# ORIGINAL ARTICLE \_

# MicroRNA-301a inhibits the progression of osteosarcoma by regulating DEC2

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# Summary

**Purpose:** The purpose of this study was to investigate the expression of microRNA (miRNA)-301a in osteosarcoma (OS) and its relationship with clinicopathological parameters and prognosis of patients with OS, and to further explore how it accelerates the progression of OS via modulating downstream target genes.

**Methods:** Quantitative real-time polymerase chain reaction (qRT-PCR) was used to examine the expression of miRNA-301a in 39 OS tumor tissue samples and adjacent ones, and the interplay between miRNA-301a and clinical indicators. The prognosis of patients with OS was analyzed. In addition, miRNA-301a overexpression vector was constructed to analyze the effect of miRNA-301a on the function of OS cells by cell counting kit-8 (CCK-8), transwell and cell wound healing assays. Finally, the potential mechanism was also investigated using luciferase reporter gene assay and cell recovery experiment.

**Results:** qRT-PCR results revealed that miRNA-301a level in OS tumor tissue specimen was remarkably lower than that in adjacent tissue. Compared with patients with high expression

of miRNA-301a, patients with low expression had a higher incidence of distant metastasis and lower overall survival. Compared with the negative control group (miR-NC group), cell proliferation and metastasis ability were remarkably decreased in the miRNA-301a mimics group. In addition, DEC2 expression was found remarkably elevated in OS cell lines and negatively correlated with miRNA-301a level. At the same time, cell recovery experiment demonstrated that there existed a mutual regulation between miRNA-301a and DEC2, the two of which could together promote the malignant progression of OS.

**Conclusions:** MiRNA-301a level was remarkably reduced both in OS tissues and cell line samples, and was confirmed to be associated with distant metastasis and poor prognosis of patients with OS. In addition, miRNA-301a was found to be able to inhibit malignant progression of OS through regulating DEC2.

*Key words: microRNA-301a*, DEC2, *osteosarcoma*, *malignant progression* 

# Introduction

Osteosarcoma (OS) is one of the most common malignant bone tumors among children and adolescents, accounting for approximately 35% of all malignant bone tumors, with a high incidence of invasion and metastasis [1-3]. Despite advances in the diagnosis and treatment of OS, the overall 5-year survival rate of patients with OS worldwide has remained stagnant in recent decades [4,5]. Pul-

monary metastasis is the most common cause of death in patients with OS, and the underlying cause is that OS cells metastasize hematogeneously to the lung at an early disease stage. Therefore, 80% of patients with OS have already pulmonary metastasis when clinically diagnosed [6,7]. Therefore, the molecular mechanism of OS is one of the focuses and hotspots in the field of OS research, which is of

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great significance for the diagnosis, treatment and prevention of disease invasion and metastasis and improvement of survival [8,9].

Tumor is a kind of genetic disease. Tumor occurrence, development and outcome involve normal gene mutations and losses, abnormal amplification and expression of oncogenes and multiple regulatory gene synergies, etc. The above factors, together with the pleiotropic genes and the body's immune factors, finally determine the tumor phenotype [10,11]. In view of the etiology of tumorigenesis, the study of tumor gene level has always been the focus of research in the field of life sciences [12,13]. In gene therapy research, the search for efficient and specific therapeutic targets will become one of the first key fields [13].

In recent years, a large number of studies have shown that miRNAs play a vital regulatory role in the occurrence, development and prognosis of a variety of tumors, which brings new light to their treatment [14,15]. MiRNAs are non-coding small molecule RNAs with a length of about 22 nucleotides, which are widely found in eukaryotes [15]. Most miRNAs originate from gene spacer sequences and are distributed in multiple forms such as single copy or multiple copies, and about 25% are co-transcribed with the host in the form of polycistrons [16,17]. The complete or incomplete complementary binding of miRNAs to the 3 'end of target gene miRNA leads to the degradation or translation inhibition of the target gene miRNA, so as to affect cell proliferation, metastasis, invasion and other biological processes [17,18]. MiRNA-301a is one of the important members of the miRNAs family. Previous studies have shown that miRNA-301a is involved in the occurrence and development of various tumors (liver cancer, cervical cancer, etc.) and can regulate the biological behavior of tumor cells. Meanwhile, it has been confirmed that miRNA-301a can play a biological regulatory role by acting on its target genes, such as FOXL1 and PTEN (gene of phosphate and tension homology deleted on chromosome ten), etc. [19,20]. However, whether it can regulate its corresponding targets in OS remains elusive.

