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MicroRNA-340-5p suppresses osteosarcoma development by down-regulating the Wnt/β-catenin signaling pathway via targeting the STAT3 gene

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Abstract. – OBJECTIVE: To study the effects of miR-340-5p on the proliferation of osteosarcoma (OS) U2OS cells.

PATIENTS AND METHODS: miR-340-5p expression was measured by quantitative Real-time Polymerase Chain Reaction (qRT-PCR) in cancer tissues and paracancerous tissues from OS patients, and in OS cell line U2OS and normal osteoblast cell line hFOB1.19. The dual luciferase reporter vector was constructed to verify whether Signal transducers and activators of transcription 3 (STAT3) were a potential target gene of miR-340-5p. MiR-340-5p mimics/inhibitor was transfected into U2OS cells using liposomes, and transfection results were verified by qRT-PCR. Cell Counting Kit-8 (CCK-8) and Annexin V/PI were used to analyze the proliferation and apoptosis of transfected cells, respectively. Western Blot was used to detect STAT3 protein expression and Wnt/β-catenin pathway-related proteins after transfection. U2OS cells were injected into nude mice to observe the effects of over expression of miR-340-5p on tumor growth *in vivo*.

RESULTS: miR-340-5p gene expression in OS tissues and U2OS cells was significantly lower than that in paracancerous tissues and hFOB1.19 cells. miR-340-5p directly interacted with the 3'-untranslated region (3'-UTR) of STAT3 gene and negatively regulated its expression. The increase of miR-340-5p expression could significantly inhibit U2OS proliferation *in vitro* and induce apoptosis, and *vice versa*. STAT3, β-catenin, c-Myc, TCF-4, CyclinD1, and ROCK1 protein expression in U2OS cells was significantly decreased after miR-340-5p over-expression, and *vice versa*. MiR-340-5p over-expression in nude mice significantly decreased tumor size and weight.

CONCLUSIONS: Low expression of miR-340-5p in OS and U2OS cells could inhibit the course of OS by negatively regulating Wnt/β-catenin signaling pathway through targeting STAT3 gene. Therefore, miR-340-5p might be a potential biomarker and target for the diagnosis and treatment of OS.

Key Words:
miR-340-5p, Osteosarcoma, U2OS cells, Wnt/β-catenin, STAT3.

Introduction

OS is the most common primary bone tumor in children and adolescents. Approximately 75% of OS patients are within the range of 15 and 25 years old. Long bones metaphysis at the distal femur and proximal tibia are the most susceptible to OS[1,2]. OS is locally highly destructive and has a strong distal metastatic potential, usually involving the lung. Despite the rapid advance in the treatment of OS, a complete recovery from this disease is still difficult[3,4]. The 5-year survival rate of patients with local OS can reach 70% using a combination of anti-OS drugs. However, the long-term survival rate of patients with metastatic or recurrent OS is only 20%, independently of the treatment regimen used[5-7]. Therefore, in-depth study of the molecular mechanism of OS may provide a new perspective in OS early diagnosis and treatment improvement. MicroRNAs (miRNAs) are small non-coding RNAs with a length of approximately 22 base pairs. Studies have shown that they regulate the expression of specific mRNA at a post-transcriptional level through complementary pairing with the 3'-UTR of the

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target gene[8,9]. miRNAs can work as proto-oncogenes or tumor suppressor genes since they can regulate proliferation, migration, invasion, and apoptosis[10-13]. Abnormal miR-340-5p expression was found in many cancer types, indicating that it plays a role in a variety of tumors. However, whether it promotes or suppresses the progression of cancer depends on the cancer type. For example, miR-340-5p inhibits the proliferation and migration of cancer cells in hepatitis B virus-related liver cancer[14], but it is upregulated in thyroid cancer and promotes its development[15]. It often regulates the expression of multiple oncogenes by adjusting the activity of signaling pathways through the interaction with target genes. Studies showed that the pathology of OS is associated with multiple miRNAs, including miR-24[16], miR-16[17], miR-183[18], miR-20a[19], but no reports are available on the role of miR-340-5p in the progression of OS. This study intends to explore the relationship between miR-340-5p expression and OS, and how it affects the proliferation and apoptosis of U2OS cells by regulating Wnt/β-catenin signaling pathway by interacting with target genes, thereby providing a theoretical basis for screening potential OS diagnostic and therapeutic targets.

Patients and Methods

Patients

Forty OS tissue samples were collected from OS patients, 22 males and 18 females, with an average age of 19.7 ± 2.4 years, treated in the Sixth Affiliated Hospital to Xinjiang Medical University from January 2013 to February 2018. All patients underwent surgery, and were not subjected to radiotherapy, chemotherapy or any other previous treatment. OS samples were analyzed by three histopathologists. After specimens' collection, the tumor tissue sample was quickly frozen in liquid nitrogen, and transferred to a -80°C refrigerator for storage. The whole collection process and follow-up study were approved by the Ethics Committee of the Xinjiang Medical University (Approval No.: 20130214-016), with confidentiality agreements signed by the patients.

