



MiR-140 targets Wnt1 to inhibit the proliferation and enhance drug sensitivity in osteosarcoma cells

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ABSTRACT

MicroRNAs (miRNAs) have been documented to function differently in numerous human cancers. Our study planned to investigate the role of microRNA-140 (miR-140) and to identify its possible target in osteosarcoma (OS) to predict their mechanism in OS. The miR-140 was down-regulated in OS, and its high expression decreased MG63 cell proliferation. At the molecular level, Wnt1 was a target of miR-140, and its expression could be suppressed by miR-140. Besides, miR-140 overexpression decreased drug resistance in OS cells treated by doxorubicin. Collectively, overexpression of miR-140 may inhibit human OS cell proliferation and may enhance drug sensitivity by direct regulation of Wnt/ β -catenin signaling.

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Introduction

Osteosarcoma (OS) is defined as a bone malignancy in orthopedics with frequent incidence and poor prognosis, which is quite common in children and adolescents. It has a great risk of destroying the surrounding tissues and a high possibility of metastasis, metastasizing to the lung, in particular, constituting the major cause of mortality in patients with OS (1, 2). In recent four decades, there is a significant improvement in the diagnostic imaging and therapeutic technologies (e.g., neoadjuvant chemotherapy and radical surgical resection jointly) for OS. It promotes a dramatic increase in the 5-year survival of OS patients (from 45% to ~71%) (3). However, there is still no effective and thorough treatment for this disease. Significantly, patients with recurrent or metastatic OS may experience a survival outcome of less than 10% in the long-term period, showing an extremely poor prognosis (4). In this regard, it is extremely important to clarify the pathogenesis of OS, to screen novel diagnostic or prognostic markers, and to find alternative and effective therapeutic strategies for such malignant

tumors, which, however, is indeed not an easy task (5, 6). With the development of tumor molecular biology, it has been recognized that the change of genetic information may play a key role in the process of cell carcinogenesis. It is generally believed that cell carcinogenesis can be primarily attributed to the activation of oncogenes and the loss of tumor suppressor genes, while osteosarcoma distant metastasis is mainly due to the abnormal expression of tumor metastasis genes and metastasis suppressor genes (6).

MicroRNAs (miRNAs) exist widely in plant and animal cells. Bioinformatics studies believe that there are about 1000 genes in the human genome (7, 8). As is known, miRNAs have diverse functions physiologically and pathologically, for instance, many physiological processes such as development, cell energy metabolism, cell differentiation, and a variety of pathological processes, such as diabetes, neurological diseases, cardiovascular diseases, tumors, etc. (9, 10). Many miRNAs can act as proto-oncogenes or tumor suppressor genes in different tumor types. Moreover, numerous miRNAs are

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differentially expressed in OS, which has been documented to be related to its occurrence, metastasis, prognosis and even drug resistance (11, 12).

Simultaneously, as for miR-140, it has been identified to be a tumor suppressor in various tumors, such as breast cancer, non-small cell lung cancer, etc. (13, 14). Moreover, miR-140 has been disclosed to be involved in the pathogenesis of osteoarthritis by regulating ADAMTS5 [15]. Nevertheless, its role remains elusive in OS that requires further comprehensive investigation. In our study, Wnt1 was a target of miR-140. Accordingly, we hope to clarify the role of miR-140 in OS and its mechanism related to Wnt1. Findings in our study are expected to provide a valuable basis for the molecular potential of miR-140 in target therapy of this cancer.

Materials and methods

Cell culture, tissue samples and cell transfection

The OS tissues were collected from 50 OS patients surgically from Beijing Tongren Hospital of Capital Medical University, and the normal bone tissues were sampled from patients who underwent joint replacement to be the control. Informed consent had been obtained prior to the initiation of this research. OS cell lines of Saos-2, MG63, U2OS and HOS as well as Normal cell line Hfob1.19 were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were culture in Dulbecco' modified Eagle' medium (DMEM, Gibco, Beijing) with the supplementation of 10 % fetal bovine serum (Gibco, Beijing) as well as streptomycin and penicillin in an incubator containing saturated humidity, the constant temperature at 37°C and 5% CO₂. The culture medium was changed once in 1 ~ 2 days. When growing to about 90% of the density at the bottom of the bottle, these cells were digested with 0.25% trypsin and subcultured once. Furthermore, cells were cultured in 6-well plates (about 100,000 cells per well). The next day, cells were observed to grow to 80 ~ 90% fusion that was well prepared for transfection using the Lipofectamine 3000. miR-140 mimics and miR-140 inhibitor were provided by Ribobio.