The purpose of this study was to investigate the expression of miRNA-301a in OS and its relationship with clinicopathological parameters and prognosis of patients with this disease, and to further explore how it accelerates the progression of OS *via* modulating downstream target genes.

#### Methods

#### Patients and OS samples

The specimens of 39 cases of OS were pathologically confirmed as bone tumors in our hospital. Gender and age of these OS patients were not different. The tissue specimens were numbered and registered, and then stored at -80°C. Specimens were obtained with informed consent from patients and their families and approval by the ethics committee of Changzhou No.2 People's Hospital.

#### Cell lines and reagents

OS cell lines including HOS, U2OS, SOSP-9607, MG63, 143B, SaOS-2 and human osteoblasts (hFOB) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C with 5% CO<sub>2</sub>.

#### Cell transfection

The negative control (NC) (miR-NC) and the vector containing miRNA-301a overexpression sequence (miR-NA-301a mimics) were purchased from Shanghai Jima Company. Cells were plated in 6-well plates and grown to a cell density of 70%, and then siRNA transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h, cells were collected for qRT-PCR analysis and cell function experiments.

#### Cell counting kit-8 (CCK-8) assay

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 6 h, 24 h, 48 h and 72 h, and then added with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 h, the optical density (OD) of each well was measured with a microplate reader at 490 nm absorption wavelength.

#### Transwell assay

After transfection for 48 h, cells were digested, centrifuged and resuspended in RPM1640 medium containing 10% FBS and without FBS to adjust the density to  $5 \times 10^5$  cells/mL. A cell suspension of 200 uL ( $15 \times 10^5$ cells) was added to the upper chamber, and 700 uL of a medium containing 20% FBS was added to the lower chamber. According to the different migration abilities of each cell line, they were put back into the incubator and continued to culture for 24h. Then, the transwell chamber was clipped, washed 3 times with 1 x phosphate buffered saline (PBS), and placed in methanol for 15 min for cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with a cotton swab. Then, the cells were observed and photographed under a microscope, and 10 fields of view were randomly selected for counting.

#### Cell wound healing assay

After transfection for 48 h, cells were digested, centrifuged and resuspended in RPMI1640 medium containing 10% FBS and without FBS to adjust the density to  $55 \times 10^5$  cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluence of the cells reached 90% or more the next day. After the scratch, cells were rinsed gently with PBS for 2-3 times and and observed again after 24 h.

#### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was extracted from OS cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse-transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Japan). QRT-PCR reactions were performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Japan), and StepOne Plus Realtime PCR System (Applied Biosystems, Foster City, CA, USA). Data analysis was performed using ABI Step One software and the relative expression levels of mRNA were calculated using the 2<sup>-ΔΔCt</sup> method.

#### Luciferase reporter gene assay

According to the instructions, the OS cell line in the logarithmic growth phase was selected and RIPA lysis buffer was prepared according to the luciferase system. The relative fluorescence values were then measured by a luminometer and compared. The detection principle is that when the selected specific miRNA is complementary to the target gene sequence in the system, the luciferase will not be expressed, and the relative fluorescence value measured in the end is remarkably lower than that in the experimental group which cannot be combined with the sequence.

#### Statistics

Statistical analysis was performed using GraphPad Prism 6 V6.01 software (La Jolla, CA, USA). Statistical differences between the two groups were analyzed using Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by *post hoc* test (least significant difference). Independent experiments were repeated at least three times and data were expressed as mean±standard deviation. Survival curves were plotted using the Kaplan-Meier method and intergroup differences were examined with log-rank test. There were three levels of p value significance i.e. p<0.05, p<0.01 and p<0.001.