Main Reagents

OS cell line U2OS and normal osteoblast cell line hFOB1.19 (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China); Dulbecco minimum essential medium (DMEM) and fetal bovine serum (Gibco, Grand Island, NJ, USA); TRIzol Reagent, Prime Script® RT reagent Kit with gDNA Eraser and SYBR®Premix Ex Taq™ II (Dalian TaKaRa, Dalian, Liaoning, China); pMIR-REPORT Luciferase Reporting Vector (Applied Biosystems, Foster City, CA, USA); Dual Luciferase Assay Kit (Promega, Madison, WI, USA); QuickChange Lighting Mutagenesis Kit (Stratagene, New York, NY, USA); Synthesis of miR-340-5p mimics/inhibitor and negative control (GenePharma, Shanghai, China); Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA); CCK-8 (Dojindo, Kumamoto, Japan); Annexin V-FITC Apoptosis Assay Kit (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA); Rabbit/mouse anti-human STAT3, β-catenin, c-Myc, TCF-4, CyclinD1, ROCK1, β-actin monoclonal antibodies and horseradish peroxidase (HRP) anti-rabbit/mouse IgG secondary antibody (CST, Boston, MA, USA), primers (Shanghai Biotech, Shanghai, China).

Methods

Cell Culture and Transfection

U2OS and hFOB1.19 cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics and incubated at 37°C under 5% CO_2. U2OS cells in the log phase were seeded into 96-well plates one day before transfection, with a cell density of $1x10^5$ cells/well. When the cell confluence reached 40-50%, 20 nM negative control (NC) mimics and miR-340-5p mimics, or 20 nM NC inhibitor and miR-340-5p inhibitor were transfected into U2OS cells using Lipofectamine 3000 reagent. The control group was treated with DMEM medium plus liposome reagent. Cells were collected for further experiments 48 h after transfection.

qRT-PCR

Total RNA from tissue or cells was extracted using TRIzol reagent. cDNA synthesis was performed using Prime Script® RT reagent Kit and gDNA Eraser Kit, under the following reaction conditions: incubation at 37°C for 15 minutes, heating at 85°C for 5 s, and storage at 4°C. qRT-PCR reaction was performed using SYBR®Premix Ex Taq™ II under the following conditions: 95°C for 30 s, 95°C for 5 s, 60°C for 30 s (40 cycles), followed by a melting curve step. CFX-96 Real-Time PCR Detection System (Bole, NY, USA) was used for detection. After amplification, data were

collected and processed by the comparative cycle threshold method. MiR-340-5p primer was a Bulge-Loop™ miRNA RT-PCR primer, with U6 snRNA as the miRNA quantitative internal reference, and β-actin as the mRNA internal reference. Primer sequences are shown in Table I.

Luciferase Reporter Gene Analysis

The 3'-UTR of STAT3 where potential binding sites of miR-340-5p could be located, was amplified. The amplified products were visualized by gel extraction, and ligated to the pMIR-REPORT luciferase reporter vector digested by XhoI and MluI. The resulting pMIR-REPORT-WT plasmid was analyzed and identified by sequencing. Subsequently, the correctly sequenced recombinant plasmid was subject to site-directed mutagenesis of the 3'-UTR of STAT3, with the help of the site mutation kit to obtain pMIR-REPORT-MUT. The primer sequences used for plasmid construction are shown in Table I. miR-340-5p mimics, or its NC mimics and pMIR-REPORT-WT, or pMIR-REPORT-MUT were co-transfected into U2OS cells, which were collected after 48 h or 72 h to analyze the activity of luciferase according to the dual luciferase assay kit, with Renilla luciferase as the internal control.

Cell Proliferation

Each group of cells transfected for 48 h were cultured in 96-well plates containing DMEM and 10% fetal bovine serum (FBS) medium, with a cell density of 2×10^3 cells/well. After the addition of 10 μL CCK-8 reagent every other day, cells were incubated for 3 h at 37°C in the dark, and the absorbance of each well was measured at 450 nm. Cell viability was normalized to the starting point and expressed as percentage.

