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Expression of miR-29 and STAT3 in osteosarcoma and its effect on proliferation regulation of osteosarcoma cells

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Abstract. – OBJECTIVE: STAT3 has been shown to be involved in the occurrence, progression, and resistance of various tumors. The abnormal expression of miR-29 is associated with the pathogenesis of osteosarcoma. The bioinformatics analysis showed a targeting relationship between miR-29 and STAT3 3'-UTR. This study investigated whether miR-29 regulates the STAT3 expression and affects the proliferation and apoptosis of osteosarcoma cells.

PATIENTS AND METHODS: The tumor tissues of osteosarcoma patients were collected, and the adjacent tissues were used as controls to detect the expression of miR-29 and STAT3. The Dual-Luciferase Gene Reporter Assay validated the regulatory relationship between miR-29 and STAT3. The expression of miR-29 and STAT3 in normal osteoblasts hFOB1.19, osteosarcoma SJSA-1, and MG-63 was measured. SJSA-1 cells were divided into miR-NC group and miR-29 mimic group. Cell apoptosis and proliferation were detected by flow cytometry.

RESULTS: Compared with adjacent tissues, miR-29 expression was decreased, and STAT3 expression was increased in osteosarcoma. There was a targeted regulation relationship between miR-29 and STAT3. Compared with hFOB1.19 cells, miR-29 expression in osteosarcoma SJSA-1 and MG-63 cells was decreased, with increased STAT3 expression. The transfection of miR-29 mimic significantly decreased the expression of STAT3 and p-STAT3 in SJSA-1 cells, inhibited cell proliferation, and increased cell apoptosis.

CONCLUSIONS: Decreased miR-29 expression plays a role in the increase of the STAT3 expression and in the promotion of the pathogenesis of osteosarcoma. Increasing the expression of miR-29 can inhibit the proliferation of osteosarcoma cells and promote apoptosis by decreasing STAT3 expression.

Key Words:
MiR-29, STAT3, Osteosarcoma, Proliferation, Apoptosis.

Introduction

Osteosarcoma is a primary malignant bone tumor originated from mesenchymal tissue. Osteosarcoma is common in children and adolescents, with high morbidity and mortality[1].

STAT is a transcription factor that involves transcriptional regulation in the signal transduction and signaling transducers of the tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT). The expression and activity of STAT3 are closely related to the occurrence, progression, invasion, and metastasis of various tumors such as breast cancer[2], colon cancer[3], and gastric cancer[4]. STAT also plays an important role in the survival and prognosis of patients. A number of studies[5-7] have shown that STAT3 expression and dysfunction are associated with the occurrence, progression, metastasis, and drug resistance of osteosarcoma.

MicroRNA is an endogenous non-coding small-molecule single-stranded RNA of approximately 22-25 nucleotides in length. MiRNA can bind to the 3'-UTR of the target gene mRNA through complementary pairing, leading to degradation or inhibition of translation, thus regulating the expression of the target genes. The role of microRNA in the pathogenesis of tumors has received increasing attention[8]. Some investigations[9-11] have shown that abnormal expression of miR-29 is associated with the occurrence, pro-

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gression, and drug resistance of osteosarcoma. The bioinformatics analysis revealed that there is a targeted complementary binding site between miR-29 and the 3'-UTR of STAT3 mRNA, suggesting a possible targeting relationship between the two. This study investigated whether miR-29 regulates STAT3 expression and its role in osteosarcoma cell proliferation and apoptosis.

