miR-429 inhibits osteosarcoma progression by targeting HOXA9 through suppressing Wnt/β-catenin signaling pathway

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Received July 12, 2019; Accepted May 20, 2020

DOI: 10.3892/ol.2020.11766

Abstract. Osteosarcoma (OS) is the most commonly diagnosed malignant cancer of bone that occurs in adolescents and children. Mounting number of studies have indicated that miRNAs are increasingly playing fundamental roles in OS development. Thus, the biological function of miR-429 in OS progression was explored. The results of RT-qPCR revealed that miR-429 was downregulated in OS tissues and OS cell lines (MG-63, U2OS, Saos-2) while homeobox A9 (HOXA9) was markedly increased. Moreover, HOXA9 was confirmed as a direct target of miR-429 by using luciferase reporter assay. It was identified that miR-429 exhibited a suppressive effect on OS progression while HOXA9 showed the oncogenic function in OS progression by using MTT and Transwell assays. More importantly, rescue assays manifested that HOXA9 can partially overturn the suppressive effect of miR-429 on OS. Overexpression of miR-429 inhibited the activation of Wnt/β-catenin signaling pathway. In conclusion, miR-429 suppressed OS progression by targeting HOXA9 through Wnt/β-catenin pathway.

Introduction

Osteosarcoma (OS), the most frequent primary bone malignancy affects adolescents and young adults, is characterized by occurrence at the extremities of long bones and originating from primitive osteogenic mesenchymal cells (1). OS is most common in pediatric bone malignancies, accounting for ~5% of pediatric tumors (2). Currently, chemotherapeutic treatments combined with surgical resection is one of the predominant treatments for patients with OS (3,4). Despite considerable progress in the diagnosis and treatment of OS, the overall clinical efficacy of OS treatment remains unsatisfactory (5,6). However, pulmonary metastasis is the predominant site of osteosarcoma recurrence and the most general cause of mortality (7). Thus, it is necessary to look for new strategies for treatment of OS.

MicroRNAs (miRNAs) are a kind of regulatory RNA, which are small endogenous non-coding RNAs consisting 18-25 nucleotides, and negatively regulate translation of the specific target gene via directly binding to 3'UTR region of target mRNAs (8). Increasing evidence has demonstrated that miRNAs display important roles in modulating the progression and development of various tumors (9-11). Accumulating evidence confirms that dysregulated miRNAs contribute to multiple physiological and pathological processes in different malignancies (including osteosarcoma), such as apoptosis, cell proliferation, and autophagy (12-14). Extensive research has shown that microRNA-429 (miR-429) deregulates and plays major roles in many tumors including melanoma, esophageal squamous cell carcinoma, renal cell carcinoma, and OS (15-18). Therefore, determining the exact role of miR-93 in OS carcinogenesis might contribute to improving the diagnosis and prognosis of patients with this tumor.

HOXA9, is a homeobox (HOX) gene, reported to regulate several malignant diseases. For instance, increasing expression of HOXA9 inhibited lung cancer cell invasion and migration (19). Makabe *et al* (20) found that HOXA9 was involved in endometrial carcinogenesis. Furthermore, HOXA9 was reported to promote leukemogenesis (21). In addition, HOXA9 was reported as the direct target of some miRNAs in regulation of tumor development and progression. A previous study reported that HOXA9 acted as the target of miR-873 in regulating the progression of OS (22). It was also demonstrated as the target of miR-182 in regulating gastric cancer cell proliferation and migration (23). However, whether HOXA9 is the target of miR-429 in modulating OS cell viability, invasion and migration has not been reported.

Wnt/β-catenin pathway is well known to participate in tumorigenesis. Previous studies have stated that HOXA9 could regulate Wnt/β-catenin pathway in glioma cell growth (24) and regulate OS cell proliferation and apoptosis (25). In this study, we aimed to test miR-429 functional role in OS progression and to confirm whether miR-429 modulate OS progression by targeting HOXA9 through Wnt/β-catenin pathway.

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Key words: osteosarcoma, miR-429, HOXA9, Wnt/β-catenin

Materials and methods

Samples. Fifty-six paired OS tissues and adjacent normal tissues were collected from the patients who underwent surgery in Weifang People's Hospitall (Weifang, China) between April 2011 and October 2017. The mean age of all patients was 9.4 years, and the age range 5-17 years. None of the patients received any treatment prior to surgery. All the tissues were collected in the same condition and were histopathologically verified carcinoma. The fresh OS tissues were confirmed by pathologists and then stored at -80˚C for further experimental use. This study was conducted in accordance with the Declaration of Helsinki. The Ethics Committee of Weifang People's Hospital approved tissue sample collection and use protocols. Written informed consent was obtained from each patient and their parents before collecting the specimens. Tables I and II show the demographic features and clinicopathological data.

Cell culture. The OS cell lines (MG-63, U2OS, Saos-2) and normal human fetal osteoblast (hFOB 1.19) cells were purchased from Tianjin Sai'er Biotechnology Co., Ltd. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) and DMEM/F-12 medium, respectively. The media contained 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 IU/ml penicillin and 100 mg/ml streptomycin (both from Baomanbio). The cells were maintained at 37˚C with 5% CO_2.

Cell transfection. Overexpression of miR-429 in MG-63 cells and knockdown of miR-429 in Saos-2 cells due to its expression was lower in Saos-2 cells than in MG-63 cells. miR-429 mimic, miR-429 inhibitor, control mimic and control inhibitor were used for increasing or decreasing miR-429, and was obtained from Shanghai GenePharma Co., Ltd. HOXA9 siRNA was synthesized by Shanghai GeneChem Co., Ltd. When the cells reached growth phase, they were collected for transfection. Transfections were finished by using the Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). miR-429 mimic, forward, 5'-UAAUACUGUCUGGUAAAACCGU-3' and reverse, 5'-GGUUUUACCAGACAGUAUUAUU-3'; NC mimic, forward, 5'-UUCUCCGAACGUGUCACGUUT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; miR-429 inhibitor, 5'-GCTGATTTAAAGGCTTAG-3'; NC inhibitor, 5'-CAAATGTAGGTAGAGGA-3'; HOXA9 siRNA, 5'-ACGGCAUUACCAGACAGUAUUA-3'; NC siRNA, 5'-AGCGUGUAGCUAGCAGAGG-3'. The efficiency of knockdown and upregulation was analyzed using qRT-PCR.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was applied for extracting total RNA from OS clinical tissues and cell lines. TaqMan MicroRNA Reverse Transcription kit (Takara) was applied for performing reverse transfection. TaqMan miRNA qRT-PCR Kit (Takara) was used for performing quantitative RT-PCR. U6 and GAPDH were applied for normalizing miR-429 level and HOXA9, respectively. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 3 min and 35 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. A melting curve analysis was performed to detect products. The relative expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (26). The primer sequences are shown in Table III.

