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Biological function of microRNA-30c/SOX9 in pediatric osteosarcoma cell growth and metastasis

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Abstract. – OBJECTIVE: Osteosarcoma is one of the commonest malignant bone tumors, which frequently occurs in children all over the world. To find out methods to improve the therapeutic effect of osteosarcoma, it is necessary to detect the functioning mechanism of miR-30c to regulate the proliferation and metastasis of osteosarcoma cell.

PATIENTS AND METHODS: In order to reveal the expression level of miR-30c, quantitative Real-time PCR (qRT-PCR) method was chosen. To evaluate cell viability and proliferation rates, colony formation and cell counting kit-8 (CCK8) assay were introduced. Based on cell migration and invasion assay, metastasis capacity of breast cancer cells was studied. Protein levels were measured by Western blotting assay and cell cycle distribution was identified by flow cytometry. Bioinformatics analysis and Luciferase assay were used to predict and verify the target gene.

RESULTS: Compared with pericarcinomatous tissues (n=38), miR-30c in osteosarcoma tissues was significantly suppressed. Overexpressed miR-30c could weaken osteosarcoma cell's abilities of viability, proliferation, migration and invasion. Moreover, it could also encourage osteosarcoma cell apoptosis and block cell cycle at G0/G1 phase. According to bioinformatics analysis and Luciferase reporter assay, SOX9 was recognized as the target gene of miR-30c. Restoration of SOX9 could make miR-30c regain the ability of suppression on tumorigenesis of osteosarcoma cells.

CONCLUSIONS: MiR-30c could play an important role in tumor suppression for pediatric osteosarcoma development and metastasis by targeting SOX9 *in vitro*. Thus, a creative and potential target was provided for diagnosis and treatment of osteosarcoma.

Key Words:
microRNAs, Metastasis, Proliferation, SOX9, Osteosarcoma.

Introduction

Osteosarcoma (OS) is a kind of malignant bone tumor, which is common in young people or children aged below 20 years old and the most common in the pediatric bone malignant tumor, about 5% of pediatric tumors[1]. OS is originated from the mesenchymal tissue with a high degree of malignancy, strong aggression and early lung metastasis, so the prognosis of patients with OS is very poor with a very low survival rate[2]. The 5-year survival rate after amputation is only 5-15%[3]. With the progress of surgery and introduction of preoperative and postoperative chemotherapy concept, the current clinical remission rate and long-term survival rate of OS have been greatly improved. However, there are still many patients with poor prognosis, mainly because of the lung metastasis and drug resistance[4,5]. The exact mechanism of the occurrence and metastasis of OS is not clear at present, so it is of significance in the diagnosis and treatment of OS to clarify the molecular mechanism of OS.

MicroRNA, which is a kind of non-coding RNA transcript with length of approximate 18 to 25 nucleotides, can suppress protein translation and gene expression by blinding to the 3'UTR of the target genes in various biological processes[6]. Many studies indicated that microRNA could become an essential factor in various cell progressions[7]. Hence, deep analysis of microRNA would be a good approach to figure out how or why tumors occur and develop.

MicroRNA-30c has been reported as an anti-oncogene in various types of cancers, such as lung cancer[8], ovarian carcinoma[9], pancreatic can-

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cer[10], and breast carcinoma[11]. Meanwhile, it was found that miRNA was a participant during the process of OS tumorigenesis[12]. However, it is still unknown the mechanism of action of miR-30c in the progression of OS.

In the present study, expression level of miR-30c would decrease in OS tissues and its overexpression could inhibit viability, proliferation, migration and invasion ability of OS cell *in vitro*, inducing cell cycle arrest at G0/G1 phase. Moreover, SOX9, the target gene of miR-30c, could be modulated to make miR-30c exert functions.

Patients and Methods

Patients

A total of 38 pairs of OS tissues and para-carcinoma tissues used in this study were obtained from routine surgeries in Xuzhou Central Hospital from 2015 to 2017. The tissue specimens were immediately put in liquid nitrogen after they were collected. All of them were diagnosed and confirmed by pathological examination. The Ethics Committee of Xuzhou Central Hospital approved this research and the informed consents were signed by all participants (or parents of the child patients) at the beginning of this research.

Cell Culture

The OS cell lines MG63, HOS, U2OS, Saos-2 and normal human osteoblast cell line, hFOB1.19, were purchased from Shanghai Cell Bank (Shanghai, China). Afterwards they were cultivated in media Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented by 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2.