Materials and Methods

Main Reagents and Materials

Osteosarcoma cells SJSA-1 and MG-63 cells were purchased from Shanghai Lianmai Bio (Shanghai, China); the human normal osteoblasts hFOB1.19 were purchased from Beijing Beina Bio (Beijing, China); Dulbecco's Modified Eagle's Medium (DMEM), Qing Streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA); Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA); RNA extraction reagent RNA iso plus, PrimeScript™ RT reagent Kit and SYBR Green dye purchased from TaKaRa (Dalian, Liaoning, China); miR-NC, miR-29 mimic, miR-29 inhibitor were purchased from Ruibo Bio (Shengzhen, Guangzhou, China); Rabbit anti-human STAT3 and p-STAT3 antibodies were purchased from Abcam (Cambridge, MA, USA); rabbit anti-human β-actin antibody and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Shanghai Biotechnology (Shanghai, China); the Luciferase gene reporter vector pGL3, double Luciferase activity assay, were purchased from Promega (Madison, WI, USA); the bicinchoninic acid (BCA) protein quantification kit and the apoptosis detection kit were purchased from Biyuntian (Nantong, Jiangsu, China); the radioimmunoprecipitation assay buffer (RIPA) lysate was purchased from Suo Labao Bio (Beijing, China).

Patients

38 patients with osteosarcoma who were treated in The Fifth Hospital of Wuhan (Wuhan, Hubei, China) from May 2018 to November 2018 were enrolled. The osteosarcoma tissues confirmed by pathological examination were collected, and the precancerous tissues which were located at least 5 cm away from the tumor tissues were collected as a control. The collection of tissue specimens has been approved by The Fifth Hospital of Wuhan (Wuhan, Hubei, China) Ethics Committee and the informed consent has been obtained from patients.

Cell Culture

SJSA-1, MG-63, hFOB1.19 cells were cultured in DMEM medium containing 10% FBS and 1% penicillin and streptomycin in a cell culture incubator containing 5% CO_2 at 37°C until the cells reached a confluence of 90%. After that, the cells were digested with 0.125% trypsin and collected by centrifugation. The subcultures at a ratio of 1:5, and the experiments were performed when the cells were in the log growth phase.

Dual-Luciferase Reporter Gene Experiment

mRNA was extracted from HEK293T cells using RNA iso plus. Then, the extracts were used as a template to amplify the fragment containing the targeted binding site in the 3'-UTR region of the STAT3 gene. The positive clones were screened by colony PCR, and the plasmids with correct sequences were picked and named as pGL3-STAT3-WT and pGL3-STAT3-MUT, respectively. pGL3-STAT3-WT (or pGL3-STAT3-MUT), and miR-29 mimic (or miR-NC, miR-29 inhibitor) were co-transfected into HEK293T cells with Lipofectamine 2000, and the relative Luciferase was detected after 48 h of culture.

Cell Transfection and Grouping

SJSA-1 cells were cultured *in vitro* and divided into two transfection groups: miR-NC transfection group and miR-29 mimic transfection group. After transfection for 72 h, the cells were collected for the analysis of relevant parameters.

QRT-PCR Detection of Gene Expression

RNA extracted from RNA iso plus was subjected to qRT-PCR analysis to detect the relative expression of genes using the PrimeScript™ RT reagent Kit. The reverse transcription reaction system was: oligdT Primer (50 μM), 0.5 μL; Random 6 mers (100 μM), 0.5 μL; PrimeScript RT Enzyme Mix, 0.5 μL; RNA, 1.0 μg; 5×PrimeScript Buffer, 2 μL; RNase Free dH2O To 10.0 μL; qPCR reaction system: SYBR Fast qPCR Mix, 10.0 μL; Forward Primer (10 μM), 0.8 μL; Reverse Primer (10 μM), 0.8 μL; cDNA, 2.0 μL; RNase Free dH2O, 6.4 μL. The reaction conditions were designed as follows: pre-denaturation 95°C, for 10 min; denaturation 95°C for 10 s; annealing 60°C for 20 s; extension 72°C for 15 s; 40 cycle,

on Bio-Rad CFX96 Real Time-PCR Detection System. The primer sequences were: STAT3-F: 5'-CAGCAGCTTGACACACGGTA-3', STAT3-R: 5'-AAACACCAAAGTGGCATGTGA-3'; β-actin-F: 5'-CATGTACGTTGCTATCCAGGC-3', β-actin-R: 5'-CTCCTTAATGTCACGCACGAT-3'.