MTT assay. 3-(4, 5-Dimethylthiazolyl-2)- 2,5-diphenyltetrazolium bromide (MTT) assay was carried out for assessing cell viability. The cells treated with miR-429 mimic, miR-429 inhibitor, control mimic and control inhibitor were seeded in 96-well plates at a density of 2,000 cells/well with three duplicate wells per group and cultured for 1, 2, 3, 4 days. MTT solution (20 μl) was added to each well and incubated for another 4 h at 37˚C. Then, the medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals by swirling gently. Finally, the absorbance was measured at 490 nm using a spectrophotometric plate reader.

Transwell assays. Transwell assays were applied for detection of cell migration and invasion. The steps performed for the migration and invasion were similar, except for the top chamber coating with or without Matrigel. The Transwell chambers (8 μm pore filter) were coated with or without 100 ml Matrigel (5 mg/ml) (BD Biosciences). In brief, cells were added into the top chamber and DMEM medium containing 20% FBS was added to the lower chamber. The upper and lower chamber was separated by polycarbonate film. After incubation for 24 h at 37˚C, the cells were invaded or migrated to the lower chamber. The non-migrated and non-invaded cells on the upper surface of the membrane were carefully scraped off using a cotton swab. Migrated and invaded cells were fixed with methanol and stained with 0.5% crystal violet for 10 min at room temperature. Then, the cells in the lower chamber were fixed, stained and finally counted with a microscope (Olympus Corporation).

Western blot analysis. Radio immunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich; Merck KGaA) was applied for lysing OS cells. The protein concentration was determined using the BCA assay, the proteins (10 μg) were separated by 15% SDS-PAGE, followed by transferred to NC membranes. Then, the membranes were blocked for 1 h at 37˚C with 5% skimmed milk overnight. Subsequently incubated with primary antibodies (HOXA9, ab140631, 1:1,000; β-catenin, ab32572, 1:1,000; c-myc, ab32072, 1:1,000; p-c-Jun, ab32385, 1:1,000; GAPDH, ab181602, 1:1,000; all from Abcam) at room temperature for 3 h, and then incubated with sheep anti-mouse or donkey anti-rabbit horseradish peroxidase-conjugated (HRP) antibody (GENXA931-1ML and GENA934-1ML, Sigma-Aldric; Merck KGaA). The enhanced chemiluminescence reagent (ECL; Pierce Biotechnology, Inc.) was used to conduct autoradiography.

Dual-luciferase reporter assay. The putative targets of miR-130a-3p were predicted using the TargetScan (http://www.targetscan.org/vert_72/). The wild-type (WT) or mutant type (MuT) of HOXA9 3'UTR fragments were sub-cloned into the pMIR-REPORT vector. Then, the MG-63 and Saos-2 cells were co-transfected with miR-429 mimic, miR-429 inhibitor, control mimic and control inhibitor along with the 3'UTR of HOXA9 using Lipofectamine 2000 reagent. After transfection for 48 h, the luciferase activity was

Table I. Association between miR-429 expression and clinicopathological characteristics of patients with OS.

		miR-429		
Characteristics	Cases	Low	High	P-value
Age, years				0.798
≥18	20	14	6	
<18	36	24	12	
Sex				0.176
Female	30	18	12	
Male	26	20	6	
Tumor size, cm				0.022[a]
≤5	31	25	6	
>5	25	13	12	
TNM stage				0.012[a]
I	26	22	4	
II/III	30	16	14	
Distant metastasis				0.013[a]
No	29	24	5	
Yes	27	14	13	
Location				0.022[a]
Femur/Tibia	28	23	5	
Elsewhere	28	15	13	

Statistical analyses were performed by the χ^2 test. [a]P<0.05 was considered significant. OS, osteosarcoma.

Table II. Association between HOXA9 expression and clinicopathological characteristics of patients with OS.

		HOXA9		
Characteristics	Cases	High	Low	P-value
Age, years				0.472
≥18	30	18	12	
<18	26	18	8	
Sex				0.264
Female	28	20	8	
Male	28	16	12	
Tumor size, cm				0.012[a]
≤5	32	25	7	
>5	24	11	13	
TNM stage				0.018[a]
I	34	26	8	
II/III	22	10	12	
Distant metastasis				0.003[a]
No	26	22	4	
Yes	30	14	16	
Location				0.439
Femur/Tibia	29	20	9	
Elsewhere	27	16	11	

Statistical analyses were performed by the χ^2 test. [a]P<0.05 was considered significant. OS, osteosarcoma; HOXA9, homeobox A9.

measured by a luciferase reporter assay system (Promega Corporation), and *Renilla* luciferase activity was used to normalize the data.

Statistical analysis. All experimental conditions were repeated in duplicate. Results are presented as mean ± SD. Statistic analysis was performed using SPSS v.19.0 software (SPSS, Inc.). Unpaired Student's t-test or Tukey's post hoc test of one-way analysis of variance (one-way ANOVA) was applied for comparing the differences between two groups or more than two groups. The clinical association was analyzed with Chi-square test. P<0.05, was considered statistically significant.

Results

miR-429 expression is decreased and HOXA9 expression is increased in OS. According to previous studies, miR-429 and HOXA9 were dysregulated in multiple tumors. Here, the aim was to test miR-429 and HOXA9 expression in OS tissues and cell lines. As illustrated in Fig. 1A, the expression of miR-429 was downregulated in OS tissues compared with that in normal tissues. Subsequently, the expression of miR-429 expression was decreased in OS cell lines compared with that in normal human fetal osteoblasts (hFOB 1.19) as shown in Fig. 1B. Moreover, miR-429 expression was lowest in MG-63 cells compared with that in U2OS and Saos-2 cells (Fig. 1B). Furthermore, the findings also showed that HOXA9 was increased in OS tissues (Fig. 1C) and cell lines (Fig. 1D). Due to the opposite expression of miR-429 and HOXA9 in OS, we detected the correlation between miR-429 and HOXA9. Results showed that their relationship was negative (Fig. 1E). To confirm their clinical importance, we divided the subgroups (high/low) according to the median value as a cutoff of miR-429 and HOXA9. In addition, the expression of miR-429 (Table I) and HOXA9 (Table II) were notably associated with tumor size, TNM stage and distant metastasis.

