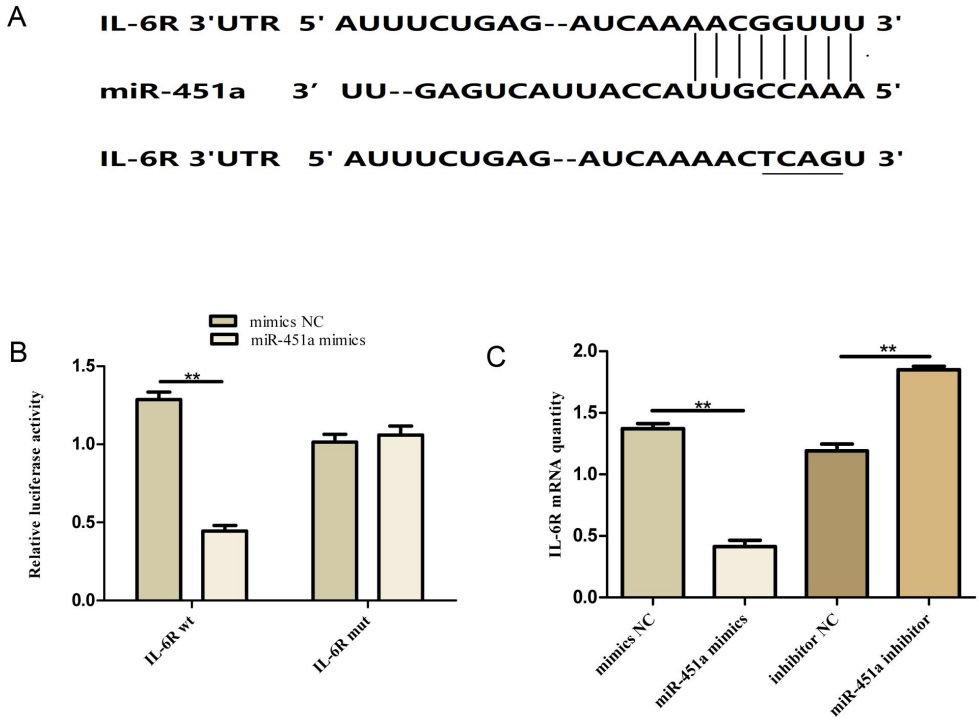

Figure 3. IL-6R may be a direct target for miR-451a. A): Prediction of targets for miR-451a with biological software TargetScan. B): Detection of cell doual-luciferase activity. C): RT-qPCR detection for IL-6R expression.

with MM and IL-6R expression is high in the plasma of patients with MM.

Overexpressing miR-451a inhibited the proliferation and promoted the apoptosis of U266 cells

To investigate the functions of miR-451a in U266 cells, miR-451a mimics and mimics Negative Control (NC) groups were transfected into U266 cells, and miR-451a expression was further examined by RT-qPCR. (Figure 2A) The results showed that miR-451a expression in U266 cells was significantly higher in the miR-451a mimics group compared with that of the mimics NC group (P<0.01). The results of Transwell analysis showed that miR-451a overexpression significantly inhibited the invasion of U266 cells (Figure 2B-2C) (P<0.01). Cell proliferation analysis revealed that miR-451a overexpression significantly inhibited the proliferation of U266 cells (Figure 2D) (P<0.05). The results of ELISA showed that miR-451a overexpression significantly promoted the apoptosis in U266 cells (Figure 2E) (P<0.01). These *in vitro* data suggest that overexpressing miR-451a can inhibit the proliferation and promote the apoptosis of U266 cells.

IL-6R may be a direct target for miR-451a

It was predicted with the biological software TargetScan that the 3′UTR of mRNA in IL-6R contained sequences complementary to the miR-451a seed sequence (Figure 3 A). To verify whether IL-6R is a direct target for miR-451a, we performed a dual-luciferase activity assay. The result showed that cell luciferase activity in the IL-6R wt+miR-451a mimics group was significantly lower than that in the IL-6R wt+mimics NC group (P<0.01). No significant difference in the luciferase activity was observed in the cells in the IL-6R mut+miR-451a mimics group compared with that in the IL-6R wt+mimics NC group (P>0.05) (Figure 3B). Then, we explored the effects of miR-451a overexpression or down-regulation on IL-6R expression and found that IL-6R expression in the miR-451a mimics group was significantly lower than that in the mimics NC group (P<0.01), and IL-6R expression in the miR-451a inhibitor group was significantly higher than that in the inhibitor NC group (P<0.01, Figure 3C). Taken together, these data suggest that IL-6R may be a direct target for miR-451a, which negatively regulates IL-6R expression.