Western Blot

After extracting the protein from RIPA lysate, the mass concentration was determined by the BCA assay and 40 μg was separated in 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim milk powder at room temperature, and incubated with the primary antibody at 4°C overnight (STAT3, p-STAT3, β-actin were diluted 1:2000, 1:800, 1:8000) followed by Phosphate-Buffered Saline and Tween 20 (PBST) washing. Then, HRP-labeled secondary antibody (1:8000 dilution) was added and incubated with the membrane at room temperature for 60 min. The protein band was visualized after addition of the enhanced chemiluminescence reagent. The protein imaging processing software and the Quantity One system were used to scan X-ray film and to measure band density. All experiments were repeated four times (n=4) for the statistical analysis.

Detection of Cell Apoptosis

The above two miR-NC and miR-29 mimic transfected cells were collected by trypsinization and resuspended in the binding buffer. After that, 5 μL of Annexin V-FITC and 5 μL of PI staining were sequentially added, and cell apoptosis was detected by Beckman Coulter FC500MCL flow cytometry (Brea, CA, USA).

Cell Proliferation Analysis

The cell proliferation was detected by the EdU Flow Cytometry Kit. The cells were transfected with the above two miR-NC and miR-29 mimic for 72 h and collected by trypsinization. The cells were resuspended in DMEM medium containing 10% FBS. After incubating with 10 μM EdU at 37°C for 2 h, the cells were inoculated into the culture plate for 48 h, and the cells were collected by trypsinization, washed by Phosphate-Buffered Saline (PBS), fixed by paraformaldehyde, lysed by permeabilization solution, and incubated with reaction the solution. After the liquid was washed by centrifugation and the cells were resuspended in the washing solution, the cell proliferation ability was measured by FC500MCL flow cytometry.

Statistical Analysis

Statistical analysis was performed using the Statistical Product and Service Solution 18.0 software (SPSS Inc, Chicago, IL, USA). The measurement data were expressed as mean ± standard deviation (SD). The comparison between the measurement data of the groups was analyzed by the Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Abnormal Expression of MiR-29 and STAT3 in Osteosarcoma

The results of qRT-PCR showed that the expression of STAT3 mRNA in tumor tissues of osteosarcoma patients was significantly higher than that in adjacent tissues (Figure 1A). Compared with adjacent tissues, the expression of miR-29 in tumor tissues of osteosarcoma patients was significantly decreased (Figure 1B).

Targeted Regulation Relationship Between MiR-29 and STAT3

The bioinformatics analysis revealed a complementary binding site between miR-29 and the 3'-UTR of STAT3 mRNA (Figure 2A). The Dual-Luciferase Gene Reporter Assay showed that miR-29 mimic significantly reduced the relative Luciferase activity in pGL3-STAT3-WT transfected HEK293T cells, and miR-29 inhibitor significantly increased the relative Luciferase activity in HEK293T cells transfected with pGL3-STAT3-WT (Figure 2B), indicating that there is a targeted regulation relationship between miR-29 and STAT3 mRNA.

Abnormal Expression of MiR-29 and STAT3 in Osteosarcoma Cells

The results of qRT-PCR showed that, compared with human normal osteogenic hFOB1.19 cells, the expression of miR-29 in osteosarcoma SJSA-1 and MG-63 cells was significantly decreased (Figure 3A), while the expression of STAT3 mRNA was significantly increased (Figure 3B). The Western blot analysis showed that the expression of STAT3 protein in osteosarcoma SJSA-1 and MG-63 cells was significantly higher than that in human normal osteogenic hFOB1.19 cells (Figure 3C).