Table III. Primer sequences for real-time fluorescence quantification PCR.

Gene	Primer sequences
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3'
	R:5'-ATGGTGGTGAAGACGCCAGT-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R:5'-AACGCTTCACGAATTTGCGT-3'
miR-429	F: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTA-3'
	R:5'-AGTGCAGGGTCCGAGGTATT-3'
HOXA9	F: 5'- CAACAAAGACCGAGCAAA-3'
	R:5'- CAACAAAGACCGAGCAAA-3'

F, forward; R, reverse; HOXA9, homeobox A9.

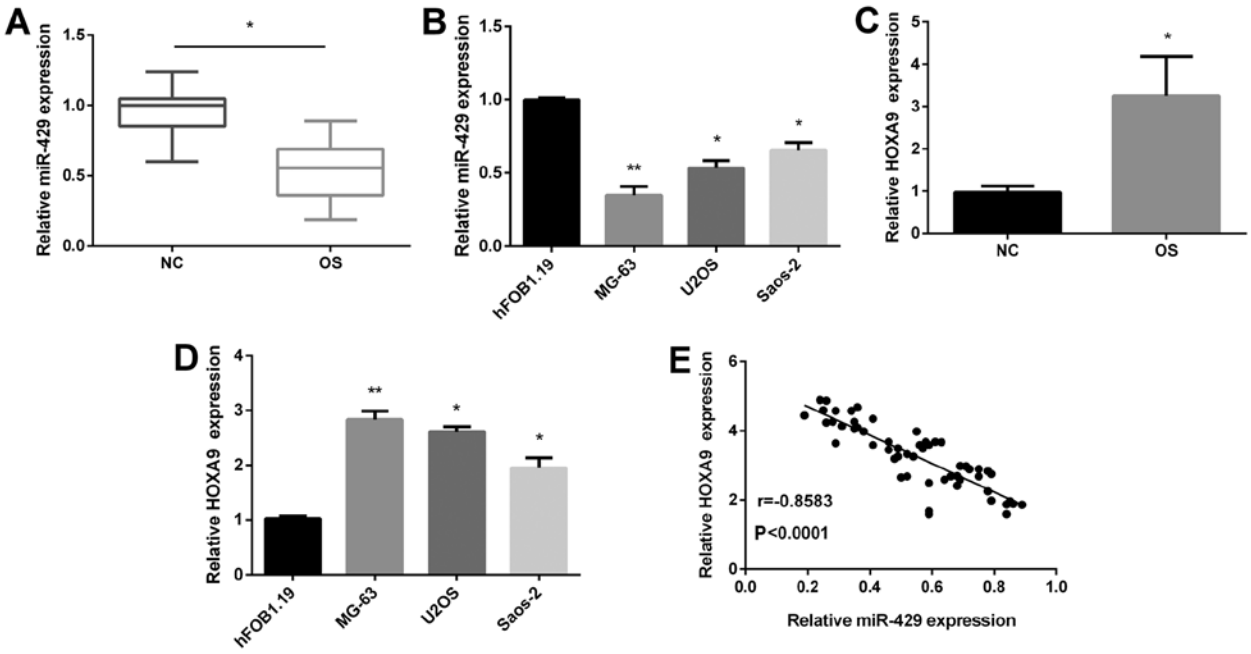

Figure 1. Downregulation of miR-429 and upregulation of HOXA9 in OS. (A) Measurement of miR-429 expression in OS tissues (n=56). (B) Measurement of miR-429 expression in OS cells. (C) HOXA9 expression measured in OS tissues (n=56). (D) HOXA9 expression measured in OS cell lines. (E) Relationship between miR-429 and HOXA9 (r=-8583; P<0.0001). *P<0.05, **P<0.01. HOXA9, homeobox A9; OS, osteosarcoma.

Collectively, the above results indicated that miR-429 might serve as a tumor suppressor while HOXA9 serves as an oncogene in OS.

miR-429 suppresses OS cell viability, invasion and migration. As stated, the expression of miR-429 was highest in Saos-2 cells, the expression of miR-429 was lowest in MG-63 cells. Thus, miR-429 inhibitor was transfected in Saos-2 cells and miR-429 mimic in MG-63 cells. Results of qRT-PCR revealed that miR-429 expression was increased by miR-429 mimic in MG-63 cells and reduced by miR-429 inhibitor in Saos-2 cells (Fig. 2A). MTT assay was applied for testing MG-63 cell viability after treated with miR-429 mimic or Saos-2 cells after treated with miR-429 inhibitor. The results of MTT assay showed that miR-429 overexpression suppressed MG-63 cell proliferation, while miR-429 knockdown remarkably promoted Saos-2 cell proliferation (Fig. 2B). Furthermore, Transwell analysis showed that miR-429 overexpression reduced MG-63 cell migration and miR-429 knockdown showed the opposite effect on Saos-2 cells (Fig. 2C). For invasion, upregulating of miR-429 expression inhibited MG-63 cell invasion, while downregulating of miR-429 expression enhanced Saos-2 cell invasion (Fig. 2D). These findings demonstrated that miR-429 inhibited OS cell proliferation, invasion and migration.

HOXA9 is the target of miR-429. As we found that HOXA9 mRNA in tissues was inversely correlated with miR-429 expression, we further investigated whether HOXA9 was the target of miR-429. By searching both TargetScan (http://www.targetscan.org) and Sanger (http://microrna.sanger.ac.uk) databases, it was found that the 3'UTR of HOXA9 contains conserved miR-429-binding sites (Fig. 3A). Then, luciferase reporter assay was applied for confirmation. The findings revealed that overexpression of miR-429 significantly suppressed firefly luciferase reporter activity of the WT HOXA9 3'UTR, while miR-429 knockdown increased its luciferase activity, however, they did not affect the luciferase activity of MuT-HOXA9 3'UTR (Fig. 3B). Next, qRT-PCR and Western blot analysis were carried out for detecting the effect of miR-429 on HOXA9 expression in mRNA and protein level, respectively. The findings demonstrated that miR-429 reversely regulated the mRNA and protein expression of HOXA9 in OS cells (Fig. 3C and D). Taken together, the above results confirmed the inverse relationship between miR-429 and HOXA9.