Plasmid and Transfection

MiR-30c mimics and miR-NC (used as related negative control) were purchased from RiboBio (Guangzhou, China). Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) was used to perform transfections based on the protocols.

For overexpression of SOX9, after synthesized the coding sequence of SOX9, we added it into vector pCDNA3.1 (Invitrogen, Carlsbad, CA, USA), and further checked its sequencing. Empty pCDNA vector was adopted as blank control.

RNA Extraction and qRT-PCR

According to the instructions, we used the TRIzol (Invitrogen, Carlsbad, CA, USA) to extract all the RNA from frozen OS and the adjacent tissues. mirVana™ qRT-PCR microRNA Detection kit (Ambion, Austin, TX, USA) was applied to evaluate the expression level of miR-30c. U6 was used as an internal reference. Afterwards, PCR reactions were performed as following primers: for miR-30c, forward, 5'- TCCTACACTCTCAGCTGTGGAAA-3' and reverse, 5'- GAAAGAGTAAACAGCCTTCTCCCA-3'. Regarding U6, forward primer is 5'-GCACCTTAGGCTGAACA-3' and reverse primer is 5'-AGCTTATGC CGAGCTCTTGT-3'. cDNA was synthesized via PrimeScript® RT reagent kit (TaKaRa, Otsu, Shiga, Japan). SYBR Green RT-PCR was adopted to detect the relative mRNA expression level of SOX9, which was normalized to GAPDH. In this study, the primers used were as follows: SOX9, forwards, 5'-AAGGACCACCCGGATTACAAG-3'; reverse, 5'-CGTTCTTCACCGACTTCCTCC-3'. GAPDH, forward, 5'-CGGAGTTGTTCGTATTCGG-3'; reverse, 5'-TACATGATGTGGACGGCATT-3'. qRT-PCR was performed on the ABI 7500 FAST Real-time PCR platform (Applied Biosystems, Foster City, CA, USA).

Cell Counting kit-8 Assay

After the transfection, the cell viability ratio was evaluated via cell counting kit-8 (CCK8) assay (Beyotime, Nanjing, China) according to the protocols. Next, the transfected cells were seeded into 96-well plates at the density of 2000 cells/well, and added 10 μl of CCK8 solution to 90 μl of Dulbecco's modified eagle Medium (DMEM). Afterwards, we recorded the absorbance at 490 nm after the 3-h incubation of the liquid.

Colony Formation Assay

After transfection, for purpose of proliferation detection, colony formation assay was applied. Firstly, cells were plated in 6-well plates at a density of 5×10^2 per well and cultured for 2 weeks. Secondly, we fixed the colonies in ice-cold 70% methyl alcohol for 10 min and kept them stained with 0.5% crystal violet for 10 min. After that, each well was carefully washed for 3 times with phosphate-buffered saline (PBS).

Detection Method of Cell Apoptosis

Double-staining process was used for the suspension of cells with 1 μl propidium iodide (PI) and 5 μl Annexin V-FITC (fluorescein isothiocyanate). The liquid was incubated for 15 min in the dark condition, then cells were counted by a flow cytometer (BD Bioscience, Detroit, MI, USA).

Afterwards, the proportion of apoptotic cells was detected by Flowjo 7.6 (TreeStar, Ashland, OR, USA).

Detection Method of Cell Cycle

After the suspension of cells was ready, it was stained with PI by the BD Cycle test Plus DNA Reagent Kit (BD Bioscience, Detroit, MI, USA). Cells were counted by a flow cytometer (BD Bioscience, Detroit, MI, USA). The relative ratio of cells in different phases was analyzed by Flowjo 7.6 (TreeStar, Ashland, OR, USA).

Wound healing

Cells in the 6-well plates were next scratched across the confluent cell layer by a pipette tip. Afterwards, cells were washed carefully and cultured for 24 h with the serum-free medium. A microscope (DFC500, München, Germany) was adopted to capture wound closure.

Transwell Assay

The cells were placed into the upper transwell chamber (BD, Detroit, MI, USA), which was coated with Matrigel and added by serum-free medium. Meanwhile, the lower transwell chamber was added with 10% fetal bovine serum (FBS) medium. After a 48-h incubation, the cells were removed on the upside of the filter. Afterwards, suspension was performed with 100% precooling methanol, cells were stained using crystal violet (0.05%) and observed by using the microscope (Olympus, Tokyo, Japan). The values for the invasion cells were calculated by mean cell numbers from counting 5 fields per membrane.