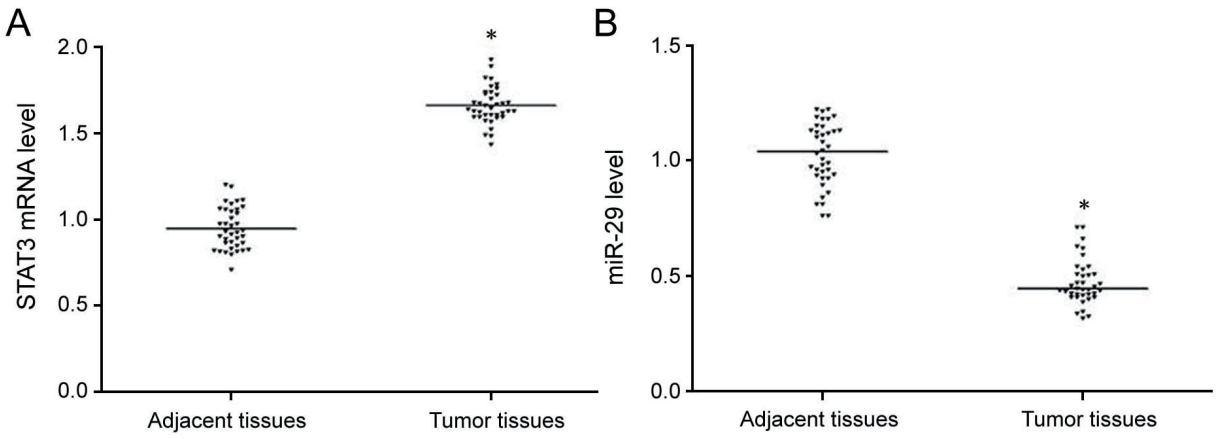

Figure 1. Abnormal expression of miR-29 and STAT3 in osteosarcoma. ***A,*** qRT-PCR detection of STAT3 mRNA expression in osteosarcoma. ***B,*** qRT-PCR detection of miR-29 expression in osteosarcoma. * represents $p<0.05$ compared with adjacent tissues.

Overexpression of MiR-29 Significantly Inhibits Osteosarcoma Cell Proliferation and Induces Apoptosis

The results of qRT-PCR showed that the expression of miR-29 in SJSA-1 cells was significantly increased in miR-29 mimic transfection group compared with that in the miR-NC transfection group (Figure 4A), while the expression of STAT3 mRNA was significantly increased (Figure 4B). The Western blot analysis showed that the expression of STAT3 and p-STAT3 protein in SJSA-1 cells was significantly decreased in miR-29 mimic transfection group compared with those in the miR-NC transfection group (Figure 4C). The flow cytometry analysis showed that the apoptosis of SJSA-1 cells in the miR-29 mimic transfection group was significantly increased compared with that in the miR-NC group (Figure 4D), and the cell proliferation ability was significantly attenuated (Figure 4E).

Discussion

Osteosarcoma refers to a primary malignant bone tumor in which tumor cells can directly produce tumor bone and bone-like tissue, which seriously affect the patients' quality of life[12,13]. Osteosarcoma is characterized by rapid disease progression, poor prognosis, and high mortality[14-16]. Therefore, to study the pathogenesis of osteosarcoma and to explore the abnormal changes in the pathogenesis of osteosarcoma is of great significance to improve the treatment and prognosis of osteosarcoma.

STAT is a transcription factor involved in the transcriptional regulation of the signal transduction and signaling transducers of the tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT). The STAT protein family consists of seven members. Among