Knockdown of HOXA9 reverses the effects of miR-429 on OS cells. The effect of HOXA9 on OS cell proliferation, invasion and migration were measured. As HOXA9 was overexpressed in OS tissues and cell lines compared with normal tissues and human fetal osteoblasts (hFOB 1.19), HOXA9 siRNA was tranfected into OS cells to inhibit HOXA9 expression. As expected (Fig. 4A), HOXA9 expression was decreased after transfection with HOXA9 siRNA. The results of MTT assay found that knockdown of HOXA9 expression inhibited OS cell viability, while decreasing miR-429 expression promoted cell expression. Furthermore, HOXA9 siRNA reversed the promotion effect of miR-429 inhibitor on OS cell viability (Fig. 4B). In addition, similar effects were observed on the migration and invasion capacity of OS cells (Fig. 4C and D). These findings demonstrated that HOXA9 is involved in miR-429-mediated regulation of OS cells.

miR-429 regulates Wnt/β-catenin pathway in vitro. To further examine the mechanism of miR-429 in modulating OS progression, the expression of the downstream genes of Wnt/β-catenin pathway was tested by Western blot

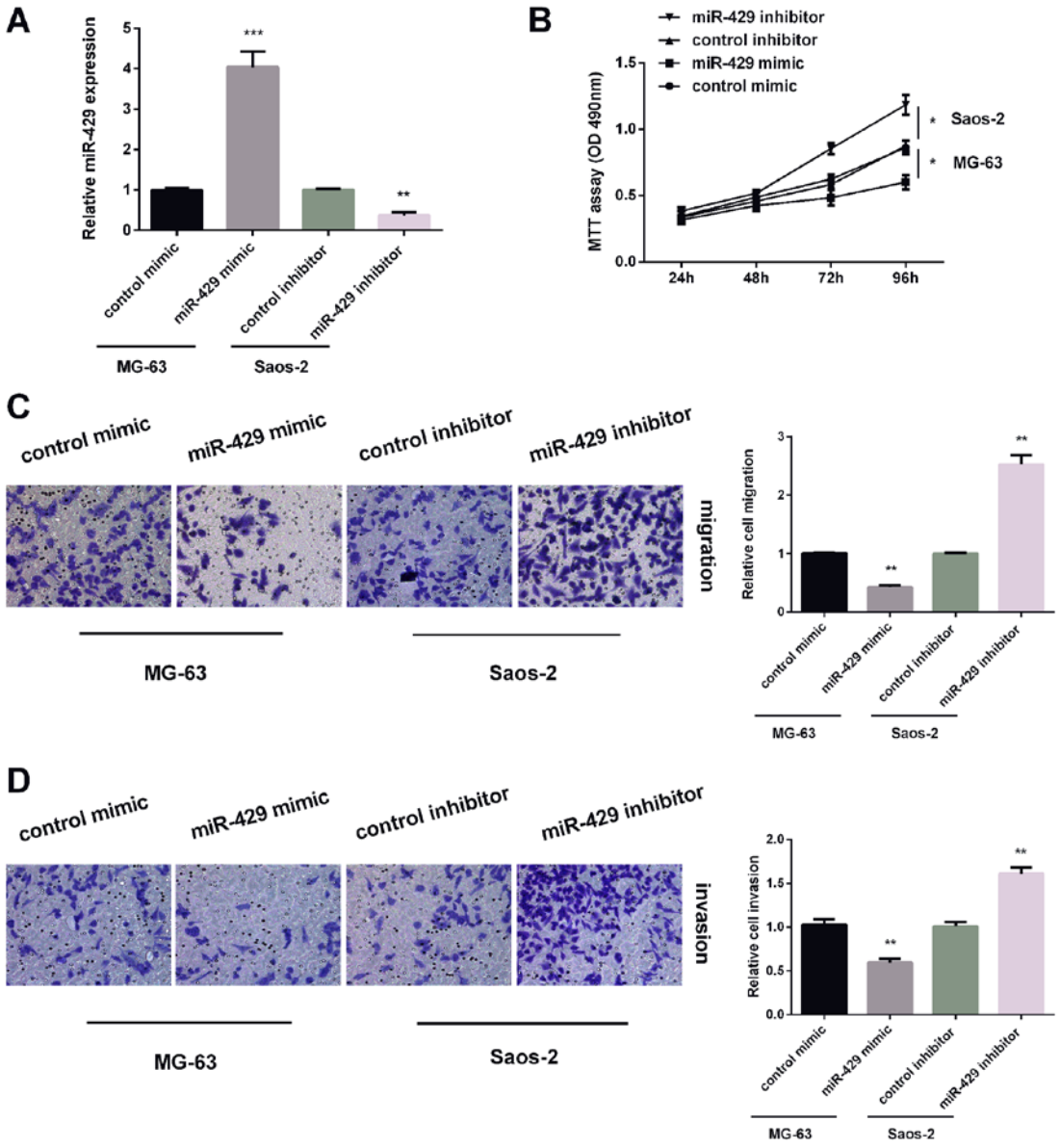

Figure 2. Impeding effect of miR-429 on OS progression. (A) miR-429 expression tested in MG-63 cells after increasing miR-429, and miR-429 expression tested in Saos-2 cells after repressing miR-429. (B) MG-63 cell viability detected after increasing miR-429 expression, Saos-2 cell viability detected after decreasing miR-429 expression. (C) MG-63 cell migration measured after increasing miR-429 expression, Saos-2 cell migration tested after decreasing miR-429 expression. (D) MG-63 cell invasion after treated with miR-429 mimic, Saos-2 cell invasion after treated with miR-429 inhibitor. *P<0.05, **P<0.01, ***P<0.001.

analysis. As shown in Fig. 5A and B, miR-429 overexpression suppressed the expression level of β-catenin, c-myc and p-c-Jun, as well as HOXA9 in MG-63 cells, while knockdown of miR-429 exhibited the opposite effect on these levels. Taken together, the findings indicated that miR-429 inhibited OS progression by targeting HOXA9 and suppressing Wnt/β-catenin pathway.

Discussion

OS is the most frequent primary bone malignancy, it affects adolescents and young adults, and is characterized by high incidence, great potential for metastasis and rapid progression (27,28). Although years of continuous progress have been obtained in OS therapy, the metastasis rates and mortality of OS are still very high, and the clinical effect of OS treatment remains unsatisfactory (29). Therefore, it is urgent to identify targets for the development of therapeutics for OS. The molecular mechanisms of OS carcinogenesis, especially regarding alterations of miRNAs in OS have attracted much attention in recent decades.