IL-6R acts U266 cells to promote proliferation of U266 cells and inhibit apoptosis of U266 cells

To investigate the functions of IL-6R in U266 cells, U266 cells were stimulated with IL-6R at 100 ng/ml, and IL-6R expression was further examined with RT-qPCR (Figure 4A). The results showed that IL-6R expression in U266 cells in the IL-6R group was significantly higher

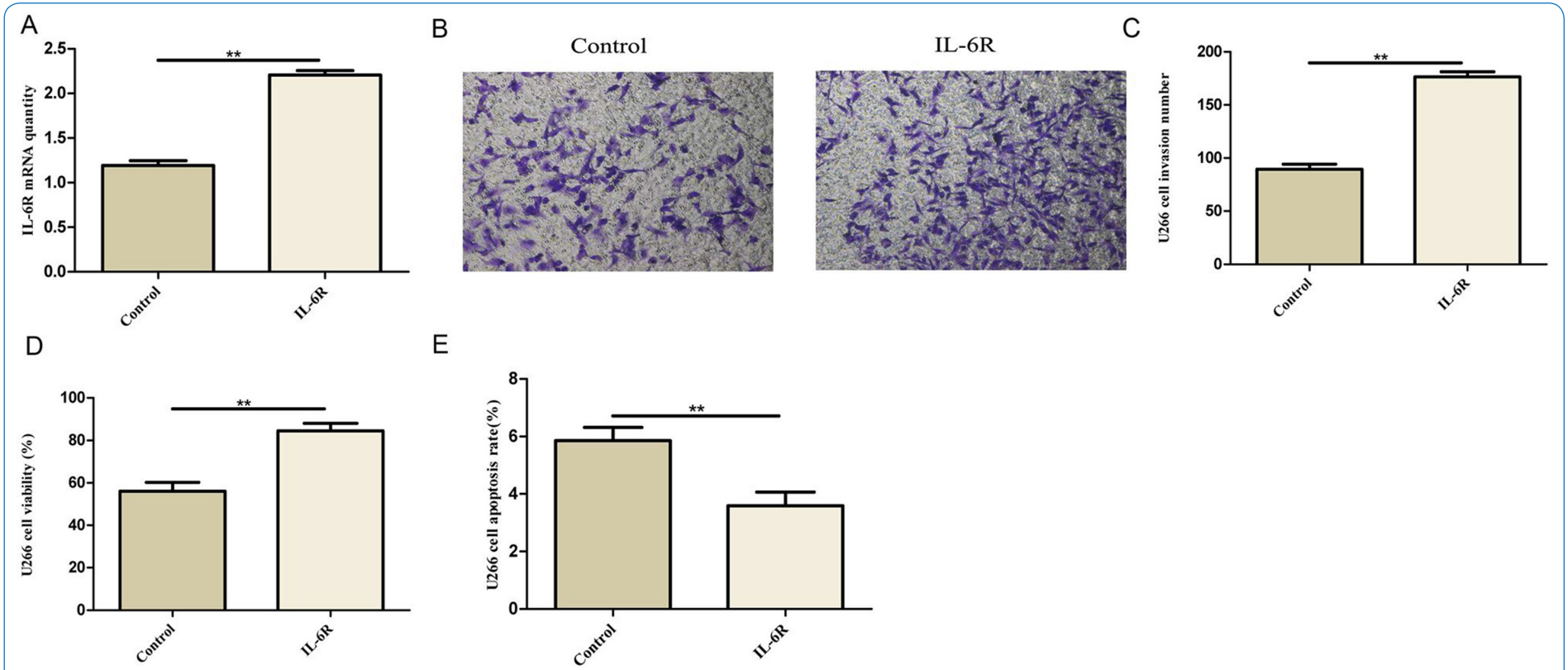

Figure 4. IL-6R acting U266 cells promoted the proliferation and inhibited the apoptosis of U266 cells. After U266 cells were stimulated with IL-6R at 100 ng/mL, IL-6R expression was further detected by RT-qPCR (A). After 48h, the invasion of cells was detected with Transwell (B-C). The proliferation vitality of cells was detected with MTT (D). The apoptosis rate of the cells was determined by ELISA (E).