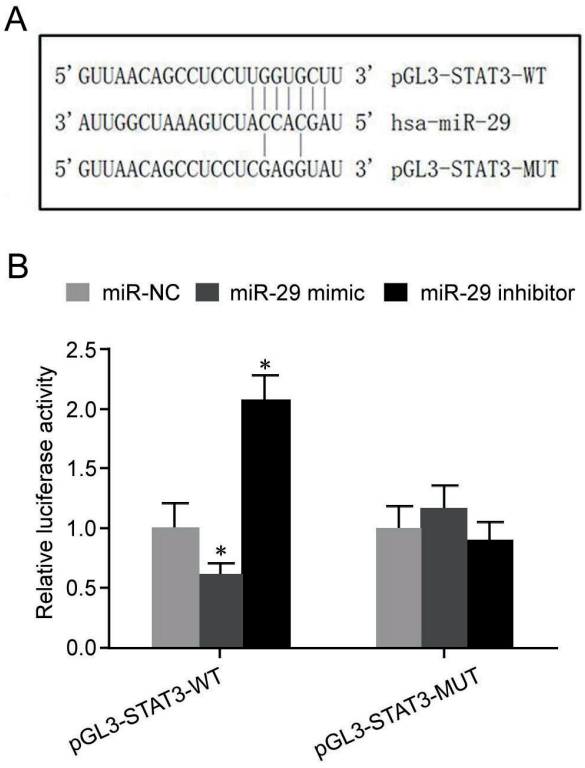

Figure 2. There is a targeted regulation relationship between miR-29 and STAT3. ***A,*** Binding site between miR-29a and the 3'-UTR of STAT3 mRNA. ***B,*** Dual luciferase gene reporter assay. *$p<0.05$ compared to miR-NC.