# Results

# MicroRNA-301a was lowly expressed in osteosarcoma tissues and cell lines

The expression of miRNA-301a in 39 pairs of OS tumor tissues and their corresponding adjacent tissues and OS cell lines was detected by qRT-PCR. The results showed that the expression level of miRNA-301a in OS tumor tissues was remarkably higher than that in adjacent tissues, and the difference was statistically significant (Figure 1A). Compared with the human normal osteoblast cell line (hFOB), miRNA-301a was remarkably downregulated in OS cell lines (Figure 1B), especially in the MG63 and U2OS cell lines, so we chose these two lines for subsequent experiments.

MiRNA-301a expression was correlated with distant metastasis and overall survival in osteosarcoma patients

39 pairs of OS tumor tissue specimens and paracancer ones were divided into high expression group and low expression group according to the expression of miRNA-301a, and the relationship between miRNA-301a level and clinicopathological parameters as well as patients' prognosis were analyzed. As shown in Table 1, low expression of miRNA-301a was positively correlated with distant metastasis of OS, but not with age, gender, and



**Figure 1.** MiR-301a was underexpressed in osteosarcoma tissues and cell lines. **A:** qRT-PCR was used to detect the differential expression of miR-301a in osteosarcoma tumor tissues and adjacent tissues. **B:** qRT-PCR was used to detect the expression level of miR-301a in osteosarcoma cell lines, which showed remarkably downregulated miRNA-301a compared with normal hFOB line. **C:** Kaplan-Meier survival of osteosarcoma patients based on miR-301a expression; the prognosis of patients with low expression was significantly worse than that of high expression group. Data are mean±SD, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Parameters	Number of cases	miR-301a expression		p value
		High (%)	Low (%)	
Age (years)				0.151
<21	16	6	10	
≥21	23	14	9	
Gender				0.648
Male	28	15	13	
Female	11	5	6	
Enneking stage				0.205
IA	6	5	1	
IIA	10	6	4	
IIB	16	7	9	
III	7	2	5	
Distant metastasis				0.037
No	23	15	8	
Yes	16	5	11	

#### Table 1. Association of miR-301a expression with clinicopathologic characteristics of osteosarcoma



**Figure 2.** Inhibition of proliferation and migration of osteosarcoma cells after overexpression of miR-301a. **A:** qRT-PCR verified the interference efficiency after transfection of miR-301a in MG63 and U2OS cell lines. **B:** The CCK-8 assay detected the effect of overexpression of miR-301a on osteosarcoma cell proliferation in MG63 and U2OS cell lines. **C:** The transwell migration assay detected the effect of overexpression of miR-301a on osteosarcoma cell proliferation in MG63 and U2OS cell migration in MG63 and U2OS cell lines. **C:** The transwell migration assay detected the effect of overexpression of miR-301a on osteosarcoma cell migration in MG63 and U2OS cell migration in MG63 and U2OS cell lines (magnification: 20x). Data are mean±SD, \*p<0.05.

Enneking stage. In addition, we collected relevant follow-up data in order to explore the correlation between miRNA-301a and OS patient prognosis. Kapla-Meier survival curves revealed that the higher the expression level of miRNA-301a, the better the prognosis (p<0.05; Figure 1C).

# Overexpression of miRNA-301a inhibited cell proliferation and metastasis

To explore the effect of miRNA-301a on OS cytology, the miRNA-301a overexpression model was first constructed and its transfection efficiency was verified by qRT-PCR (Figure 2A). CCK8 result revealed that the cell proliferation rate of miRNA-301a mimics group was remarkably decreased compared with the miRNA-NC group (Figure 2B). Similarly, the transwell migration assay also suggested that overexpression of miRNA-301a could inhibit OS cell migration and thus inhibited OS metastasis (Figure 2C).

# Overexpression of miRNA-301a inhibited cell migration ability

Cell wound healing assay was performed to explore the effect of miRNA-301a on OS cell migration ability. In the MG63 cell line, it was found that the migration ability of OS cells in miRNA-301a

mimics group was remarkably decreased (Figure 3), suggesting that cell growth and metastasis were inhibited, and similar result was observed in the U2OS cell line.