Bioinformatics Analysis

StarBase v2.0 (http://starbase.sysu.edu.cn/index.php) and TargetScan (http://www.targetscan.org/vert_71/) were applied to predict the target genes. qRT-PCR was performed to detect whether there was a negative correlation between the expression level of SOX9 and miR-30c in OS cells.

Luciferase Reporter Assay

For luciferase reporter, pGL3 luciferase expression construct containing the 3'UTR of SOX9 the Renilla luciferase plasmid (pRL-TK) (Promega, Madison, WI, USA), and miR-30c mimics or miR-NC (RiboBio, Shanghai, China) was applied to transfect cells. Duo-Glo luciferase assay kit (Promega, Madison, WI, USA) was adopted to monitor the luciferase signal following the protocols. Furthermore, the predicted miR-30c binding site on 3'UTR of SOX9 was mutated, and the luciferase activity was detected by miR-30c mimics.

Western Blot Analysis

Briefly, after cells were lysed, protein concentration was detected by a protein assay kit from Beyotime (Nanjing, China). A total of 20 μg protein was degenerated and chilled on ice for several minutes' standing. Then, proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and were shifted to polyvinylidene difluoride (PVDF) membranes purchased from Millipore (Billerica, MA, USA). To block non-specific protein interactions, 5% fat-free milk was used at 4°C for 1 h. Next, the membranes that loaded with proteins were soaked in fat-free milk overnight with the primary SOX9 antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4°C. The unbound antibody was washed using the Tris-buffered saline-Tween (TBST) (10 min each time, 3 times). Then, secondary antibody was incubated conjugated with horseradish peroxide (HRP) at room temperature for 1 h. Afterwards, we washed the membranes in TBST buffer for 3 times, and the membranes were developed by enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) following the protocols.

Statistical Analysis

SPSS statistics version 17.0 software (IBM, Armonk, NY, USA) was applied for statistical analysis, and Graph PAD prism software (Version X; La Jolla, CA, USA) was used to present statistical data. Quantitative data was exhibited as mean ± SD. The relative expression of mRNA was measured by the method of $2^{-\Delta\Delta CT}$. The regression and correlation analysis were conducted based on the x^2-test and Spearman's correlation test. $p<0.05$ was considered as statistically significant.

Results

Expression Level of miR-30c was Reduced in OS Tissues

Expression level of miR-30c was observed in 38 pairs of OS and pericarcinomatous tissues by qRT-PCR. The mRNA expression level of miR-30c was remarkably decreased in OS tissues when compared with the para-carcinoma tissues (Figure 1A). This result implied that miR-30c might participate in OS tumorigenesis.

Afterwards, qRT-RCR was adopted to detect the expression level of miR-30c in a number of