Increasing evidence has indicated that miRNAs play vital roles as diagnostics biomarkers and therapeutic targets in human malignancies, and expression of miRNAs are dysregulated, including OS (30,31). miR-429, in combination with the other four members (miR200a, miR-200b, miR-200c and miR-141), forms the miR-200 family (32). miR-429 mostly took part in the development of many tumors as a tumor suppressor. For example, miR-429 expression was downregulated in human thyroid cancer and it inhibited cell growth and promoted cell apoptosis (33). Moreover, miR-429 served as a suppressor in cervical cancer (34) and in pancreatic cancer (35). However,

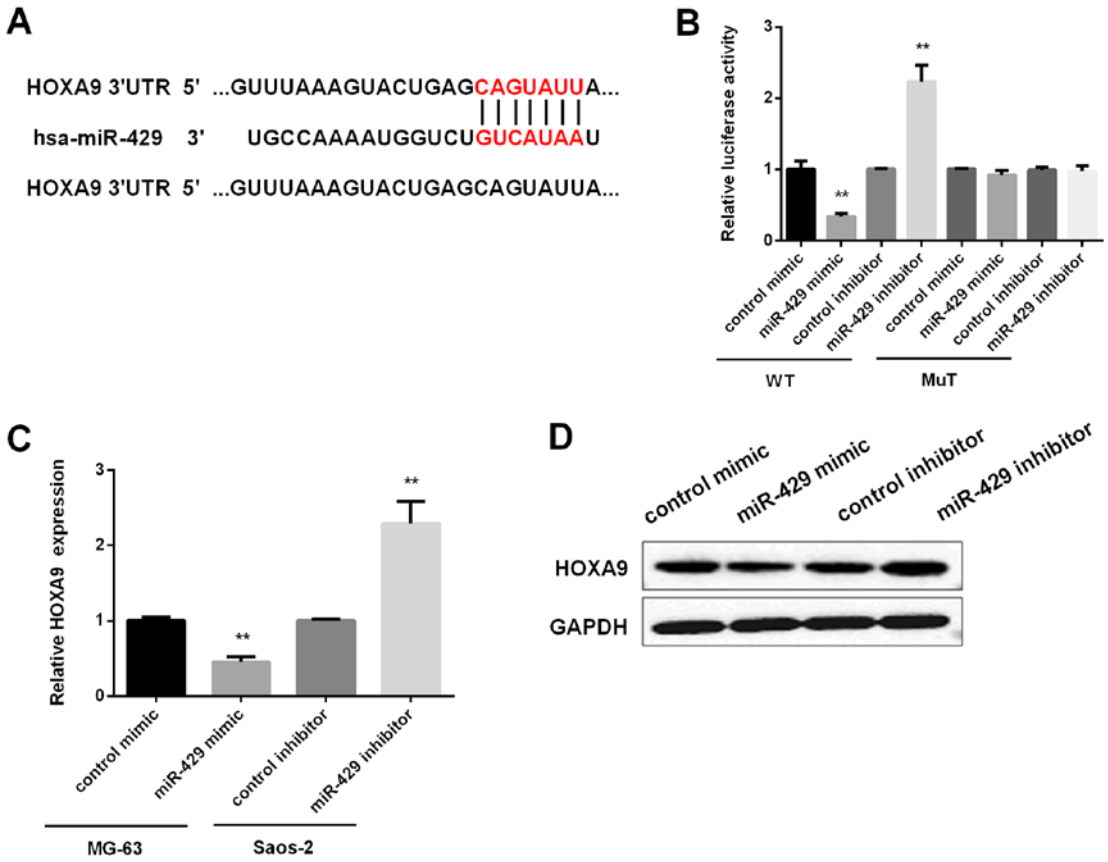

Figure 3. HOXA9 is the direct target of miR-429. (A) The binding site of miR-429 with HOXA9 3'UTR. (B) Detection of HOXA9 3'UTR luciferase activity in MG-63 and Saos-2 cells after increasing or decreasing miR-429. (C) Detection of HOXA9 protein level in MG-63 and Saos-2 cells after increasing or decreasing miR-429. (D) HOXA9 mRNA expression in MG-63 and Saos-2 cells after increasing or decreasing miR-429. **P<0.01. HOXA9, homeobox A9.

studies concerning miR-429 expression and its functional effect in OS are still rare and it is therefore necessary to investigate the underlying mechanisms of miR-429 in OS. Herein, it was shown that miR-429 was decreased in OS tissues and cells, which was in line with the studies reported by Liu *et al* and Deng *et al* (18,36). Based on previous studies, we further found that miR-429 suppressed OS cell proliferation, invasion and migration. The low expression of miR-429 was obviously associated with adverse clinical pathological issues of OS patients, including OS tumor size, TNM stage and clinical stage.

Combined with the bioinformatic analysis tool and dual-luciferase activity reporter assay, HOXA9 was validated a putative target for miR-429. HOXA9 is a member of the HOX gene family reported to have crucial roles in regulating hematopoiesis (37). Previous studies have shown that HOXA9 was the target of miRNAs in acute myeloid leukemia (38), in cutaneous squamous cell carcinoma (39) and in ovarian cancer (40). Therefore, we investigated whether HOXA9 was a mediator of miR-429 tumor inhibition in OS cells. In this study, it is first displayed that HOXA9 is a direct target of miR-429 in OS progression. Moreover, it was found that HOXA9 acted as an oncogene in OS development. These findings are consistent with previous research that HOXA9 level is increased markedly in ovarian cancer patients (41) and it was upregulated in colon cancer (42). The higher expression of HOXA9 was associated with OS tumor size, TNM stage and clinical stage. Furthermore, we showed that inhibiting HOXA9 suppressed OS cell viability, invasion and migration and it could overturn miR-429 inhibitor effect on OS progression. The above findings suggested miR-429 may act as a tumor suppressor by increasing HOXA9 expression in OS progression. HOXA9 overexpression reverses the inhibition effects of miR-429 on OS cells.

Increasing evidence shows that Wnt/β-catenin axis was regulated, for example, by miR-25, -376c, -21, -34a miRNAs in OS progression (43-45). Wnt/β-catenin signaling pathway modulates various genes that in turn regulate a diversity of cell functions such as proliferation, differentiation and morphogenesis, and β-catenin was proved to enhance progression, invasion and tumorigenesis in cancers (46). Increasing evidence has suggested that the signaling pathway was activated in OS cells and the aberrant regulation of the signaling pathway results in tumorigenesis of OS cells (47-49). To confirm the involvement of Wnt/β-catenin signaling pathway in regulating migration of OS cells, Western blotting was performed to analyze the nuclear fractions of β-catenin, c-myc and p-c-Jun. In this study, results show that miR-429 upregulation deactivated the Wnt/β-catenin pathway, while suppressing miR-429 promoted the activation of this pathway.