#### MiRNA-301a could combine with DEC2

A possible relationship between DEC2 and miR-NA-301a was found through related bioinformatics analysis. Subsequently, luciferase reporter gene assay was performed and the results demonstrated that overexpression of miRNA-301a remarkably attenuated the luciferase activity of the wild-type DEC2 vector, further confirming that DEC2 could be targeted by miRNA-301a through this binding site (Figure 4A). Furthermore, after miRNA-301a overexpressing vector was constructed, qRT-PCR was used to detect DEC2 level in MG63 and U2OS cell lines after miRNA-301a was up-regulated, suggesting that miRNA-301a mimics could inhibit DEC2 expression (Figure 4B).

Subsequently, DEC2 expression levels were found remarkably increased in OS tumor tissues compared with adjacent tissues (Figure 4C). Furthermore, as shown in Figure 4D, DEC2 was also found highly expressed in OS cells compared to hFOB, and the difference was statistically significant.



**Figure 3.** Inhibition of osteosarcoma cell migration ability after overexpression of miR-301a. The cell scratch assay validated the osteosarcoma cell migration ability (magnification: 20x) after transfection of miR-301a in MG63 and U2OS cell lines. Data are mean±SD, \*\*p<0.01.



Figure 4. Direct targeting of miR-301a and DEC2. A: The results of the dual luciferase reporter gene assay showed that overexpression of miR-301a significantly attenuated the luciferase activity of the wild-type DEC2 vector (p<0.001) without attenuating the luciferase activity of vector containing the mutant vector (p>0.05) or empty vector (p>0.05). B: Overexpression of miR-301a significantly reduced DEC2 expression levels in MG63 and U2OS cell lines. C: qRT-PCR was used to detect the difference in expression of DEC2 in osteosarcoma tumor tissues and adjacent tissues and showed DEC2 expression levels remarkably increased in osteosarcoma tissues compared with adjacent tissues. D: qRT-PCR was used to detect the expression level of DEC2 in osteosarcoma cell lines and showed remarkably highly expressed DEC2 in osteosarcoma cells compared to hFOB. Data are mean±SD, \*p<0.05, \*\*\*p<0.001.

DEC2 modulated miRNA-301a expression in osteosarcoma cells

To further explore the interaction between miRNA-301a and DEC2 in OS cell lines, DEC2 overexpression plasmid was constructed and qRT-PCR was used to verify the transfection efficiency (Figure 5A). Subsequently, CCK-8, transwell migration and cell wound healing assays demonstrated that overexpression of DEC2 could counteract the influence of miRNA-301a mimics on the proliferative capacity and metastasis of OS cells (Figure 5B-5D).

# Discussion

OS is a common malignant bone tumor prone to occur in adolescents, with a high tendency of multi-factor and multi-step process, and high evisystemic metastases. At the initial diagnosis, about dence shows that the occurrence and development

JBUON 2020; 25(2): 1018

20% of the patients have developed lung metastasis, and another 40% will develop distant organ metastasis in their late stage. Therefore, OS is a disease with high grade of malignancy and poor prognosis [1-3]. In recent years, due to the promotion and treatment of neoadjuvant chemotherapy, the current 5-year survival rate of patients with OS has increased from about 20% in the 1970s to 70% now, but some patients still die rapidly due to drug resistance [4,5]. Therefore, to develop new, effective and specific prevention and treatment measures, improve the survival and quality of life of patients, and prevent or delay the metastasis of OS, have become the primary topic of contemporary bone tumor research [6-9].

The occurrence and development of tumor is a



Figure 5. MiR-301a regulated the mechanism of action of DEC2 in inhibiting the malignant progression of osteosarcoma. A: DEC2 expression levels were detected by co-transfection of miR-301a and DEC2 in MG63 and U2OS cell lines by qRT-PCR, showing remarkably downregulated expression in miRNA-301a+ DEC2 compared with miRNA-301a+NC. B: CCK-8 assay detected the effect of MiR-301a and DEC2 co-transfection on osteosarcoma cell proliferation in MG63 and U2OS cell lines and showed overexpression of DEC2 could counteract the influence of miRNA mimics on the proliferation capacity of osteosarcoma cells. C: The transwell migration assay detected the effects of MiR-301a and DEC2 co-transfection on osteosarcoma cell migration in MG63 and U2OS cell lines and showed overexpression of DEC2 could counteract the influence of miRNA-301a mimics the metastasis of osteosarcoma cells. D: Cell wound healing assay demonstrated the ability of osteosarcoma cells to migrate after co-transfection of MiR-301a with DEC2 in MG63 and U2OS cell lines. Data are mean ± SD, \*#p<0.05.