Cell Apoptosis

Cells transfected for 48 h were subject to Annexin V-FITC/PI double staining in accordance to the apoptosis assay kit, and early and late apoptosis were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot Analysis

Cells transfected for 72 h were collected and lysed with Radio Immunoprecipitation Assay (RIPA) buffer to extract and quantify total proteins. An amount of 60 μg of protein was separated by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and electrotransfered onto a polyvinylidene difluoride (PVDF) membrane. Subsequently, the membrane was blocked with Tris-Buffered Saline and Tween-20 (TBST) containing 5% skim milk for 1 hour at room temperature, and subsequently incubated overnight at 4°C with β-catenin, c-Myc, TCF-4, CyclinD1, ROCK1, and β-actin primary antibodies (1:1000). The next day, the membrane was washed 3 times with TBST and incubated for 2 h at room temperature with HRP-conjugated secondary antibody (1:5000). The membrane was washed with TBST, and developed using the ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA). Band intensity was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA). Protein expression was normalized with β-actin.

Animal Experiments

Sixteen male BALB/c nude mice (20 ± 2 g, 5 weeks old) (Xinjiang Medical University, Urumqi, Xinxiang, China) were housed in the nude mouse breeding room of the animal center in Xinjiang Medical University, under a 12-hour light/dark cycle, with food and water *ad libitum*. They were randomly divided into two groups: miR-340-5p mimics and NC mimics (n= 8 each group). U2OS cells (suspended in 100 μL DMEM supplemented with 10% FBS) stably transfected with miR-340-5p mimics and NC mimics were subcutaneously injected at a dose of 1×10^6 cells into the mice scapula region. The tumor size was measured every two days and calculated using the formula: 1/2

Table I. Primer sequences used for qRT-PCR and plasmid construction.

Primer	Sequence (5'-3')
STAT3-qF	GGCCCAATGGAATCAGCTACAG
STAT3-qR	GAAGAAACTGCTTGATTCTTCG
ACTB-F	AGTCCTGTGGCATCCACGAAA
ACTB-R	GTCATACTCCTGCTTGCTGA
STAT3-3'UTR-F	ATCCGCTCGAGATGGCCCAATGGAATC
STAT3-3'UTR-R	CGACGCGTGAGGTCAACTCCATGTCAAAG

× (length × width × width). Five weeks after the injection, mice were sacrificed and the tumors were collected and weighed. The animal experiment was approved by the Ethics Committee of the Xinjiang Medical University (Approval No.: 20130214-017).

Statistical Analysis

Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All experiments were repeated three times, and data were expressed as mean ± standard deviation. Two-tailed t-test was used to compare two groups. One way ANOVA test with Tukey-Kramer multiple comparisons test was used to assess differences for multiple groups. Statistical significance was set as $p<0.05$.

Results

Expression of miR-340-5p in OS Tissue and Cells

miR-340-5p expression in cancer and paracancerous tissues of OS patients, and in U2OS and hFOB1.19 cells was detected by qRT-PCR (Figure 1a and 1b). miR-340-5p was significantly under-expressed in the cancer tissue of OS patients compared with its expression in the normal paracancerous tissue. In addition, miR-340-5p expression in U2OS cells was significantly lower than that in hFOB1.19 cells. To further explore the relationship between miR-340-5p and the pathogenesis of OS, U2OS cells were transfected with miR-340-5p mimics, inhibitor, and its negative control, and the expression of miR-340-5p was quantified by qRT-PCR. As shown in Figure 1c, miR-340-5p level after its over-expression was significantly higher than that in the NC mimics group, while it was significantly lower than that of the NC inhibitor group after suppression.

Regulatory Relationships Between miR-340-5p and its Potential Target Gene

STAT3 was predicted by TargetScan as a potential target gene of miR-340-5p. qRT-PCR and Western blot were performed to detect STAT3 mRNA and protein expression, respectively, in U2OS cells after miR-340-5p over-expression or suppression. As shown in Figure 2a, 2b and 2c, STAT3 mRNA and protein expression was significantly lower than that in the NC mimics group after miR-340-5p over-expression, while STAT3 mRNA and protein expression was significantly increased than that in the NC inhibitor group after suppression of miR-340-5p. Moreover, to verify that miR-340-5p binds directly to the 3'-UTR of STAT3 to regulate its expression, a dual luciferase reporter vector was constructed and transfected into U2OS cells together with mimics or its NC mimics, to analyze the relative luciferase activity. As shown in Figure 2d, miR-340-5p over-expression significantly inhibited the activity of luciferase compared with NC mimics, while such inhibition was significantly weakened after site-directed mutagenesis of STAT3 3'-UTR (Fi-