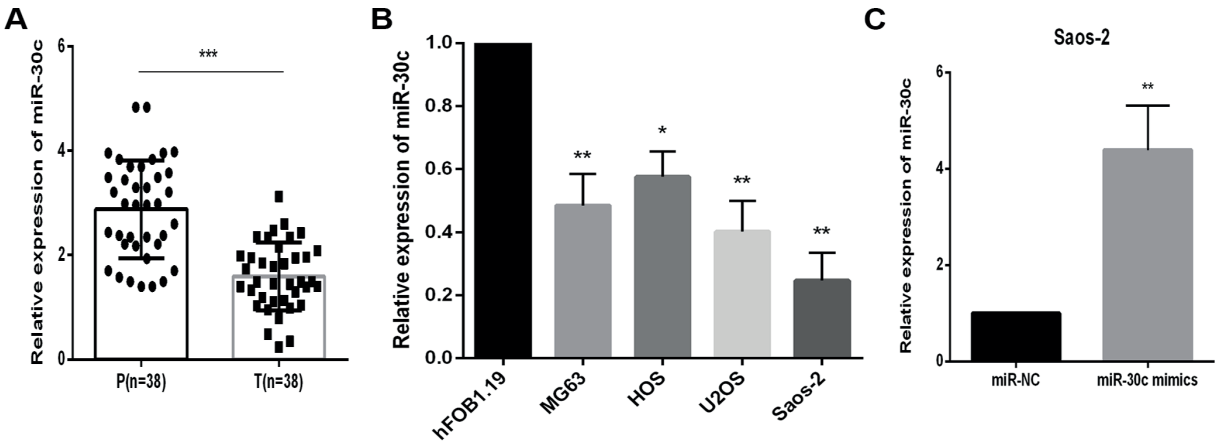

Figure 1. MiR-30c expression was decreased in OS tissues and cell lines. ***A,*** Analysis of miR-30c expression in para-carcinoma tissues (P) and tumor tissues (T); ***$p<0.001$. ***B,*** Analysis of miR-30c expression in several OS cell lines and normal cell line; *$p<0.05$ and **$p<0.01$ compared with normal human osteoblast cell (hFOB1.19). **C,** Analysis of transfection efficiency in Saos-2 cells transfected with miR-30c mimics and miR-NC. **$p<0.01$ compared with miR-NC. Total RNA was detected by qRT-PCR and GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments.

OS cell lines and normal breast cells. The results indicated that miR-30c was expressed on a comparatively lower expression level in the OS cell lines. Meanwhile, Saos-2 expressed the relatively lowest (Figure 1B). Then, miR-30c mimics and miR-NC were used to transfect Saos-2 cell line (Figure 1C) to further analyze the action mode of miR-30c in OS tumorigenesis *in vitro*.

MiR-30c Inhibited OS cell Proliferation and Viability

As results of CCK8, the Saos-2 cells viability was distinctly suppressed after transfected by miR-30c mimics in a time-dependent manner (Figure 2A).

Moreover, colony formation assay was carried out to explore proliferation ability of OS cell, the results of which showed less formed colonies of Saos-2 cells transfected with miR-30c mimics than the ones transfected with miR-NC (Figure 2B). We can figure out from these results that miR-30c could suppress the proliferation and viability of OS cells.

MiR-30c Induced Cell Apoptosis and Arrested Cell Cycle

After we found that miR-30c could affect the cell proliferation ability of miR-30c, we supposed that cell apoptosis and cell cycle progression could also be influenced by it. As shown in flow cytometry analysis, the apoptotic rate of miR-30c mimics transfected cells was higher than the one transfected with miR-NC (Figure 2C). Furthermore, transfection with miR-30c mimics could have a significantly higher proportion of Saos-2 cells at G0/G1 phase and lower proportion at S phase than the ones of transfection with control plasmid (Figure 2D). The results revealed that miR-30c might attenuate cell proliferation ability of OS by inducing cell apoptosis, as well as block cell cycle profession at G0/G1 phase.

MiR-30c Attenuated OS Cell Migration and Invasion Ability

Furthermore, we also analyzed the effect of miR-30c on OS cell metastasis *in vitro*. As results, overexpression of miR-30c could inhibit migration of OS cell compared with cells transfected with miR-NC (as shown in Figure 2E). Moreover, the effect of miR-30c on cell invasion was evaluated by transwell and the same trend was presented (Figure 2F). These results exhibited the inhibition role of miR-30c in metastasis of OS cell.

MiR-30c Directly Regulated SOX9

SOX9 was predicted as the potential downstream of miR-30c from the application and analysis realized by StarBase and TargetScan database (Figure 3A). Based on the results, miR-30c and SOX9 3'UTR Luciferase reporter genes were transfected into Saos-2 cell. As results, OS cell transfected with both wild-type SOX9 and miR-30c mimics showed attenuated luciferase activity than mutated SOX9 and miR-NC transfected cells (Figure 3B), revealing that SOX9 might be a direct downstream target of miR-30c. We next ex-