In conclusion, miR-429 was underexpressed in OS and repressed OS progression, whereas, HOXA9 was overexpressed in OS and facilitated OS progression. Moreover, HOXA9 was shown as the direct target of miR-429 in OS. Taken together, miR-429 suppressed OS progression by targeting HOXA9 via Wnt/β-catenin signaling pathway.

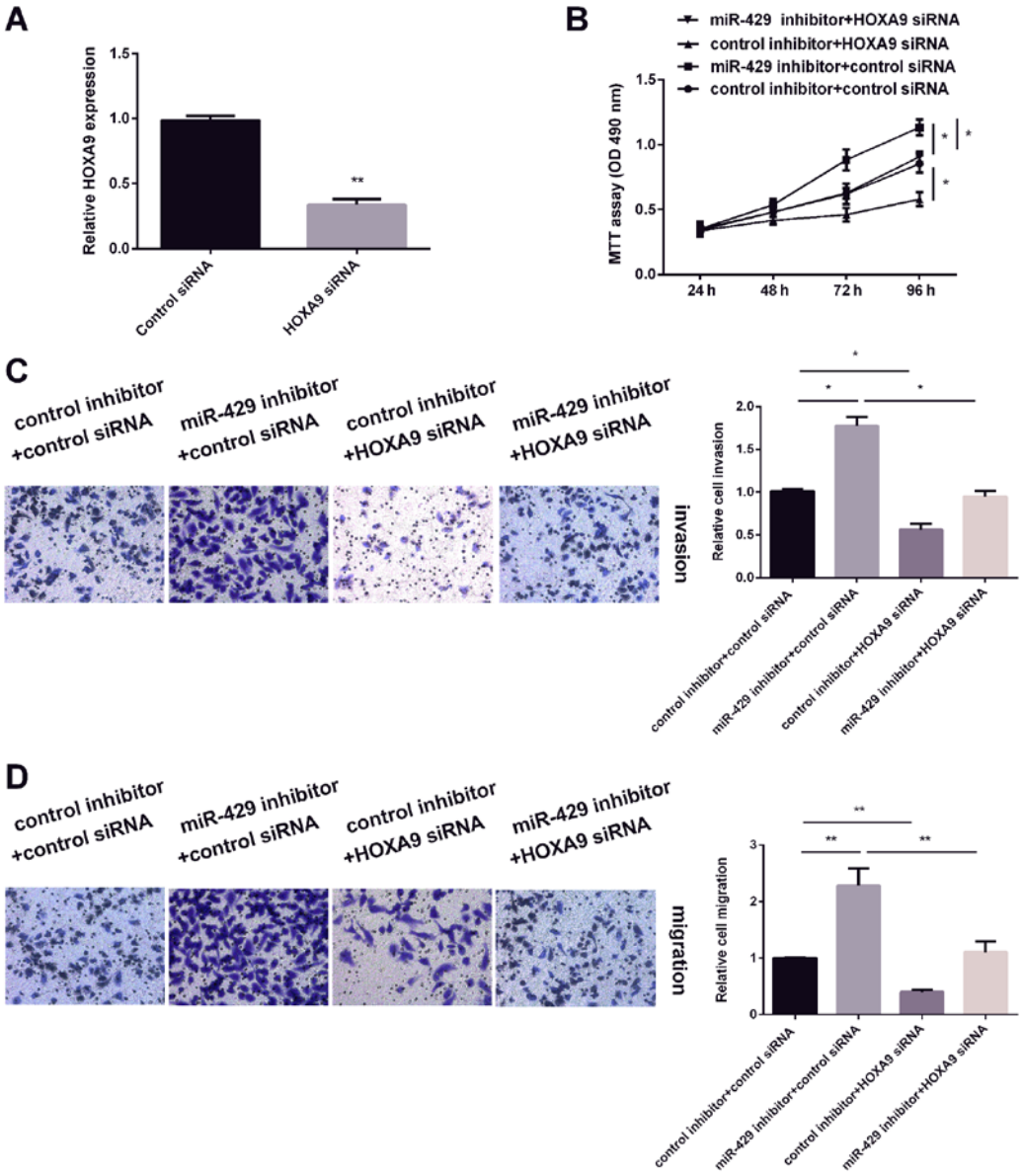

Figure 4. Inhibitory effect of HOXA9 siRNA on OS progression. (A) Measurement of HOXA9 mRNA expression in Saos-2 cells after inhibition of HOXA9. (B) Measurement of Saos-2 cell viability after treated with miR-429 inhibitor, HOXA9 siRNA, or miR-429 along with HOXA9 siRNA. (C) Measurement of Saos-2 cell migration after treated with miR-429 inhibitor, HOXA9 siRNA, or miR-429 along with HOXA9 siRNA. (D) Measurement of Saos-2 cell invasion after treated with miR-429 inhibitor, HOXA9 siRNA, or miR-429 along with HOXA9 siRNA. *P<0.05, **P<0.01. HOXA9, homeobox A9.