of tumor is related to the activation or inactivation of tumor suppressor genes caused by epigenetic mechanisms [10,11]. Epigenetics, especially the abnormality of miRNA regulation, leads to the abnormal expression of oncogenes or tumor suppressor genes [12,13]. MiRNAs are a class of small non-coding RNAs, which are highly conserved in evolution and widely exist in various eukaryotes, and play an important regulatory role in various biological activity processes [14-16]. MiRNAs can pair with complete or incomplete bases in the 3 'non-coding region of the target mRNA, leading to degradation of the target mRNA or inhibition of protein translation, so as to regulate the expression, transcription or translation of the target gene and achieve the regulation of cell life activities [16]. They play a pivotal regulatory role in almost all life processes, involving cell growth, differentiation, apoptosis, cell cycle regulation and cell stress response [16,17]. A large number of studies has shown that miRNAs are also involved in the occurrence and development of human diseases [18]. Genes are the basic elements and determinants of lieved that miRNA-301a may play a role of tumor

life activities, while proteins are the expression forms of specific functions. Whether miRNA-301a, as one of the expression forms of genes, is involved in the occurrence and development of tumors and regulates the growth and prognosis of tumors has attracted extensive attention of researchers [19,20]. Numerous studies have shown that miRNA-301a is involved in the formation of a variety of human tumors, regulates the growth of tumors, and is closely related to their outcome [19,20]. The effect of miRNA-301a on OS cells and the specific mechanism have not been reported at yet home and abroad. The invasion and metastasis ability of OS is very strong, which is the main reason for its poor prognosis. Therefore, in this study, in addition to the effect of miRNA-301a on the proliferation of OS cells, the effect of miRNA-301a on the invasion and metastasis ability of OS cells was also studied. The results of this experiment showed that the expression of miRNA-301a was remarkably downregulated and positively correlated with distant metastasis and poor prognosis. Therefore, we beinhibition in OS. In order to further explore the effect of miRNA-301a on the biological function of OS, the miRNA-301a overexpression model was constructed, and the results of CCK-8, transwell migration and cell scratch experiments revealed that miRNA-301a could inhibit the occurrence and development of OS and play a critical role in OS, but the specific molecular mechanism remains unclear.

DEC2 is expressed in various embryonic and adult tissues and plays an inhibitory role in the transcriptional activity [21]. Accumulated evidence shows that DEC2 inhibits the development of gastric cancer, prostate cancer, breast cancer and other tumors, and plays a role in multiple cell activities including cell apoptosis, proliferation, metastasis and invasion [21-23]. In this experiment, bioinformatics analysis revealed that miRNA-301a and DEC2 had mutual regulatory effects. Subsequently, we confirmed through luciferase reporter gene experiment that DEC2 can be targeted by miRNA-301a through this binding site. With the deepening of research, the understanding of the biological function of DEC2 gene and its role in the development of tumor is further elucitated. In order to prove whether miRNA-301a inhibits the occurrence and development of OS via regulating DEC2, we detected the change of DEC2 expression

using qRT-PCR after overexpression of miRNA-301a, and found that miRNA-301a could inhibit the proliferation and metastasis of OS through modulating the DEC2 level. In addition, the results also showed that the expression level of DEC2 was remarkably down-regulated after overexpression of miRNA-301a, further suggesting that miRNA-301a may inhibit the progression of OS by regulating DEC2. The above results are conducive to the diagnosis, treatment and prognosis evaluation of OS, which will undoubtedly bring good news to many patients with OS and their families, and offer new hope and dawn for the human conquest of OS.

# Conclusions

In summary, microRNA-301a was remarkably decreased in OS tissues as well as cells and was remarkably associated with distant metastasis and poor disease prognosis. What's more, miRNA-301a might inhibit the progression of OS through modulating DEC2 expression.

# **Conflict of interests**

The authors declare no conflict of interests.

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