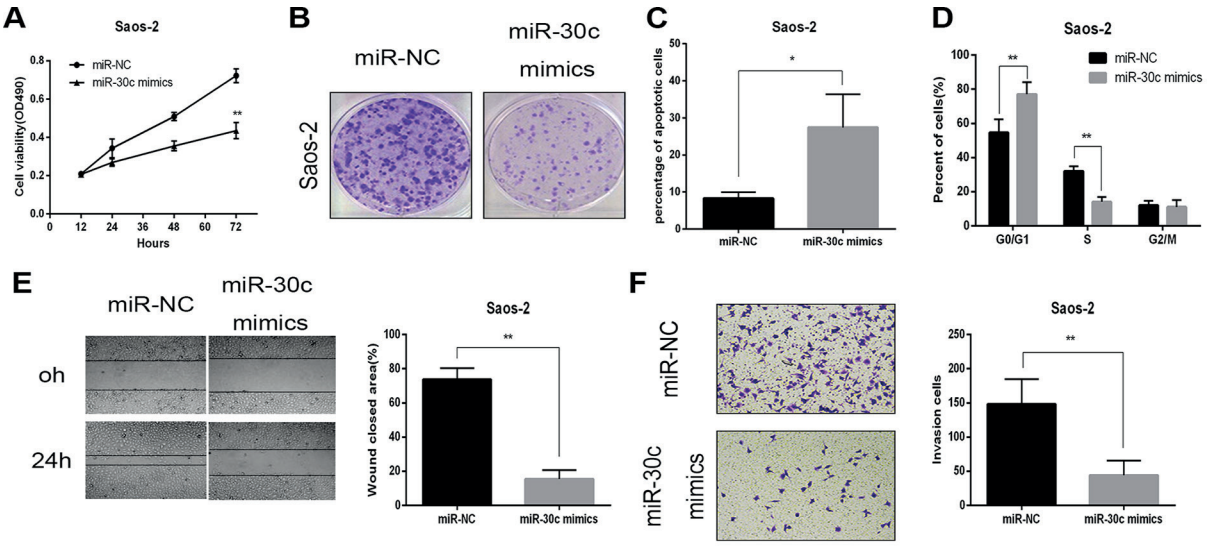

Figure 2. MiR-30c inhibited OS cell growth and metastasis *in vitro*. ***A,*** CCK8 assay was performed to determine the viability of transfected Saos-2 cells; ***B,*** Colony formation assay was performed to determine the proliferation of transfected Saos-2 cells; ***C,*** Flow cytometric analysis was performed to detect the apoptotic rates of transfected Saos-2 cells; ***D,*** Flow cytometric analysis was performed to detect cell cycle progression of transfected Saos-2 cells; ***E,*** Wound healing assay was performed to determine the migration of transfected Saos-2 cells; ***F,*** Transwell assay was performed to determine the invasion of transfected Saos-2 cells. *$p<0.05$; **$p<0.01$.

plored the expression of SOX9 in miR-30c mimic or miR-NC transfected OS cells. The results revealed that both the mRNA and protein level of SOX9 were downregulated in OS cells transfected with miR-30c mimics compared with that in miR-NC transfected cells (as shown in Figure 3C-D). The results also indicated that miR-30c could directly target SOX9.

Tumor Suppression Role of miR-30c could be rescued by SOX9

To explore the interrelation between miR-30c and SOX9 *in vivo*, the expression of SOX9 in OS tissues was measured. The results revealed the overexpression of SOX9 in OS tissues compared with that in para-carcinoma tissues on mRNA level (Figure 4A), meanwhile SOX9 expression was negatively correlated with miR-30c (Figure 4B). Afterwards, we studied if SOX9 is related to the roles of miR-30c in OS tumorigenesis. Thus, SOX9 expression was overexpressed by transfected with pCDNA3.1-SOX9 to obtain miR-30c-overexpressing Saos-2 cells (Figure 4C). The restoration of SOX9 aggrandized the proliferation ability of cells transfected with miR-30c (as shown in Figure 4D), inhibited cell apoptosis and arrested cell cycle at G0/G1 phase (Figure 4E-F), meanwhile it increased cell migration and invasion ability when compared with cells transfected with miR-NC (Figure 4G-H). These results revealed that miR-30c could suppress the OS tumorigenesis by regulating SOX9.

Discussion

Emerging researches have shown that microRNAs exert important functions in formation and development of different kinds of cancers. For instance, Xiang et al[13] indicated that miR-93-5p could inhibit tumor cell apoptosis, invasion and phenotype switch between EMT and MET based on regulation of MKL-1and STAT3 in breast cancer. Xia et al[14] demonstrated that the growth, as well as the differentiation of colorectal cancer cells could be suppressed by miR-22; therefore, miR-22/ PTEN/AKT pathway might service as a potential therapeutic target for colorectal cancer. A study conducted by Xiao et al[15] found that miR-100 could suppress the development and increase chemosensitivity of osteosarcoma cell by targeting ZNRF2. Another study[16] demonstrated that targeting RKIP could make miR-543 expedite the development and metastasis of prostate cancer cell.