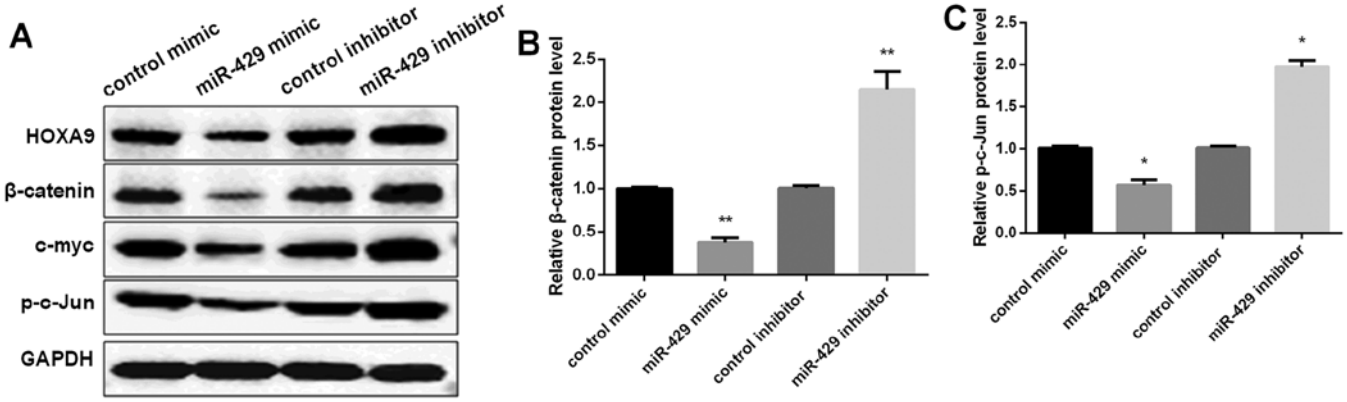

Figure 5. miR-429 regulates activation of Wnt/β-catenin *in vitro*. (A) Detection of the expressional level of HOXA9, β-catenin, c-myc and p-c-Jun in MG-63 cells after miR-429 overexpression and in Saos-2 cells after miR-429 silencing. (B) Detection of relative β-catenin protein level in MG-63 cells after re-expression of miR-429 and in Saos-2 cells after silencing miR-429. (C) Detection of relative p-c-Jun level in MG-63 cells after increasing miR-429 and in Saos-2 cells after decreasing miR-429. *P<0.05, **P<0.01. HOXA9, homeobox A9.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study areavailable from the corresponding author on reasonable request.

Authors' contributions

LS and LW contributed to the conception of the study. SL contributed significantly to the data analysis and study preparation. YJ and QW performed the data analyses and wrote the study. LS helped perform the analysis with constructive discussions. All authors read and approved the final study.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China). Written informed consent was obtained from each patient and their parents before collecting the specimens.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Li J, Yang Z, Li Y, Xia J, Li D, Li H, Ren M, Liao Y, Yu S, Chen Y, *et al*: Cell apoptosis, autophagy and necroptosis in osteosarcoma treatment. Oncotarget 7: 44763-44778, 2016.
2. Endo-Munoz L, Cumming A, Sommerville S, Dickinson I and Saunders NA: Osteosarcoma is characterised by reduced expression of markers of osteoclastogenesis and antigen presentation compared with normal bone. Br J Cancer 103: 73-81, 2010.
3. Vos HI, Coenen MJ, Guchelaar HJ and Te Loo DM: The role of pharmacogenetics in the treatment of osteosarcoma. Drug Discov Today 21: 1775-1786, 2016.
4. Amankwah EK, Conley AP and Reed DR: Epidemiology and therapies for metastatic sarcoma. Clin Epidemiol 5: 147-162, 2013.
5. Bishop MW, Janeway KA and Gorlick R: Future directions in the treatment of osteosarcoma. Curr Opin Pediatr 28: 26-33, 2016.
6. Isakoff MS, Bielack SS, Meltzer P and Gorlick R: Osteosarcoma: Current treatment and a collaborative pathway to success. J Clin Oncol 33: 3029-3035, 2015.
7. Mayer MN and Grier CK: Palliative radiation therapy for canine osteosarcoma. Can Vet J 47: 707-709, 2006.
8. Croce CM: Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10: 704-714, 2009.
9. Ning Q, Liu YF, Ye PJ, Gao P, Li ZP, Tang SY, He DX, Tang SS, Wei H and Yu CY: Delivery of liver-specific miRNA-122 using a targeted macromolecular prodrug toward synergistic therapy for hepatocellular carcinoma. ACS Appl Mater Interfaces 11: 10578-10588, 2019.
10. Lin X, Wang S, Sun M, Zhang C, Wei C, Yang C, Dou R, Liu Q and Xiong B: miR-195-5p/NOTCH2-mediated EMT modulates IL-4 secretion in colorectal cancer to affect M2-like TAM polarization. J Hematol Oncol 12: 20, 2019.
11. Zuo S, Dai G, Wang L, Wen Y, Huang Z, Yang W, Ma W and Ren X: Suppression of angiogenesis and tumor growth by recombinant T4 phages displaying extracellular domain of vascular endothelial growth factor receptor 2. Arch Virol 164: 69-82, 2019.
12. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
13. Weidhaas J: Using microRNAs to understand cancer biology. Lancet Oncol 11: 106-107, 2010.
14. Nana-Sinkam SP and Croce CM: MicroRNA regulation of tumorigenesis, cancer progression and interpatient heterogeneity: Towards clinical use. Genome Biol 15: 445, 2014.
15. Huang D, Wang F, Wu W, Lian C and Liu E: MicroRNA-429 inhibits cancer cell proliferation and migration by targeting the AKT1 in melanoma. Cancer Biomark 26: 63-68, 2019.
16. Zong M, Liu Y, Zhang K, J Y and Chen L: The effects of miR-429 on cell migration and invasion by targeting Slug in esophageal squamous cell carcinoma. Pathol Res Pract 215: 152526, 2019.
17. Su Z, Jiang G, Chen J, Liu X, Zhao H, Fang Z, He Y, Jiang X and Xu G: MicroRNA-429 inhibits cancer cell proliferation and migration by targeting AKT1 in renal cell carcinoma. Mol Clin Oncol 12: 75-80, 2020.
18. Liu X, Liu Y, Wu S, Shi X, Li L, Zhao J and Xu H: Tumor-suppressing effects of miR-429 on human osteosarcoma. Cell Biochem Biophys 70: 215-224, 2014.
19. Yu SL, Koo H, Lee HY, Yeom YI, Lee DC and Kang J: Recombinant cell-permeable HOXA9 protein inhibits NSCLC cell migration and invasion. Cell Oncol (Dordr) 42: 275-285, 2019.
20. Makabe T, Arai E, Hirano T, Ito N, Fukamachi Y, Takahashi Y, Hirasawa A, Yamagami W, Susumu N, Aoki D, *et al*: Genome-wide DNA methylation profile of early-onset endometrial cancer: Its correlation with genetic aberrations and comparison with late-onset endometrial cancer. Carcinogenesis 40: 611-623, 2019.
21. Sun Y, Zhou B, Mao F, Xu J, Miao H, Zou Z, Phuc Khoa LT, Jang Y, Cai S, Witkin M, *et al*: HOXA9 reprograms the enhancer landscape to promote leukemogenesis. Cancer Cell 34: 643-658, e5, 2018.
22. Liu Y, Wang Y, Yang H, Zhao L, Song R, Tan H and Wang L: MicroRNA 873 targets HOXA9 to inhibit the aggressive phenotype of osteosarcoma by deactivating the Wnt/β catenin pathway. Int J Oncol 54: 1809-1820, 2019.
23. Yu J, Tian X, Chang J, Liu P and Zhang R: RUNX3 inhibits the proliferation and metastasis of gastric cancer through regulating miR-182/HOXA9. Biomed Pharmacother 96: 782-791, 2017.
24. Zheng DH, Wang X, Lu LN, Chen DL, Chen JM, Lin FM and Xu XB: MiR-638 serves as a tumor suppressor by targeting HOXA9 in glioma. Eur Rev Med Pharmacol Sci 22: 7798-7806, 2018.
25. Zhang ZF, Wang YJ, Fan SH, Du SX, Li XD, Wu DM, Lu J and Zheng YL: MicroRNA-182 downregulates Wnt/β-catenin signaling, inhibits proliferation, and promotes apoptosis in human osteosarcoma cells by targeting HOXA9. Oncotarget 8: 101345-101361, 2017.
26. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
27. Gianferante DM, Mirabello L and Savage SA: Germline and somatic genetics of osteosarcoma - connecting aetiology, biology and therapy. Nat Rev Endocrinol 13: 480-491, 2017.
28. Lindsey BA, Markel JE and Kleinerman ES: Osteosarcoma overview. Rheumatol Ther 4: 25-43, 2017.
29. Gorlick R and Khanna C: Osteosarcoma. J Bone Miner Res 25: 683-691, 2010.
30. Liu X, Zhang C, Wang C, Sun J, Wang D, Zhao Y and Xu X: miR-210 promotes human osteosarcoma cell migration and invasion by targeting FGFRL1. Oncol Lett 16: 2229-2236, 2018.
31. Wang L, Hu K and Chao Y: MicroRNA-1301 inhibits migration and invasion of osteosarcoma cells by targeting BCL9. Gene 679: 100-107, 2018.
32. Alvarez-Garcia I and Miska EA: MicroRNA functions in animal development and human disease. Development 132: 4653-4662, 2005.
33. Wu G, Zheng H, Xu J, Guo Y, Zheng G, Ma C, Hao S, Liu X, Chen H, Wei S, *et al*: miR-429 suppresses cell growth and induces apoptosis of human thyroid cancer cells by targeting ZEB1. Artif Cells Nanomed Biotechnol 47: 548-554, 2019.