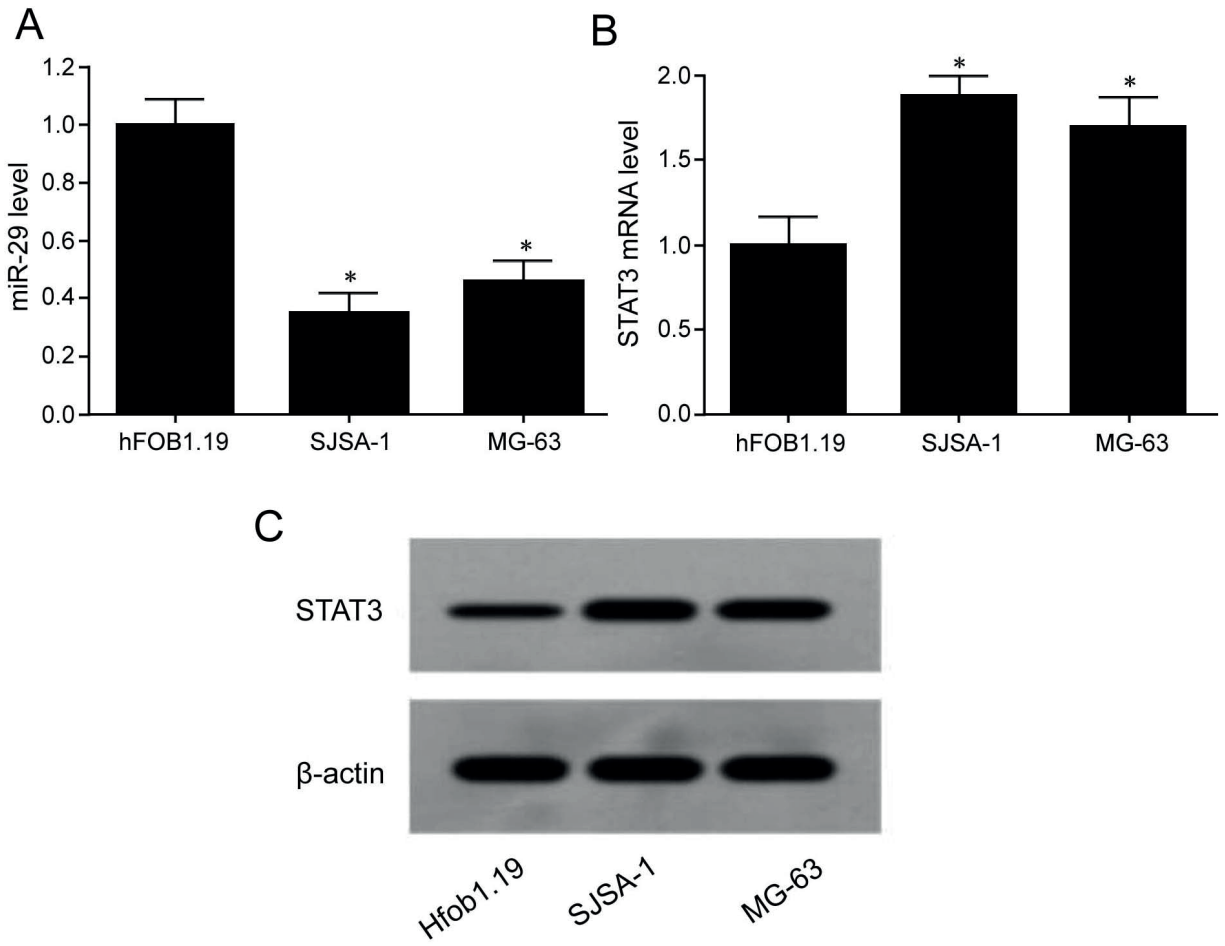

Figure 3. Abnormal changes in the expression of miR-29 and STAT3 in osteosarcoma cells. ***A,*** qRT-PCR detection of miR-29 expression in osteosarcoma cells. ***B,*** qRT-PCR detection of STAT3 mRNA expression in osteosarcoma cells. ***C,*** Western blot was used to detect the expression of STAT3 protein in osteosarcoma cells. * represents $p<0.05$ compared to HEEC cells.

them, STAT3 and STAT5 are closely related to the development of human tumors[17-19]. When the JAK-STAT3 signaling pathway is activated, the dimerized cell membrane receptor phosphorylates JAK kinase, which in turn is phosphorylated by the receptor, leads to the recruitment of STAT3 to the membrane receptor. The tyrosine of STAT3 is phosphorylated by the action of JAK kinase. Then, the activated STAT3 is disassociated from the receptor and dimerized, and then, translocated from the cytoplasm to the nucleus to act on a DNA fragment of a specific gene, thus regulating the expression of the corresponding genes[20,21]. STAT3 can promote cell proliferation and inhibit cell apoptosis. The expression and activity of STAT3 are closely related to the occurrence, progression, invasion, and metastasis of various tumors such as breast cancer[2], colon cancer[3], and gastric cancer[4]. Many investigations[5-7] have shown that STAT3 expression and dysfunction are associated with the occurrence, progression, metastasis, and drug resistance of osteosarcoma.

Many studies[22-25] have shown that the abnormal expression of miR-29 is involved in the occurrence, progression, metastasis, and drug resistance of various tumors such as breast cancer, ovarian cancer, gastric cancer, and liver cancer. Some reports[9-11] have shown that the abnormal expression of miR-29 is associated with the occurrence, progression, and drug resistance of osteosarcoma. This report investigated whether miR-29 plays a role in regulating the expression of STAT3 and affecting the biological effects of osteosarcoma cell proliferation and apoptosis.

This work showed that, compared with the adjacent tissues, the expression of miR-29 in