than that in the control group (P<0.01). The results of Transwell analysis showed that IL-6R acting on U266 cells promoted the invasion of U266 cells (Figure 4B-4C) (P<0.01). Cell proliferation analysis showed that IL-6R acting on U266 cells promoted the proliferation of U266 cells (Figure 4D) (P<0.01). The results of ELISA showed that apoptosis was significantly inhibited after IL-6R acting on U266 cells (Figure 4E) (P<0.01). These *in vitro* data suggested that IL-6R can promote the proliferation and inhibit the apoptosis of U266 cells.

Overexpressing miR-451a inhibited the activation of JAK2/STAT3 pathway and down-regulating miR-451a promoted the activation of JAK2/STAT3 pathway

To investigate the effects of miR-451a on JAK2/STAT3 pathway in U266 cells, miR-451a mimics, mimics NC, miR-451a inhibitor, inhibitor NC groups were transfected into U266 cells, and the gene and protein expression of JAK2/STAT3 pathway was further examined by Western blot and RT-qPCR. The results showed that overexpressing miR-451a significantly decreased the ratio of p-JAK2/JAK2 to p-STAT3/STAT3,

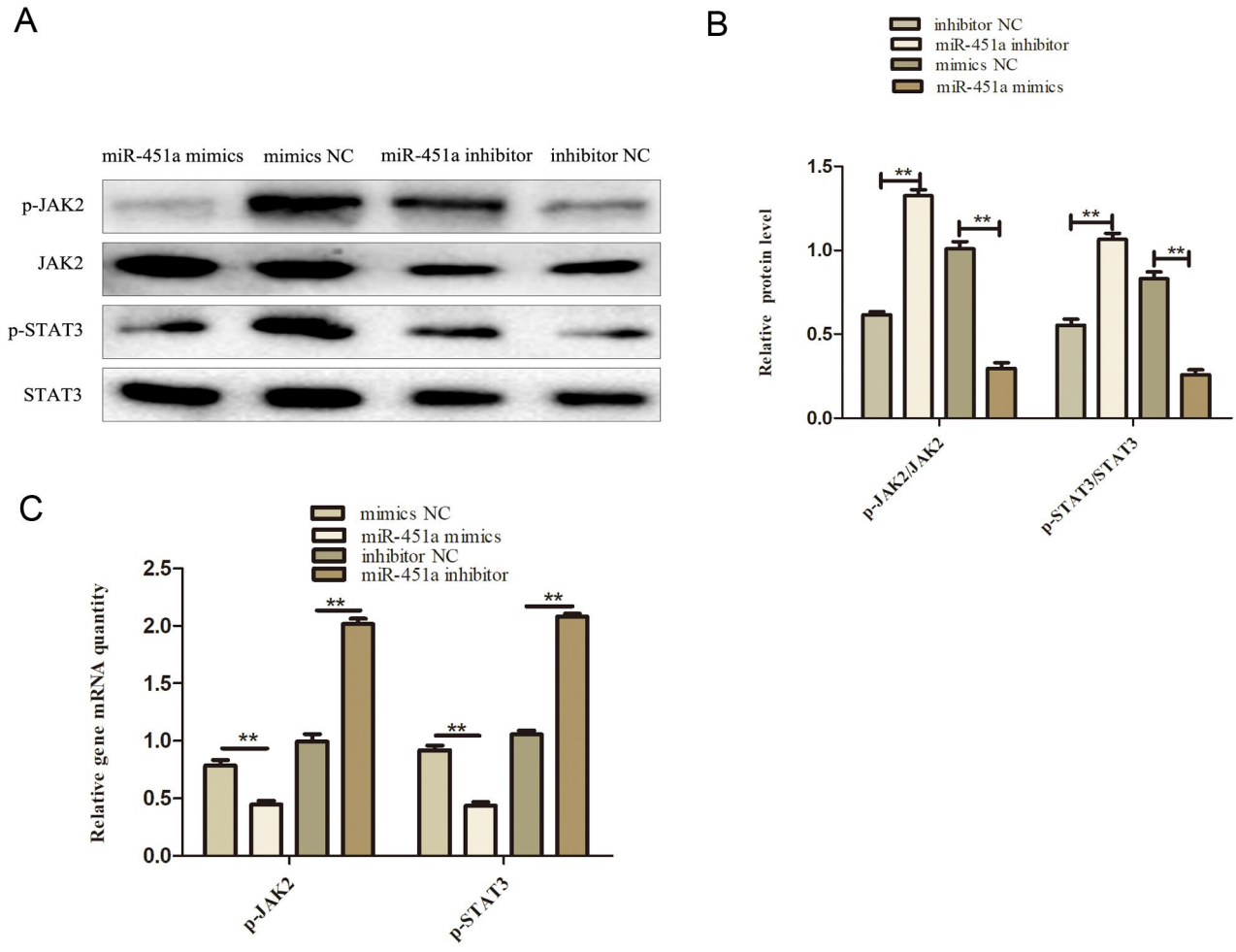

Figure 5. Overexpressing miR-451a inhibited the activation of JAK2/STAT3 pathway and down-regulating miR-451a promoted the activation of JAK2/STAT3 pathway. A-B) Western blot for protein expression of JAK2/STAT3 pathway and RT-qPC R for gene expression of p-JAK2 and p-STAT3.

and inhibiting miR-451a expression significantly increased the ratio of p-JAK2/JAK2 to p-STAT3/STAT3 (Figure 5A) ($P<0.01$). The results showed that miR-451a overexpression significantly decreased gene expression of p-JAK2 and p-STAT3 ($P<0.01$) and inhibition of miR-451a expression significantly increased p-JAK2 and p-STAT3 (Figure 5C) ($P<0.01$). These *in vitro* data suggested that overexpressing miR-451a can inhibit the activation of JAK2/STAT3 pathway and down-regulating miR-451a can promote the activation of JAK2/STAT3 pathway.