34. Shen F, Zheng H, Zhou L, Li W and Xu X: Overexpression of MALAT1 contributes to cervical cancer progression by acting as a sponge of miR-429. J Cell Physiol 234: 11219-11226, 2018.
35. Liu D, Song L, Dai Z, Guan H, Kang H, Zhang Y, Yan W, Zhao X and Zhang S: MiR-429 suppresses neurotrophin-3 to alleviate perineural invasion of pancreatic cancer. Biochem Biophys Res Commun 505: 1077-1083, 2018.
36. Deng Y, Luan F, Zeng L, Zhang Y and Ma K: MiR-429 suppresses the progression and metastasis of osteosarcoma by targeting ZEB1. EXCLI J 16: 618-627, 2017.
37. Pineault N, Helgason CD, Lawrence HJ and Humphries RK: Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. Exp Hematol 30: 49-57, 2002.
38. Schneider E, Staffas A, Röhner L, Malmberg ED, Ashouri A, Krowiorz K, Pochert N, Miller C, Wei SY, Arabanian L, *et al*: Micro-ribonucleic acid-155 is a direct target of Meis1, but not a driver in acute myeloid leukemia. Haematologica 103: 246-255, 2018.
39. Zhou L, Wang Y, Zhou M, Zhang Y, Wang P, Li X, Yang J, Wang H and Ding Z: HOXA9 inhibits HIF-1α-mediated glycolysis through interacting with CRIP2 to repress cutaneous squamous cell carcinoma development. Nat Commun 9: 1480, 2018.
40. Chong GO, Jeon HS, Han HS, Son JW, Lee YH, Hong DG, Park HJ, Lee YS and Cho YL: Overexpression of microRNA-196b accelerates invasiveness of cancer cells in recurrent epithelial ovarian cancer through regulation of homeobox A9. Cancer Genomics Proteomics 14: 137-141, 2017.
41. Thomsen CB, Andersen RF, Steffensen KD, Adimi P and Jakobsen A: Delta tocotrienol in recurrent ovarian cancer. A phase II trial. Pharmacol Res 141: 392-396, 2019.
42. Bhatlekar S, Ertel A, Gonye GE, Fields JZ and Boman BM: Gene expression signatures for HOXA4, HOXA9, and HOXD10 reveal alterations in transcriptional regulatory networks in colon cancer. J Cell Physiol 234: 13042-13056, 2018.
43. Yoshida A, Fujiwara T, Uotani K, Morita T, Kiyono M, Yokoo S, Hasei J, Nakata E, Kunisada T and Ozaki T: Clinical and functional significance of intracellular and extracellular microRNA-25-3p in osteosarcoma. Acta Med Okayama 72: 165-174, 2018.
44. Kureel J, John AA, Prakash R and Singh D: MiR 376c inhibits osteoblastogenesis by targeting Wnt3 and ARF-GEF-1-facilitated augmentation of beta-catenin transactivation. J Cell Biochem 119: 3293-3303, 2018.
45. Kushlinskii NE, Fridman MV and Braga EA: Molecular mechanisms and microRNAs in osteosarcoma pathogenesis. Biochemistry (Mosc) 81: 315-328, 2016.
46. Liu X, Li L, Lv L, Chen D, Shen L and Xie Z: Apigenin inhibits the proliferation and invasion of osteosarcoma cells by suppressing the Wnt/β-catenin signaling pathway. Oncol Rep 34: 1035-1041, 2015.
47. Zhang J, Yu XH, Yan YG, Wang C and Wang WJ: PI3K/Akt signaling in osteosarcoma. Clin Chim Acta 444: 182-192, 2015.
48. Graziano AC, Cardile V, Avola R, Vicario N, Parenti C, Salvatorelli L, Magro G and Parenti R: Wilms' tumor gene 1 silencing inhibits proliferation of human osteosarcoma MG-63 cell line by cell cycle arrest and apoptosis activation. Oncotarget 8: 13917-13931, 2017.
49. Perry JA, Kiezun A, Tonzi P, Van Allen EM, Carter SL, Baca SC, Cowley GS, Bhatt AS, Rheinbay E, Pedamallu CS, *et al*: Complementary genomic approaches highlight the PI3K/mTOR pathway as a common vulnerability in osteosarcoma. Proc Natl Acad Sci USA 111: E5564-E5573, 2014.

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