OS has become a severe health threat to worldwide children and more and more researches have been developed. In recent years, some of the

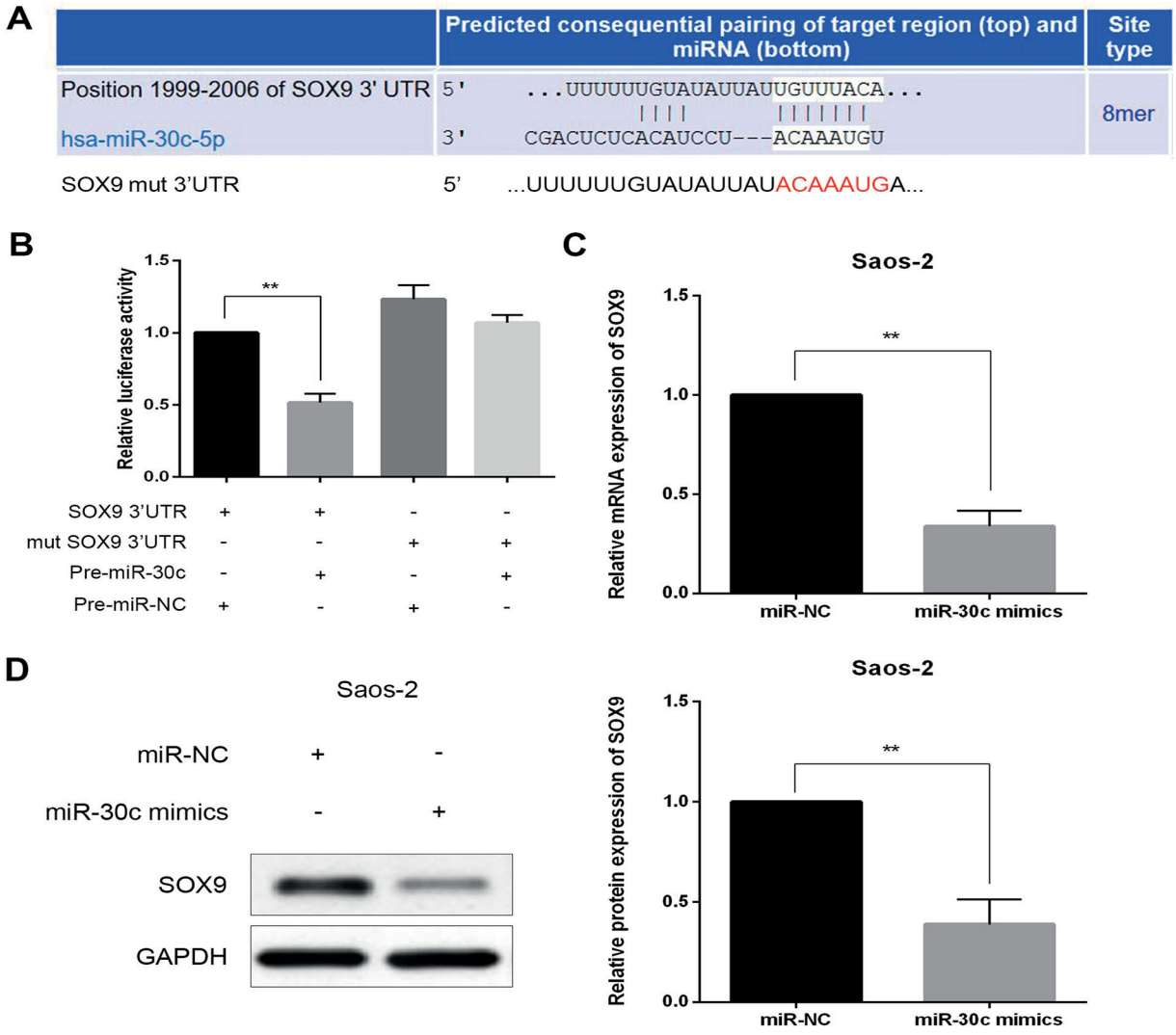

Figure 3. SOX9 is the target gene of miR-30c. ***A,*** SOX9 was selected as the potential downstream of miR-30c via using bioinformatics analysis; ***B,*** Luciferase activities of Saos-2 cells transfected with the wild-type or the mutated SOX9 3'UTR together with miR-30c mimics or miR-NC; ***C,*** Analysis of SOX9 mRNA expression level of Saos-2 cells transfected with miR-30c mimics or miR-NC; ***D,*** Analysis of SOX9 protein expression level of Saos-2 cells transfected with miR-30c mimics or miR-NC. Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$.