Real-time quantitative PCR

For this experiment, cells after 48 h of transfection were washed twice with PBS to wash away the mixed serum in the culture medium. With the removal of PBS,

a Lysis/Binding Buffer was added to obtain and collect the lysis solution. With the harvest of total RNA, the content and purity of RNA were detected by UV spectrophotometer, and the obtained RNA solution was stored in a refrigerator at - 80°C. The cDNA was synthesized by reverse transcription for subsequent quantitative real-time PCR (qRT-PCR). PCR amplification conditions were: 95°C for 10min, and then 40 cycles of at 95°C for 15s, and at 60°C for the 60s. All the primers are described in Table 1. Data were analyzed with the 2^{-ΔΔCt} method and corresponding results were normalized to U6 and GAPDH, respectively, with three repeated times of this experiment.

Table1. Primers used in qRT-PCR.

Primer	Sequence (5'-3')
miR-140-F	ACACTCCAGCTGGGCAGTGGTTTTACCCTA
miR-140-R	TGGTGTCGTGGAGTCG
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
GAPDH-F	GAAGGTGAAGGTCGGAGTC
GAPDH-R	GAAGATGGTGATGGGATTG
Wnt1-F	TGGTTTGCAAAGACCACCTCCA
Wnt1-R	TGATTCCAGGAGGCAAACGCAT

Luciferase activity assay

Through TargetScan (<http://www.targetscan.org>), Wnt1 was a potential target of miR-140. Cells were cultured routinely, and those in the logarithmic growth stage were added with 1 mL of 0.25% trypsin solution for cell digestion in the culture flask for 2-3 minutes. The round-shaped adherent cells were then transferred to a 1.5 ml centrifuge tube for centrifugation (1000r, 3min) to obtain cell precipitation. After cell re-suspension and cell counting, the transfected cells were cultured routinely for 48h continuously. As described in the previous researches (15, 16), a dual-luciferase reporter assay was performed with its steps described briefly as follows. Cells were cultured in a 12-well plate (1×10⁵cells/well). The next steps were co-transfection with prepared wild-type or mutated 3'-UTRs of Wnt1 luciferase reporter vectors and miR-140 or control mimic with Lipofectamine 3000. Luciferase Assay Reagent II (100μl) was added in each test well to detect Firefly luciferase; similarly, Stop&Glo Reagent (100μl) was supplemented for detecting Renilla luciferase value. In the final step, taking firefly luciferase value as the reference, the luciferase activity

of 3'-UTR-Wt and miR-140 NC co-transfection group was normalized to 1 for the calculation of the relative luciferase activity.

Cell viability assay

The Cell Counting Kit-8 (CCK-8) assay was conducted to quantify cell viability, which was reflected by measuring the absorbance of each well at 450 nm. Cells in logarithmic growth were used for the following experiments. After routine digesting processing, cells were subject to centrifugation to obtain cell precipitation. The cells were re-suspended with a culture medium and counted by a cell counting chamber. According to the results of cell counting, the cell suspension was diluted to the concentration of about 5000 cells/100 μ l, which was then plated in 96-well micro plates. After incubation in a 37°C incubator for an appropriate period, each well of the 96-well plate was added with 100 μ l of 10% CCK-8 solution (i.e. 90 μ l of basic medium + 10 μ l of CCK-8 solution) for 1 h of incubation.