Discussion

Multiple myeloma (MM) is the second most common hematologic malignancy, which is usually caused by the proliferation of malignant plasma cells in the bone marrow, and patients show non-specific symptoms, such as fatigue, anemia and bone pain. At the time MM is diagnosed, patients are generally in the advanced stage, which mainly affects the elderly (average 70 years old) and is rarely diagnosed in patients under 40[16,17]. Since MM is a relatively rare disease and its etiology has been difficult to assess, a deeper understanding of its pathogenesis is important. It is shown that IL-6 plays a crucial role in the pathogenesis of MM, which is involved in the development of MM, as reported in the literature that ZFP91 regulates the proliferation of MM cells through IL-1β-mediated IL-6 in the tumor microenvironment[18]. In addition, it was found that long-chain non-coding RNA CRNDE regulates the growth of polymyeloma cells by IL-6 signaling[19]. Several studies have found that IL-6 is essential for the development of multiple myeloma cells, and some factors and mechanisms can increase the levels of IL-6[20]. However, when IL-6R transduce the IL-6 signal, IL-6 must bind to the receptor to play its role. However, the molecular mechanisms regulating IL-6R expression have not been fully

elucidated. In this study, we highlighted that miR-451a, as an important anti-tumor factor in MM, was shown to target IL-6R to participate in the development of MM.