studies reported that microRNAs played a significant role in suppression of the tumorigenesis and development of OS. Zhao et al[17] stated that miR-19a played a role of a key regulator in OS via targeting PTEN. Jin et al[18] pointed out that miR-610 could work as a new tumor suppressor and reversed drug resistance by targeting TWIST1. Gai et al[19] stated that miR-22 could block OS cell cycle progression at G0/G1 phase to promote OS cell death. Li et al[20] suggested that miR-150 and its target gene ROCK1 could be key to OS. Until now the interaction between miR-30c and OS tumorigenesis has not been analyzed. In this study, the relationship proved that, compared with pericarcinomatous tissues, miR-30c was significantly down-regulated in OS tissues. This implied that miR-30c might potentially have an essential role in the tumorigenesis and development of OS. Moreover, overexpressed miR-30c could attenuate OS cell's abilities of viability, proliferation, invasion and migration, inducing cell apoptosis and blocking cell cycle at G0/G1 phase. The results implied that miR-30c was an important regulator to suppress the proliferation and metastasis of OS cell. In order to detect the underlying mechanism of how miR-30c suppressed tumorigenesis and

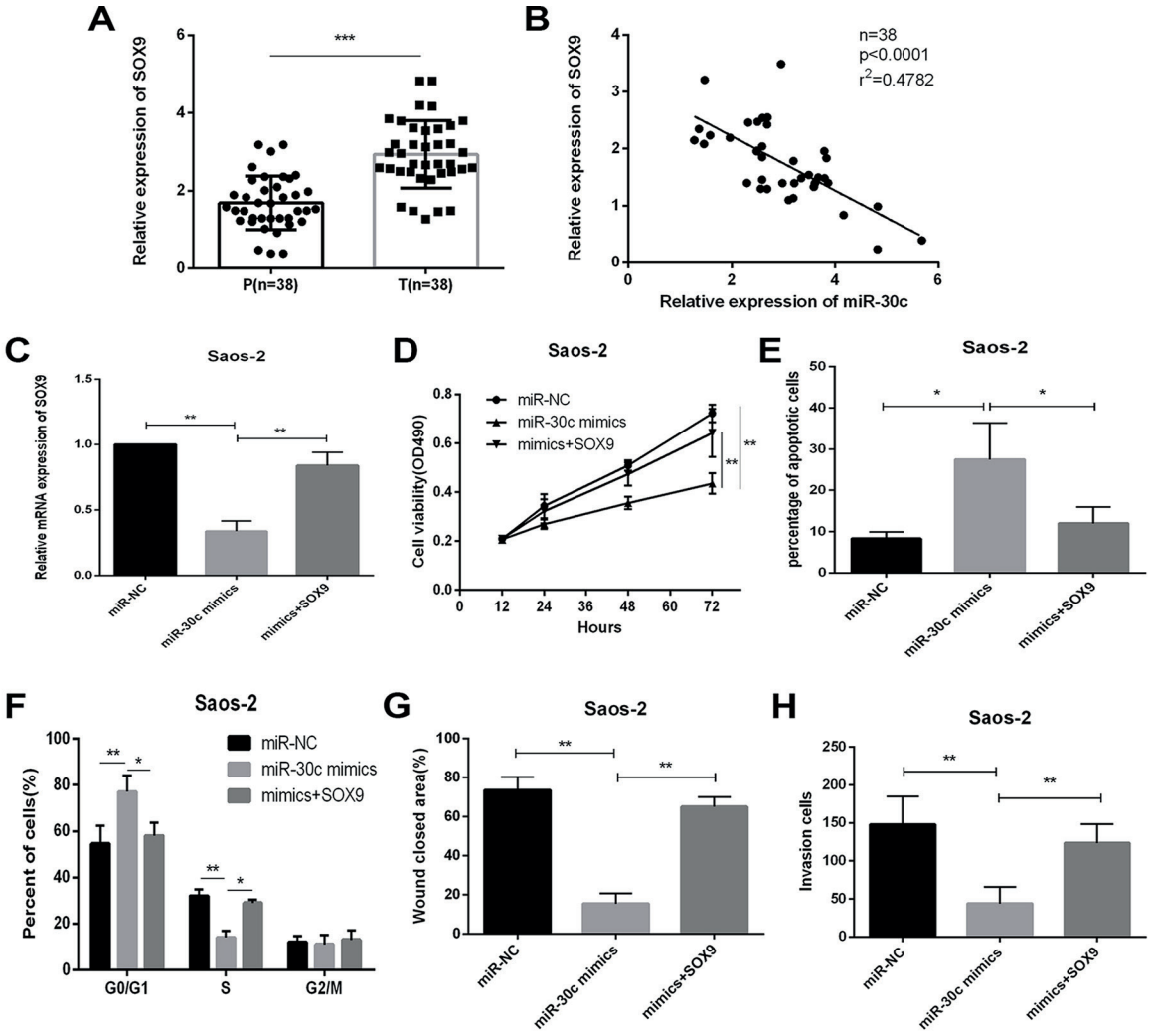

Figure 4. Restoration of SOX9 rescued tumor suppression role by miR-30c. ***A,*** Analysis of SOX9 expression level in OS tissues (T) and matched para-carcinoma tissues (N), n=38; ***B,*** Correlation between miR-30c and SOX9 expression in OS tissues (n=76); ***C,*** Analysis of transfection efficiency in Saos-2 cells transfected with miR-30c negative control (NC), mimics and/or pCDNA3.1-SOX9; **D,** Overexpressed SOX9 rescued suppressed cell proliferation by miR-30c; ***E,*** Overexpressed SOX9 attenuated cell apoptosis; ***F,*** Overexpressed SOX9 attenuated cell cycle distribution at G0/G1 phase; ***G,*** Overexpressed SOX9 increased cell migration of miR-30c-transfected cells; ***H,*** Overexpressed SOX9 increased cell invasion of miR-30c-transfected cells. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

metastasis of OS cell, SOX9 was selected as the novel target gene of miR-30c depending on bioinformatics analysis. SOX9, the abbreviation of sex determining region Y (SRY) related high-mobility group box 9, in humans is encoded by the SOX9 gene[21]. SOX9 is a member of E subfamily of SOX gene family, a nuclear transcription factor associated with early embryonic development, involved in many physiological and pathological