Cell cycle assay

Similarly, cells in the logarithmic phase were collected and added with 1 mL of 0.25% trypsin solution for cell digestion in the culture flask for 2-3 minutes. The round-shaped adherent cells were then transferred to a 1.5 ml centrifuge tube for centrifugation (1000r, 3min) to obtain cell precipitation. After cell re-suspension and cell counting, the transfected cells were cultured routinely for 48h continuously. Then, 1 mL of PBS was supplemented to the cell precipitation, washed twice, added with 1 mL of pre-cooled 70% ethanol and fixed at 4°C for 12-24 h. After centrifugation (1000r, 5min) and removal of the supernatant, freshly prepared Propidium Iodide (PI) buffer was used for warm incubation of cells for 30 min at 37°C. Finally, cell cycle distribution was observed by flow cytometry within 24 h.

Colony formation assay

Cells were digested as above and blown into a single-cell suspension. Then, cells were suspended for further use. After dilution, the cell suspension was inoculated into a dish containing 10ml of pre-heated DMEM culture solution, and cultured in an incubator

containing saturated humidity, the constant temperature at 37 °C and 5% CO₂ for 14 days. If visible clones appeared in the dish, the culture can be terminated and the DMEM culture medium can be absorbed. After washing with PBS (twice), colonies were fixed in methanol for 15 min, and the next step was Giemsa staining for 30 min. The staining solution was removed by running water and dried in the air. Colony numbers were counted under Leica optical microscope.

Western blotting assay

Before extracting the cell protein, cells were washed twice with PBS solution to wash the mixed serum in the culture medium. With the removal of PBS solution, 100 μ l of protein lysate containing PMSF was used to lyse the cells, and the scraped cell lysate was transferred into the EP tube for a subsequent ice bath for 30-45 minutes, with intermittent shocking of the lysis. The operation of detecting protein concentration shall be carried out following the guidance of the BCA kit. After the preparation of separation gel (80-100VmA) and concentrated gel (150-200mA) for electrophoresis, the next step was transferring to the PVDF membrane. The 5% milk prepared with TBST was to seal the PVDF membrane on a shaking table for 30min. Then, overnight incubation of the membranes with the primary antibodies was performed at 4°C. After another two times of washing with TBST, the diluted HRP-conjugated secondary antibody (1:10000) in blocking buffer was supplemented for further incubation of the membranes for 1 h at 37°C. After that, ECL (HRP substrate) was added for subsequent exposure in dark. All antibodies were purchased from Abcam (Wnt1, GAPDH, β -catenin, Myc, CyclinD1 and secondary antibody). Through photographing with a gel image system, protein expressions were analyzed and normalized to that of GAPDH protein.

Statistical analysis

In our study, SPSS 20.0 and GraphPad Prism 7.0 were used to analyze the relevant data in the format of mean \pm standard deviation by using ANOVA or two-tail Student's t-test. The value of P < 0.05 was accepted to indicate the presence of statistical difference statistically during analysis.

Results and discussion

Downregulation of miR-140 in OS tissue and cell lines

As displayed in Figure 1A, qRT-PCR detection of miR-140 expression in tissue samples indicated obviously decreased levels in OS tissues compared to normal tissues ($P < 0.001$). Next, as for the detection in cell lines, in relative to that in a normal cell line of hFOB1.19, its expression was evidently down-regulated in OS cell lines, including Saos-2, MG63, U2OS, HOS, especially in MG63 was the suitable cell line for subsequent experiments (Figure 1B).

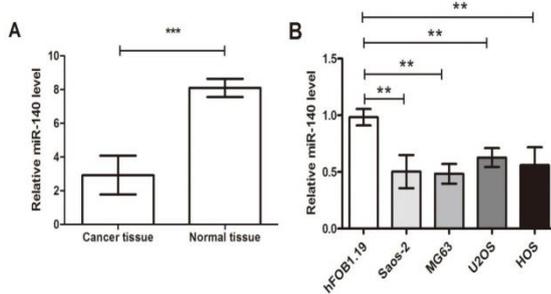


Figure 1. Detection of miR-140 expression in OS tissue and cell lines; A: qPCR quantitative analysis of miR-140 expression at the tissue level (Cancer tissue vs. Normal tissue). B: qPCR quantitative analysis of miR-140 expression at the cell level (OS cell lines of Saos-2, MG63, U2OS and HOS vs. Normal cell line Hfob1.19). ** $P < 0.01$, *** $P < 0.001$