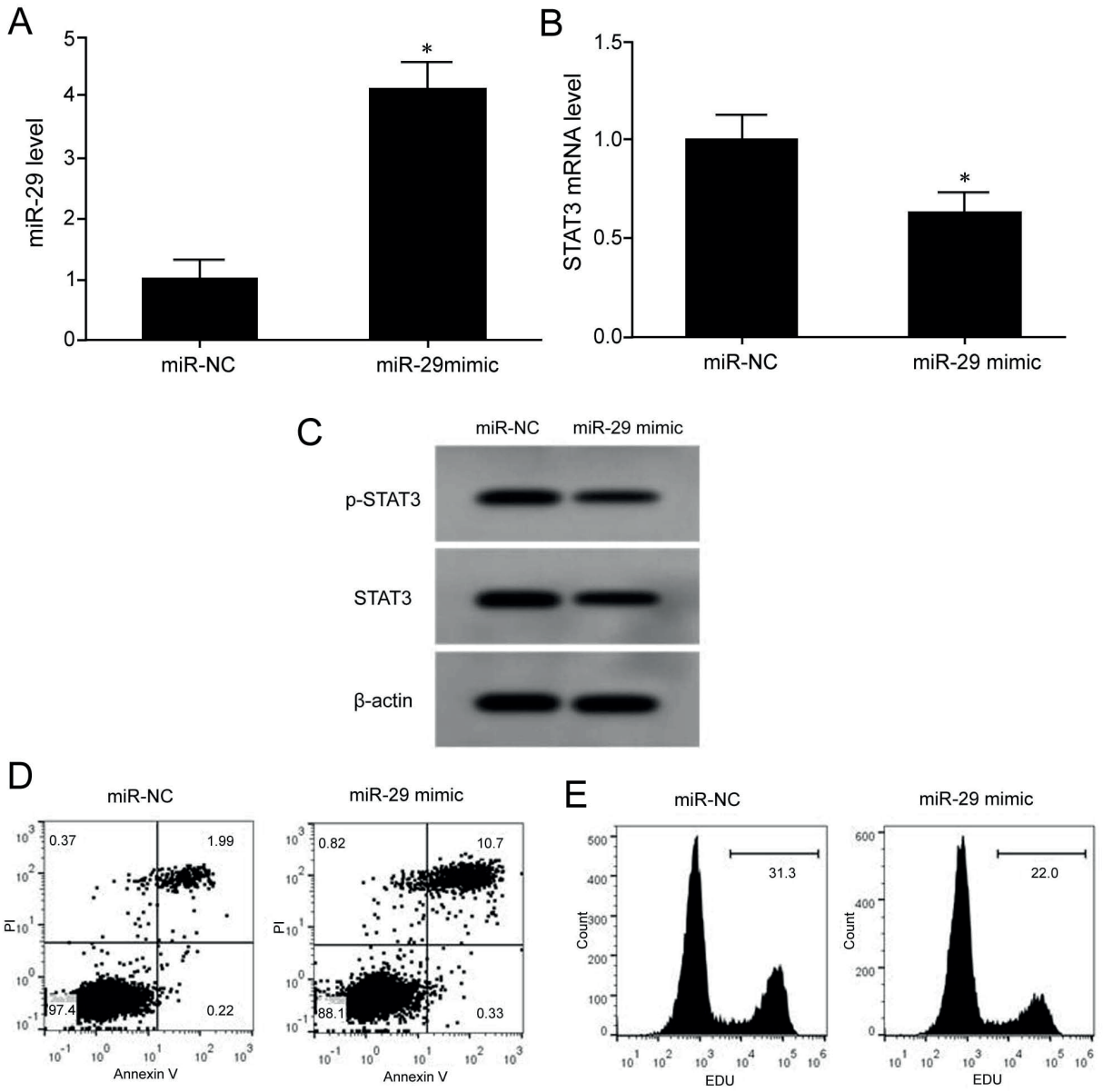

Figure 4. Overexpression of miR-29 can significantly inhibit osteosarcoma cell proliferation and induce apoptosis. ***A,*** qRT-PCR detection of miR-29 expression in SJSA-1 cells. ***B,*** qRT-PCR was used to detect STAT3 mRNA expression in SJSA-1 cells. ***C,*** Western blot was used to detect the expression of STAT3 and p-STAT3 protein in SJSA-1 cells. ***D,*** Flow cytometry to detect apoptosis in SJSA-1 cells. ***E,*** EdU staining for detection of SJSA-1 cell proliferation. * represents $p<0.05$ compared to miR-NC.

the tumor tissues of osteosarcoma patients was significantly decreased, while the expression of STAT3 was abnormally elevated, suggesting that miR-29 and STAT3 abnormalities might play a role in the pathogenesis of osteosarcoma. The results of the Dual-Luciferase Gene Reporter Assay showed that the relative Luciferase activity in the miR-29 mimic transfection group was significantly lower than that in the miR-NC group, whereas the relative Luciferase activity in the miR-29 inhibitor transfection group was significantly increased. The cells transfected miR-29 mimic, or miR-29 inhibitor, had no significant effect on the relative Luciferase activity in pGL3-STAT3-MUT transfected HEK293T cells, indicating the presence of the regulatory relationship between miR-29 and STAT3. The results of the comparative analysis showed that, compared with