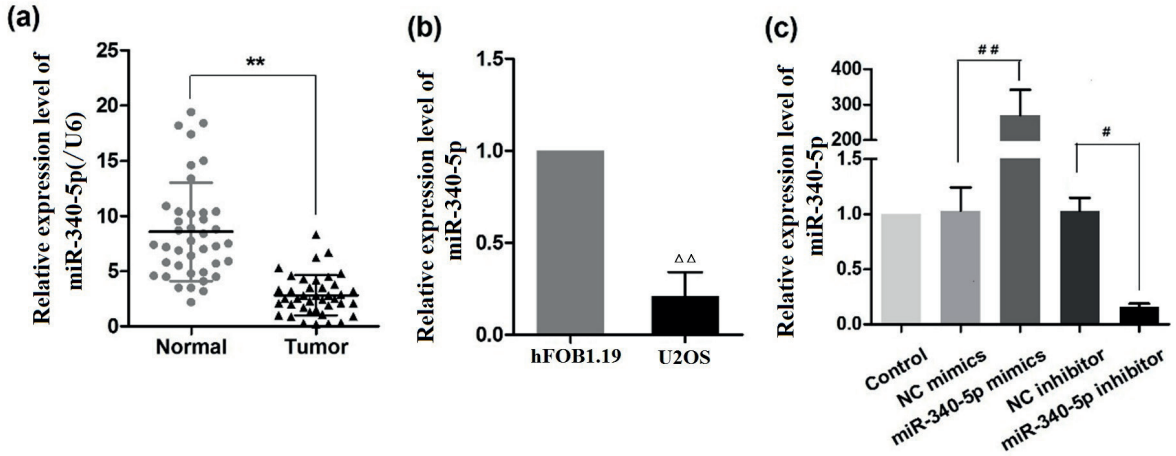

Figure 1. Changes in miR-340-5p expression in OS tissue and U2OS cells before and after mimics/inhibitor transfection. a: miR-340-5p levels in cancer and normal paracancerous tissue in patients with OS; b: miR-340-5p levels in U2OS and hFOB1.19 cells; c: miR-340-5p level after mimics/inhibitor transfection. $^{**}p<0.01$ *vs.* Normal, $^{\Delta\Delta}p<0.01$ *vs.* hFOB1.19, $^{\#}p<0.05$ *vs.* NC inhibitor; $^{\#\#}p<0.01$ *vs.* NC mimics.