Inhibitory activity of miR-140 in MG63 cell proliferation and invasion

To examine the role of miR-140 in OS cell growth, overexpressing or control plasmids were transfected into MG63 cells. Consequently, the results of qRT-PCR indicated the miR-140 expression level in miR-140 mimic transfection cells was about 5 times that in the mimic control (Figure 2A). Next, according to the measurement of CCK-8 assay, the number of cells in the miR-140 mimic group was much lower than in the mimic control group (Figure 2B), especially at 48h and 72h after transfection. In addition, the miR-140 group also showed a reduced proliferative index and colony-forming efficiency rate than those in the mimic control group (Figure 2C-D). At the same time, as reflected by the visualized electrophoretic band, the protein expressions of Myc and CyclinD1 were decreased after miR-140 mimic transfection than those in the mimic control group (Figure 2E).

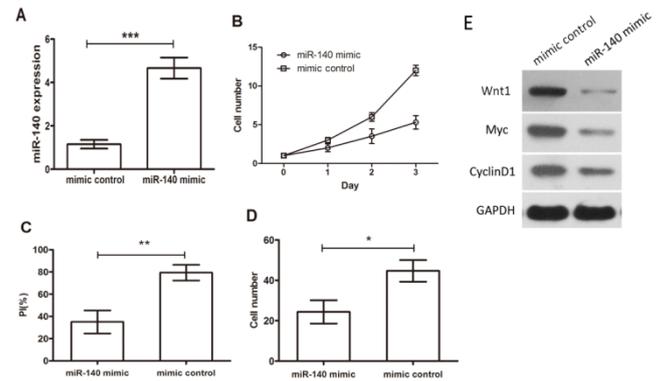


Figure 2. Inhibitory roles of miR-140 in the proliferation and invasion of MG63 cells; A: miR-140 expression level after mimic transfection in MG63 cell. B: MG63 cell viability by CCK-8 assay. C: Cell cycle analysis by flow cytometry. D: Colony-forming efficiency detection by colony-forming assay. E: Western blot detection of Wnt1, Myc, and CyclinD2 expression after the overexpression of miR-140. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

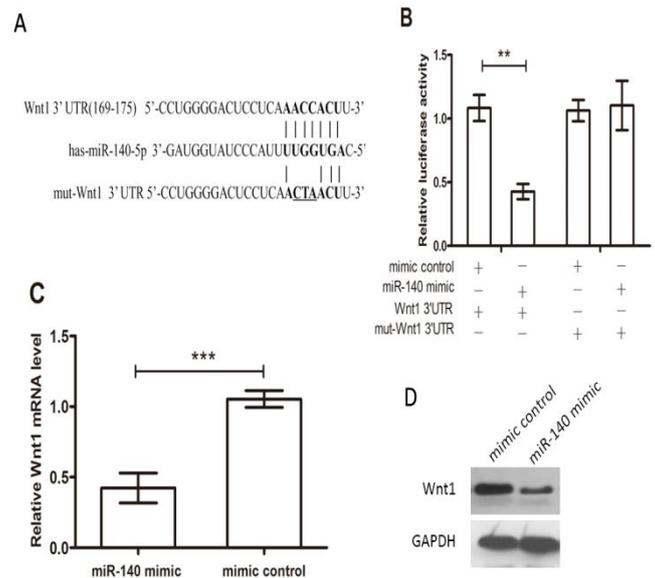


Figure 3. Target relationship confirmation between Wnt1 and miR-140; A: Online TargetScan prediction of Wnt1 mRNA as a target for miR-140. B: Dual-luciferase reporter assay to confirm that miR-140 could regulate Wnt1 3'UTR. C: Wnt1 mRNA expression following miR-140 overexpression. D: Wnt1 protein expression by overexpressing miR-140. ** $P < 0.01$, *** $P < 0.001$

MiR-140 directly inhibited Wnt1

After the prediction of potential target mRNA of miR-140 based on online TargetScan, *Wnt1* was predicted to share the sequence AACCACU in *Wnt1* 3'UTR (Figure 3A), which facilitated our subsequent exploration of the potential regulatory mechanism.