normal hFOB1.19 osteoblasts, the expression of miR-29 in osteosarcoma SJSA-1 and MG-63 cells was significantly decreased, while the expression of STAT3 mRNA and protein was significantly increased, suggesting that the abnormal reduction of miR-29 may play a role in increasing STAT3 expression and promoting the pathogenesis of osteosarcoma. In the study on the relationship between miR-29 and osteosarcoma, the results of Xu et al[11] showed that the amount of miR-29 was significantly decreased in chemotherapy-resistant osteosarcoma tissues compared with tumor tissues of chemotherapy-sensitive osteosarcoma patients. The result indicated that the expression of miR-29 was decreased in the pathogenesis of osteosarcoma. In this analysis, the expression of miR-29 was significantly decreased in osteosarcoma and osteosarcoma cell lines, and miR-29 played a role as a tumor suppressor gene in osteosarcoma, and these results were confirmed by Xu et al[11]. However, in addition to the anti-cancer effect of miR-29 in osteosarcoma, Hong et al[9] have shown that an elevated expression of miR-29 plays a role in promoting cancer in osteosarcoma, and they showed that, compared with the healthy group, the expression of miR-29 in tumor tissues and peripheral blood of patients with osteosarcoma was significantly increased. Also Cheng et al[26] showed that the expression of miR-29 in tumor tissues of patients with osteosarcoma was abnormally elevated, and the abnormal expression of miR-29 was associated with poor prognosis. This is inconsistent with the results of this work and may be the result of inconsistent analyses of the test samples.

This report further explored the effect of miR-29 on the biological effects of osteosarcoma cells. The results showed that the transfection of miR-29 mimic significantly downregulated the expression of STAT3 in osteosarcoma SJSA-1 cells and decreased the expression of the p-STAT protein. The proliferative capacity of the cells is weakened, and the number of apoptosis of the cells is increased. The results showed that in osteosarcoma, miR-29 exerts a tumor suppressor effect of inhibiting cell proliferation and promoting apoptosis by targeting STAT3 expression. Xu et al[11] showed that the overexpression of miR-29 in drug-resistant osteosarcoma MG-63/MTX and U2OS/MTX cells can be targeted. It inhibits the expression of COL3A1 and MCL1, promotes apoptosis of MG-63/MTX and U2OS/MTX cells, inhibits cell proliferation activity, and reduces drug resistance to MTX. Gao et al[27] showed that in osteosarcoma, IGF1 has a cancer-promoting effect that promotes angiogenesis and osteosarcoma, and miR-29 can inhibit angiogenesis by inhibition of IGF1 expression in osteosarcoma. In the work on the relationship between STAT3 and osteosarcoma, Wang et al[28] showed that the inhibition of STAT3 function can significantly inhibit the proliferation of osteosarcoma cells, reduce the ability of osteosarcoma cell colony formation, and induce apoptosis. Zhang et al[6] showed that Toosendanin, an active ingredient of the Chinese medicine, can inhibit the proliferation of the osteosarcoma cells and attenuate the metastasis ability of the osteosarcoma cells by inhibiting the STAT3 expression and functional activity. Zuo et al[7] showed that Napabucasin can inhibit the proliferation of osteosarcoma cells, induce apoptosis, and attenuate the growth and tumorigenicity of osteosarcoma cells in animals by inhibiting the expression and phosphorylation of STAT3, thus inhibiting the distant metastasis of the transplanted tumors. This report combines the targeting relationship between miR-29 and STAT3 and reveals that a decreased expression of miR-29 plays a role in increasing STAT3 expression and promoting osteosarcoma.

Conclusions

The decreased expression of miR-29 plays a role in increasing the expression of STAT3 and promoting the pathogenesis of osteosarcoma. The increase of the expression of miR-29 can inhibit the proliferation of osteosarcoma cells and promote apoptosis by decreasing the expression of STAT3.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Acknowledgments

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