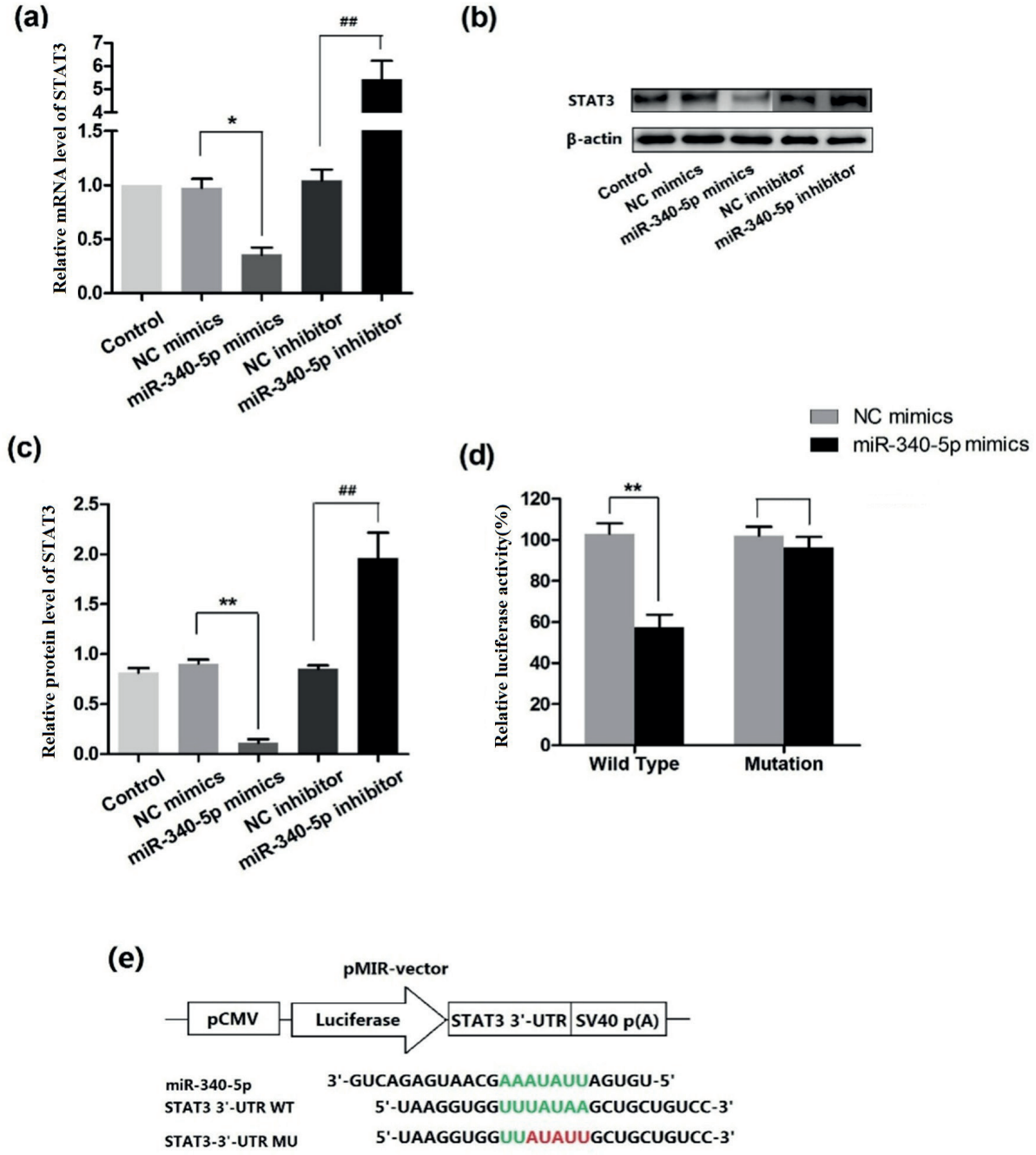

Figure 2. Effect of miR-340-5p on the expression of its target gene STAT3. a: STAT3 mRNA expression after over-expression and suppression of miR-340-5p; b: STAT3 protein expression after over-expression and suppression of miR-340-5p; c: Relative protein level of STAT3 after over-expression and suppression of miR-340-5p; d: luciferase activity after transfection with different reporter vector and mimics; e: Construction of dual luciferase reporter vector. * $p<0.05$ *vs.* NC mimics; ** $p<0.01$ *vs.* NC mimics; $^{\#\#}$ $p<0.01$ *vs.* NC inhibitor.

gure 2e), and no differences were observed when compared with NC mimics. The results of the dual luciferase reporter assay indicated that miR-340-5p negatively regulated STAT3 expression by complementary pairing with its 3'-UTR.

Effects of miR-340-5p on U2OS Cell Proliferation in vitro

CCK-8 was used to analyze the proliferation of U2OS cells after transfection with miR-340-5p mimics/inhibitor. Cell proliferation was signifi-

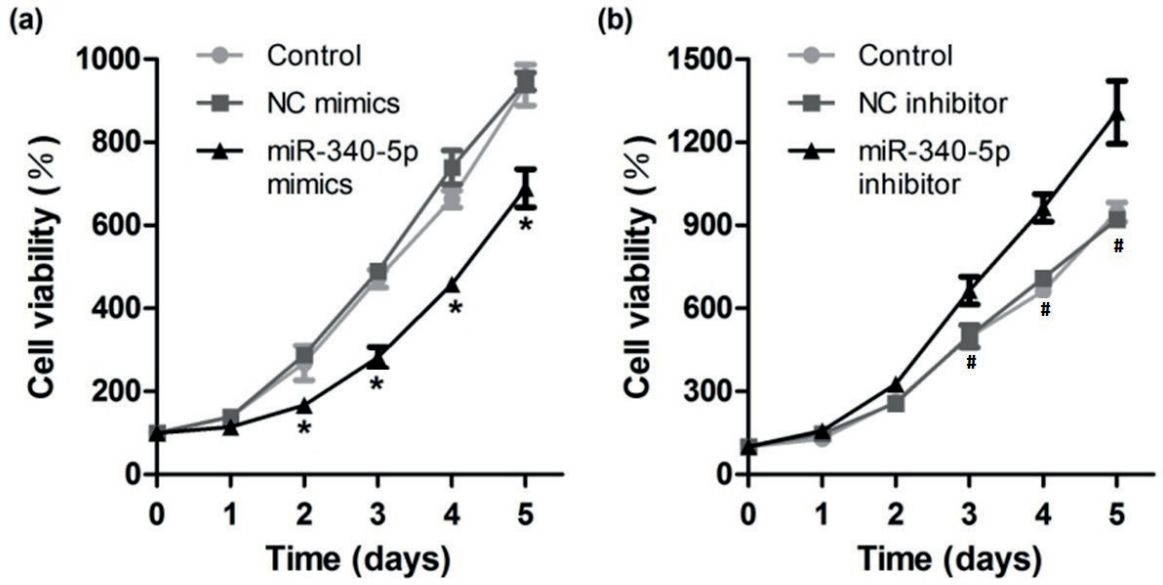

Figure 3. Effects of miR-340-5p over-expression and suppression on U2OS cells proliferation. a: U2OS cell proliferation after miR-340-5p over-expression; b: U2OS cell proliferation after suppression of miR-340-5p. $^{*}p<0.05$ *vs.* NC mimics; $^{\#}p<0.05$ *vs.* NC inhibitor.