Based on the above, taking into consideration the results of dual-luciferase reporter assay, miR-140

mimic inhibited the luciferase activity of vector containing wild-type *Wnt1* 3'UTR ($P < 0.01$, Figure 3B), yet without obvious suppressing role in the mutant type ($P > 0.05$). For further confirmation, the expressions of *Wnt1* mRNA and its protein were both tested to be decreased by miR-140 overexpression ($P < 0.001$, Figure 3C-D).

Overexpressing miR-140 could reduce the drug resistance of OS cells

In the consideration of the thorny problem of the existence of drug resistance in OS, the aforementioned results might suggest that miR-140 was a protective factor against OS. Therefore, this experiment continued to evaluate the influence of overexpressing miR-140 in MG63 cell sensitivity to doxorubicin at different doses. In this experiment, the treatment was lasted for less than 24 h to minimize the effect of miR-140 produced to cell viability (Figure 2B).

Comparison based on different treatment doses revealed that in relative to non-treated control cells treatments at 0.05, 0.1 and 0.2 $\mu\text{g/ml}$ resulted in the inhibited cell viability ($P < 0.05$, Figure 4A). Furthermore, in view of the comparison between groups at each dose, no significant difference was found in cell viability between the miR-140 mimic group and the mimic control group at 0 $\mu\text{g/ml}$ of doxorubicin ($P > 0.05$); while in other doses, suppressed cell viability was detected in miR-140 mimic group than that in the mimic control group ($P < 0.05$). For further verification, expressions in ABCC1 and ABCG2 (17, 18) were tested and compared between the miR-140 mimic group and the mimic control group. Both two protein expressions were inhibited in the former group in relative to those of the latter group (Figure 4B).

OS is a primary malignant tumor of bone, with a high incidence only inferior to plasma cell myeloma. Indeed, neoadjuvant chemotherapy combined with surgery (amputation or limb salvage) can improve the survival of OS patients greatly (5). However, there is still no continuous improvement in the survival rate in recent decades.

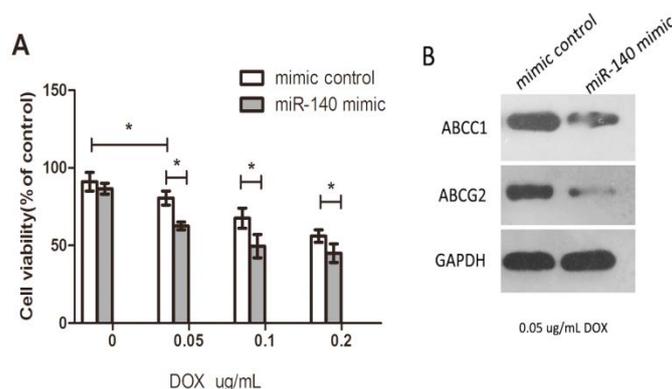


Figure 4. Impact of overexpressing miR-140 on drug resistance of OS cells; A: Comparison of cell viability by CCK-8 in cells treated by doxorubicin at the doses of 0.05, 0.1, and 0.2 $\mu\text{g/ml}$ between miR-140 mimic group and the mimic control group. * $P < 0.05$. B: Comparison of the protein expressions of ABCC1 and ABCG2 (normalized to GAPDH) in cells treated by doxorubicin at the doses of 0.05 $\mu\text{g/ml}$ between miR-140 mimic group and the mimic control group by Western blot. DOX – doxorubicin. ABCC1, ATP-binding cassette sub-family C member 1; ABCG2 – ATP-binding cassette sub-family G member 2

More importantly, high-dose chemotherapy can also cause serious side effects such as gastrointestinal reaction, bone marrow suppression, nephrotoxicity, myocardial toxicity, etc.; it also has the risk of leading to malignant tumors in other parts such as leukemia, lung cancer, colon cancer, etc. In view of the high possibility of metastasis and recurrence of OS, the treatment of OS has reached a bottleneck state, showing an urgent requirement for novel therapies (12). In recent decades, with the development of molecular biology, it is recognized that gene changes may be critical in the occurrence and development of OS and gene therapy may have great potential in the clinical application (19).