A growing number of studies have found that miRNA can take part in the biological processes of various diseases by targeting mRNA. Nie Y L et al. found that miRNA-191 promotes the proliferation, migration and invasion of prostate cancer by targeting PLCD1[21], and microRNA3 inhibits the proliferation, migration and invasion of hepatocellular cancer cells by targeting PHF8. In addition, they found that microRNA-518-3p inhibits the proliferation invasion and migration of colorectal cancer cells by targeting TRIP4[22]. Studies by Nayan, J et al. showed that hepatitis C virus-induced miRNA-107 and miRNA-449a regulate the changes in CCL2 by targeting the interleukin 6 receptor complex[23]. Yan H et al. found that microRNA-22 regulates thyrocyte growth and lipid accumulation by IL-6R[24]. Furthermore, it has been shown that aberrant expression of miR-451a is inversely associated with tumor malignancy, for example, miR-451a ameliorates alcoholic hepatitis by inhibiting the pro-inflammatory response mediated by HDAC8[25]. Zhao et al. demonstrated that exosome miR-451a plays a role as a tumor inhibitor in HCC by targeting LPIN1[26]. It has also been shown that miR-101-2, miR-125b-2 and miR-451a play potential roles as tumor inhibitors in gastric cancer by regulating PI3K/AKT/mTOR pathway[27]. It has also been reported that miR-451a may be a potential biomarker for the diagnosis of papillary thyroid cancer. miR-451a inhibits the proliferation of breast cancer cells and enhances tamoxifen sensitivity through macrophage migration inhibitors. Recently, miR-451a has been shown to be significantly down-regulated in patients with MM and associated with a poor clinical prognosis, which is consistent with the results of this study. In this experiment, we showed that miR-451a expression was lower in the plasma of patients with MM, and we speculated that abnormal expression of miR-451a may be involved in the development of MM cells and require further investigation.

It has been found that in osteosarcoma, miR-451a inhibits the proliferation, migration and angiogenesis of cancer cells by silencing IL-6R. However, the relationship between miR-451a and IL-6R during the development of MM remains unknown, so we further explored the potential acting downstream sites of miR-451a and found that IL-6R was confirmed to be a target for miR-451a in U266 cells. The direct relationship between miR-451a luciferase and IL-6R was confirmed by the dual-luciferase reporter assay, and the results suggest that the regulatory effects of miR-451a on the proliferation and apoptosis of U266 cells may be achieved by IL-6R. It has been found that IL-6R causes the activation of JAK-STAT signaling to regulate the proliferation and apoptosis in myeloma cells. However, the mechanism by which IL-6R is up-regulated in MM remains unclear. Over these years, more studies have shown that JAK-STAT signaling is involved in the development of diseases, for example, miR-769-5p inhibits the progression of oral squamous cell carcinoma by directly targeting JAK1/STAT3 pathway[28]. It has also been shown that FGFR2 promotes PD-L1 expression in colorectal cancer via JAK/STAT3 signaling pathway[29]. In ovarian cancer, JQ1 combined with cisplatin inhibits tumor growth and inhibits JAK/STAT signaling. Lv J et al. demonstrated that LNK promotes the growth and metastasis in triple-negative breast cancer via the activation of JAK/STAT3 and ERK1/2 pathways. Study by Yao et al. showed that B7-H4 (B7x) -mediated cross-dialogue between glioma-initiating cells and macrophages through IL6/JAK/STAT3 pathway leads to poor prognosis in patients with glioma[30]. Given the critical role of JAK/STAT3 signaling in the development of diseases, we examined the effects of overexpression and down-regulation of miR-451a on JAK/STAT3 signaling-related proteins in the study with Western blot, and confirmed that overexpression or down-regulation of miR-451a altered the expression of p-JAK2 and p-STAT3 (active forms) to induced the apoptosis in myeloma cells, without changes in levels of total JAK2 and STAT3, which could be used for clinical transformation. Thus, miR-451a can be used as a potential biomarker for predicting the prognosis in patients. In addition to signaling through the JAK2/STAT3 pathway, IL-6 can also promote the proliferation of myeloma cells through the Ras/MAPK pathway and reduce the apoptosis in MM cells by regulating PI3K/AKT pathway. In addition, IL-6 can interact with VEGF to promote angiogenesis, migration and invasion, and it can also lead to the activation of NF-κB pathway[31]. Thus, miR-451a may act through other mechanisms and signaling pathways, which may be tumor-type-dependent. However, these hypotheses have to be tested in future studies.

In conclusion, this study demonstrated that miR-451a specifically silenced the IL-6R-induced apoptosis in myeloma cells and promoted the inactivation of JAK2/STAT3 pathway, to play its anti-tumor role. Thus, miR-451a may be an attractive alternative target for mapping the tumor burden in real time, as well as for the treatment of MM through novel strategies.

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Authors' contributions

LX and LJ conceived and designed the study, and drafted the manuscript. LX, YY, TL and LJ collected, analyzed and interpreted the experimental data. YY and LJ revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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