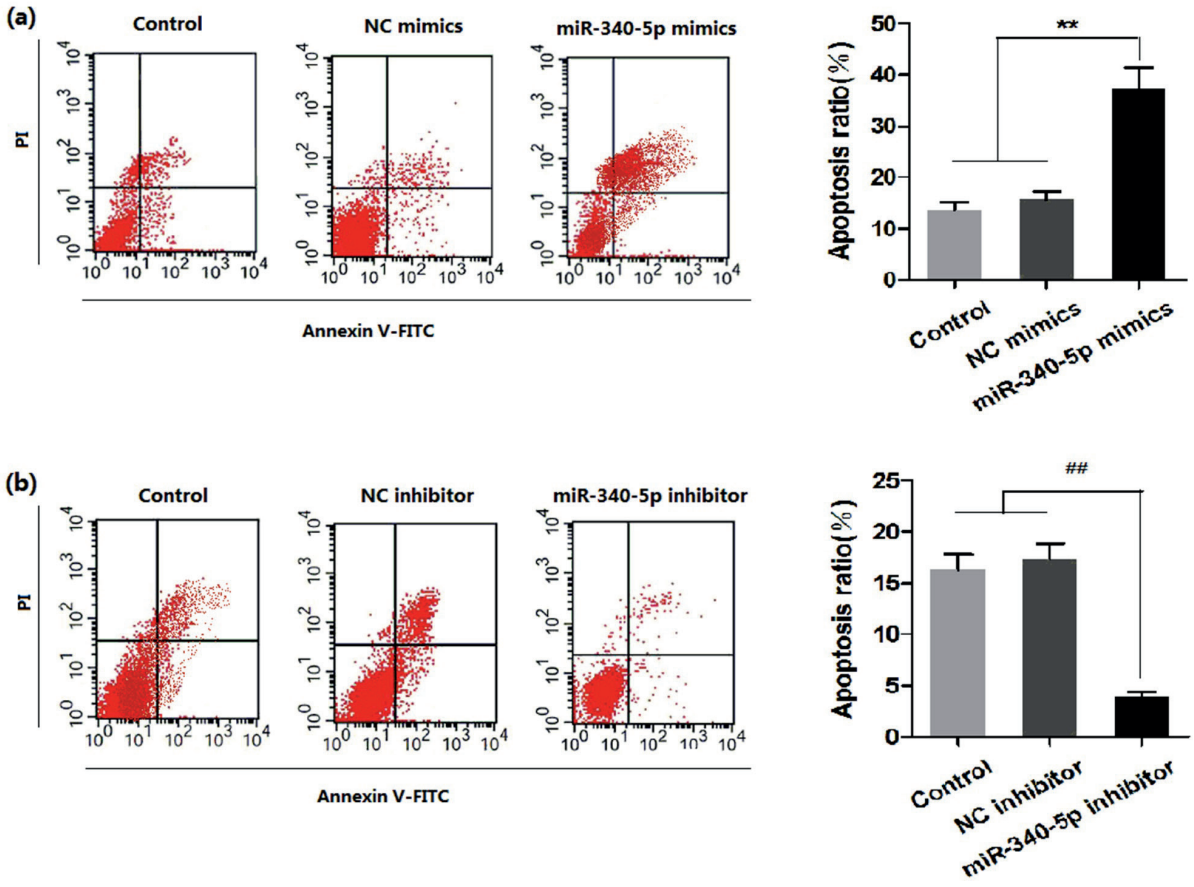

Figure 4. Effects of miR-340-5p over-expression and suppression on apoptosis of U2OS cells. a: U2OS cell apoptosis after miR-340-5p over-expression; b: U2OS cell apoptosis after suppression of miR-340-5p. $^{**}p<0.01$ *vs.* NC mimics; $^{\#\#}p<0.01$ *vs.* NC inhibitor.