MicroRNAs are small but powerful molecules that can mediate the expression of genes extensively (20). Its mechanism is disclosed that the target miRNA completely or incompletely matches the 3'-UTR of the target gene mRNA, regulates the degradation of the target mRNA or inhibits its post-transcriptional translation, so as to regulate biological behaviors of cells, and participate in ontogeny, metabolism, tumorigenesis, etc (21). The miRNAs can down-regulate the activity of tumor suppressor gene as oncogene and proto-oncogene as tumor suppressor gene, and can also regulate the expression of tumor-related genes. Moreover, its alteration via mutation,

deletion and abnormal mutual regulation can also lead to abnormal expression of related genes (12). There exist significant differences in the expressions of miRNAs between tumor tissues and corresponding normal tissues. At the same time, it has been demonstrated widely that the expression abnormalities of various miRNAs can produce a significant impact on the progression of human malignancies. As for OS, which was reported in our study, there was a reduced miR-140 expression, supported by the detection of qRT-PCR both in tissues and cells (22). Based on its low expression in OS, we hypothesized and confirmed that its overexpression can exert an anti-tumor effect of inhibiting the proliferation of OS cells via targeting *Wnt1*, and key member of WNT signaling pathway that promotes cell proliferation and metastasis. Meanwhile, miR-140 may improve the drug sensitivity of OS cells to doxorubicin.

It is well known that the formation of a tumor is a complex process with multiple factors at different stages of development, including malignant transformation from the initial genetic change to the final distant metastasis. In these processes, each tumor has its unique molecular events, and eventually, the interaction between different genes may affect and determine the development process of different tumors. In our study, one of the significant discoveries is the anti-proliferative role of miR-140 in OS cells. MiR-140 can mediate cell activities (23) and can inhibit EMT and cell invasion (24). Simultaneously, CyclinD1, a mediator of the cell cycle was suppressed by overexpressing miR-140, which in turn supports its anti-proliferative activity in OS cells.

Our next emphasis was the underlying mechanism, which was initiated by predicting the potential target of miR-140. *Wnt1* belongs to the WNT gene family, and members in this family can mediate the oncogenesis, and regulate cell fate and patterning during embryogenesis (25). For example, HDAC4 was the target mRNA of miR-140 in OS cells, and miR-140 mimic treatment was found to inhibit the protein expression but produce no significant impact on the mRNA level of HDAC4 (26). It reveals that miR-140 may participate in controlling the development of OS through negatively regulating HDAC4 protein expression. Similarly, in this study, it was observed that miR-140 overexpression could down-regulate *Wnt1* at both the mRNA and protein levels, which is possibly

attributed to the effect of miR-140 on *Wnt1* mRNA stability and translation.

Our subsequent experiment also explored the effect of miR-140 on drug resistance in MG63 cells. As a common clinical issue, the resistance of tumor cells to drugs may seriously weaken the therapeutic role of certain drugs. For example, in gastric cancer, miR-508-5p can mediate ABCB1 and ZNRD1, and miR-497 can target Bcl-2, both of which could modulate drug resistance (27, 28). In this study, a more evident inhibitory effect of doxorubicin (a widely used drug for solid tumors) on inhibiting MG63 cell viability was observed after the overexpression of miR-140, suggesting the beneficial role of miR-140 in decreasing drug resistance of OS cells. In this process, there were down-regulated expressions of ABCC1 and ABCG2, which also support the protective role of miR-140 in improving drug sensitivity.

In conclusion, the findings of this study provide certain evidence that miR-140 overexpression may suppress the proliferation of human OS cells and may enhance drug sensitivity by directly regulating *Wnt*/ β -catenin signaling. It may be important to provide an additional reference for broadening molecular targets that may be available to be used for target therapy of OS and other cancers. While the results of our study still require further validation both in vivo and in vitro, so as to enhance the reliability and assist the development of gene therapy.

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None.

Conflict interest

The authors declare no conflict of interest.

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