processes, such as cell growth, apoptosis, differentiation and tumor cell infiltration and metastasis[22]. Its main physiological functions are cartilage formation, determination of gender differentiation and glial and heart development[23-25]. In addition, it has also been found that its abnormal expression is related to many human diseases[26]. The abnormal expression of SOX9 may promote the tumorigenesis and development of a variety of human tumors. SOX9 expression is abnormally increased in some tumors playing as a cancer gene, such as lung cancer[27], colorectal cancer[28], pancreatic cancer[29], and prostate cancer[30]. The effects of SOX9 on tumors include the tumor cell proliferation, differentiation and apoptosis, tumor inva-

sion and metastasis, etc. The abnormal expression of SOX9 in tumors is associated with the low survival rate, poor prognosis and decreased susceptibility to some chemotherapeutic drugs[31,32]. Furthermore, dysregulation of SOX9 also plays an important role in OS, whereas the underlying mechanism of how SOX9 exerts function in OS has not been adequately studied and confirmed. In the present work, SOX9 was predicted as the potential downstream target gene of miR-30c by prediction software, and the luciferase reporter gene analysis also showed that there was a true binding relationship between them. Meanwhile, the re-validation of RNA and protein levels in OS cells also proves that there is a negative effect between miR-30c and SOX9. At the level of OS tissue, we also found a negative correlation between the expression of miR-30c and SOX9. Thus, we can underline the target relationship between miR-30c and SOX9. Furthermore, tumor suppression role of miR-30c could be rescued by restoration of SOX9 on the growth and metastasis of OS cell. For example, it promotes the recovery of the capacity of cell invasion, migration, and proliferation, reduces the level of cell apoptosis, and weakens the distribution of cell cycle in G0/G1 phase. Evidence showed that miR-30c might locate upstream of SOX9 and be involved in tumorigenesis and metastasis of OS.

Conclusions

We demonstrated that MiR-30c could play an important role in tumor suppression for pediatric osteosarcoma progression and metastasis by targeting SOX9 *in vitro*. This research may be helpful for elucidating the underlying mechanisms of OS progression, providing a creative and potential target for diagnosis and treatment of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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