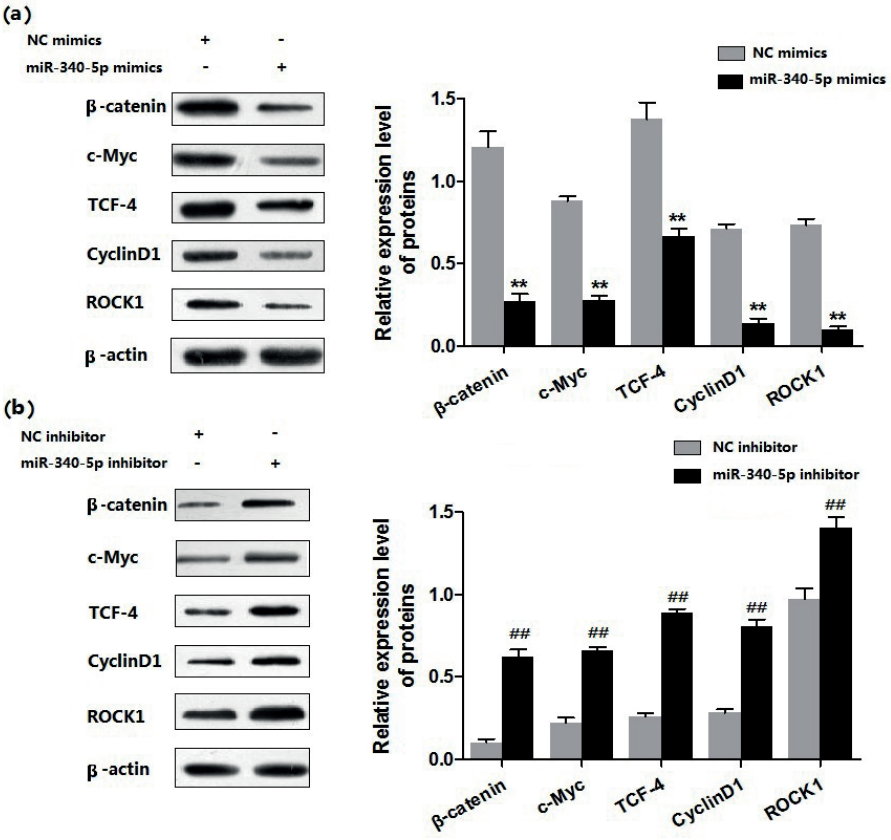

Figure 5. Effects of miR-340-5p over-expression and suppression on Wnt/β-catenin-related proteins. a: Expression of Wnt/β-catenin-related proteins after miR-340-5p over-expression; b: Expression of Wnt/β-catenin-related proteins after miR-340-5p suppression. $^{**}p<0.01$ vs. NC mimics; $^{\#\#}p<0.01$ vs. NC inhibitor.

cantly inhibited after miR-340-5p over-expression compared with NC mimics, but significantly promoted after suppression of miR-340-5p compared with NC inhibitor (Figure 3).

Effects of miR-340-5p on OS Cell Apoptosis

Annexin V/PI double staining was performed to detect the apoptosis of U2OS cells after 48 h transfection with miR-340-5p mimics/inhibitor. As shown in Figure 4, U2OS early and late apoptosis after miR-340-5p over-expression was significantly higher than that in NC mimics, while the early and late apoptosis after miR-340-5p suppression was significantly lower than that in NC inhibitor.

Molecular Mechanism of miR-340-5p in OS Regulation

Western blot was used to detect the level of Wnt/β-catenin-related proteins in U2OS cells after transfection with miR-340-5p mimics/inhibitor (Figure 5). β-catenin, c-Myc, TCF-4, CyclinD1, and ROCK1 protein expression was significantly lower after miR-340-5p over-expression compared with NC mimics. However, the expression of the proteins above mentioned was significantly increased when the expression of miR-340-5p was suppressed compared with NC inhibitor.

Effects of miR-340-5p on the Tumorigenic Ability of U2OS Cells in Nude Mice

U2OS cells were injected into nude mice to observe the effect of miR-340-5p on tumor growth (Figure 6). Tumor size and weight were both significantly reduced in the miR-340-5p mimics group compared with the group treated with NC mimics.

Discussion

OS is a highly aggressive tumor that mainly affects children and adolescents[20]. Patients with

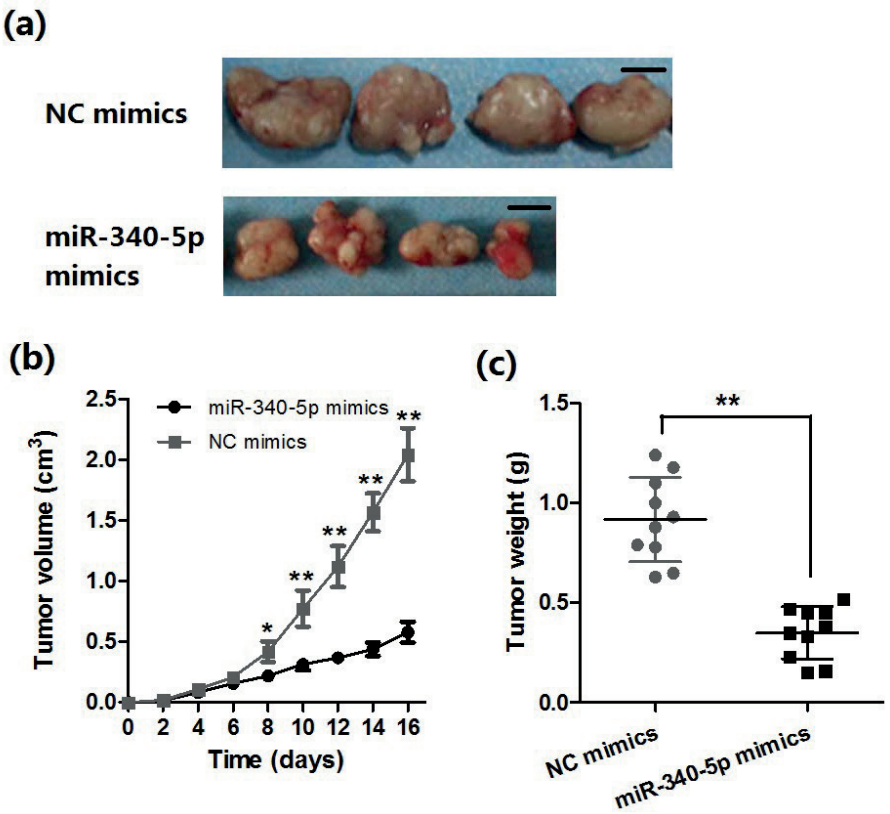

Figure 6. Effect of miR-340-5p on tumor growth in nude mice. a: Anatomical drawing of tumors in mice injected with cells treated with NC mimics and miR-340-5p mimics, the back ruler is 5 mm; b: tumors size in mice injected with cells treated with NC mimics and miR-340-5p mimics; c: tumor weight in mice injected with cells treated with NC mimics and miR-340-5p mimics after 5 weeks. $^{*}p<0.05$ vs. NC mimics; $^{**}p<0.01$ *vs.* NC mimics).

advanced, metastatic or recurrent OS have a very low five-year survival rate (about 20%) and a median survival of only 23 months[21-23]. As indicated by current clinical trials, treatments such as chemotherapy with cytotoxic drugs and targeted drug therapy are only effective for some OS patients, and cannot improve overall survival [24-26]. The etiology of OS is rather complicated and involves a variety of factors, which are characterized by genomic instability, abnormal karyotype, and genomic aberration with increased or decreased copies of multiple chromosomes[27, 28]. At present, studies on the molecular mechanisms revealing the incidence and development of OS, and the discovery of effective therapeutic target against OS are of great significance for the diagnosis, treatment and prognosis of this disease. miRNAs are closely related to the formation and development of multiple human tumors. As new regulatory molecules, they can act as a proto-oncogene or tumor suppressor gene, and regulate cancer cells through the binding to target genes[29,30]. Studies showed that miR-129-5p inhibits the proliferation of OS cells[31], while miR202-5P inhibits the migration and invasion of OS cells by targeting ROCK1[32]. miRNA-3200-5p promotes the invasion of OS cell by inhibiting Breast cancer metastasis suppressor 1 (BRMS1)[33], and miR214-3P promotes the proliferation, migration, and invasion of OS cells by targeting cell adhesion molecule 1 (CADM1)[34]. miR-340-5p, a small RNA molecule commonly found in normal human cells, is abnormally expressed in various tumor tissues, and involved in the progression of cancer[14]. Up-regulation of miR340-5P promotes the progression of thyroid cancer[15] and affects the proliferation of melanoma stem cells[35]. STAT3 is highly expressed in a variety of cancer tissues. Its over-expression is closely related to OS clinical stages and may be the main determinant in the progression of OS[36-38]. Multiple miRNAs can target the 3'-UTR of STAT3 thus influencing the activity of cancer cells by regulating signaling pathways[39]. The

Wnt/β-catenin pathway is an important pathway in the cell signal transduction process. β-catenin accumulates in the cytoplasm and enters into the nucleus to activate the expression of downstream target genes, thus playing a vital role in cell proliferation and differentiation, and in tumor formation[40-42]. Currently, no studies have been reported yet on miR-340-5p regulate target genes in OS cells to affect specific signaling pathways and participate in the pathogenesis of OS. In this study, we found that miR-340-5p was abnormally under-expressed in tissues and cells of patients with OS, indicating that the low expression of miR-340-5p might be closely associated with OS. By analyzing the effects of miR-340-5p up- and down-regulation in U2OS cells on proliferation and apoptosis *in vitro*, we discovered that miR-340-5p over-expression could significantly inhibit the proliferation of U2OS cell and induce apoptosis. Therefore, it acted as a tumor suppressor gene in the pathology of OS. By verifying whether STAT3 is the potential target gene of miR-340-5p and detecting the level of Wnt/β-catenin pathway-related proteins, we found that miR-340-5p directly interacted with the 3'-UTR of STAT3, and up-regulation of miR-340-5p significantly inhibited the expression of β-catenin, c-Myc, TCF-4, CyclinD1, and ROCK1 proteins in U2OS cells, suggesting that miR-340-5p regulated the activity of Wnt/β-catenin pathway by binding to the target gene STAT3, thus inhibiting the proliferation of U2OS cells *in vitro* and promoting apoptosis. Animal experiment results indicated that miR-340-5p over-expression significantly reduced the size and weight of the tumor in nude mice, indicating that miR-340-5p inhibited tumor growth and progression *in vivo*.

Conclusions

We indicated that miR-340-5p might be used as a potential target for the clinical diagnosis and treatment of OS.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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