

Overexpression of miR-214 promotes the progression of human osteosarcoma by regulating the Wnt/ β -catenin signaling pathway

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Abstract. The aberrant expression of microRNA (miR)-214 contributes to the regulation of normal and cancer cell biology, and is associated with human malignancies, however, it can operate in a contradictory manner. The role of miR-214 in osteosarcoma remains to be fully elucidated. The aim of the present study was to investigate the effects of miR-214 on osteosarcoma progression and tumor cell proliferation, and examine the molecular mechanism underlying osteosarcoma. The level of miR-214 was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis in osteosarcoma and matched paracancerous tissues, and in human osteosarcoma cancer cell lines. The roles of miR-214 in cell proliferation, survival and cell cycle were analyzed using miR-214 lentivirus (LV-miR-214)-infected osteosarcoma cells. In addition, the downstream target proteins in the Wnt/ β -catenin signaling pathway were evaluated using western blot analysis in the LV-miR-214-infected cells. The LV-miR-214-infected MG63 cells were also treated with exogenous β -catenin for 24, 48 and 72 h, respectively, following which the expression of β -catenin was measured using western blot analysis and survival was determined using a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results of the RT-qPCR analysis showed that the expression level of miR-214 was significantly higher in the osteosarcoma tissues, compared with that in the matched paracancerous tissues, and the same was observed in the osteosarcoma cell lines. The MG63, Saos-2 and U2OS cells were infected with the hsa-mir-214 lentivirus for 48 h, and the levels of miR-214 were significantly upregulated in the human osteosarcoma cancer cells. The overexpression of miR-214 in the MG-63 and Saos-2 cells promoted cell growth,

and treatment of the cells with specific antisense-microRNA oligonucleotides (AMOs) for miR-214 for indicated durations reversed the effects of miR-214. Additionally, the AMO-treated MG63 cells showed G0/G1 phase arrest, suggesting that miR-214 contributed to regulation of the cell cycle. In addition, the results of western blot analysis showed that, in the miR-214 lentivirus-infected cells, the levels of cyclin-D1, c-myc and lymphoid enhancer-binding factor-1 were significantly increased, compared with those in the control lentivirus-infected cancer cells. Of note, infection with the miR-214 lentivirus did not affect the levels of Wnt1, Wnt2, Wnt4, Axin or glycogen synthase kinase β in the U2OS cells, whereas the expression levels of β -catenin in the MG63 cells and Saos-2 cells were significantly increased. The addition of exogenous β -catenin effectively reversed the efficiency of miR-214-specific AMOs, which was detected using an MTT assay. These data suggested the critical role of miR-214 in human osteosarcoma via regulation of the Wnt/ β -catenin signaling pathway and demonstrated that miR-214 is as an oncogene for human osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant bone cancer, which occurs most frequently in adolescents and its peak incidence is 15-19 years (1,2). Osteosarcoma usually occurs in the long bones of limbs, particularly in the distal femur and proximal tibia. Osteosarcoma is a locally destructive tumor and it usually has a high tendency for systemic metastasis (3). It is reported that ~20% of patients have lung metastases at the time of initial diagnosis and 40% of patients have advanced distant organ metastases (4,5). Although substantial basic investigations and clinical trials have been performed to elucidate the molecular mechanisms underlying the process of osteosarcoma carcinogenesis, the precise and clear mechanisms remain to be fully elucidated, which leads to the poor prognosis and therapeutics of osteosarcoma. The 5-year survival rate of patients with osteosarcoma remains at 60-70% (6,7). Therefore, it is important to determine the molecular mechanisms underlying osteosarcoma carcinogenesis.

MicroRNAs (miRNAs or miRs) constitute a recently discovered class of small, non-coding RNAs molecules, which are able to post-transcriptionally regulate gene expression (8,9).

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In a variety of organisms, they are widespread and show a high level of conservation throughout evolution (10,11). Several studies have reported that miRNAs are usually abnormally expressed in a number of cancer cells, and they are often important in the progression of cancer by regulating tumor suppressor genes and oncogenes (12,13). In previous years, miR-214 has been located inside the sequence of the long noncoding Dnm3os transcript and acts as an important regulator in carcinogenesis, although it may operate in a context-dependent manner and can have contradictory effects during tumor progression (14). Yang *et al* (15) reported that miR-214 regulates gastric cancer cell proliferation, migration and invasion by targeting phosphatase and tensin homolog. Zhang *et al* (16) found that hemolysis-free plasma miR-214 works as a novel biomarker of gastric cancer and is correlated with distant metastasis. These studies demonstrated that miR-214 may be a novel potential therapeutic agent for human gastric cancer. In addition, decreased expression of miR-214 has been found to contribute to liver metastasis in patients with colorectal cancer, which may be involved in determining the metastatic niche (17). Xu *et al* (18) reported that miR-214 is critical in ovarian cancer stem cells via regulation of the p53-Nanog axis. It can also be used as a therapeutic target for ovarian cancer. However, Kalniete reported that patients with triple-negative breast cancer exhibiting a high level of miR-214 showed significantly poorer disease-specific survival rates, compared with patients with a low level, suggesting that miR-214 may be used as a potential prognostic biomarker for patients with triple-negative breast cancer.

Until now, the role and cellular effects of miR-214 in osteosarcoma cancer have not been fully clarified. In the present study, the expression levels of miR-214 were examined in clinical specimens from patients with osteosarcoma and in osteosarcoma cell lines. The molecular mechanism underlying the involvement of miR-214 in osteosarcoma was also examined. The data obtained in the present study may provide novel clues for improving the clinical therapy of osteosarcoma.

Materials and methods

Patients. A total of 36 patients (20 male and 16 female) were recruited and normal human skeletal muscle cells (HskMCs) were isolated from the skeletal muscle of limbs from adult or child donors from the Second Affiliated Hospital of Bengbu Medical College (Bengbu, China). The median age of the patients was 16.87 years (range 6-21 years). The specimens were collected between April 2013 and March 2015. All patients were diagnosed with osteosarcoma and underwent surgery. All tissue samples were analyzed and confirmed by postoperative histopathological examination of the specimens. The osteosarcoma tissues were collected and frozen at -80°C until they were analyzed using western blot analysis. The present study was performed in compliance with the Declaration of Helsinki and approved by the ethics committee of the Second Affiliated Hospital of Bengbu Medical College. All patients were well informed and provided signed consent prior to the experiments.

Cell lines. The MG63, Saos-2 and U2OS human osteosarcoma cell lines were obtained from the American Type

Culture Collection (Manassas, VA, USA). The HSkMCs were purchased from Tongpai Biological Technology Co., Ltd. (Shanghai, China). Cells were maintained at 37°C in a 5% CO₂ atmosphere.

Reagents. Dulbecco's modified Eagle's medium and RPMI-1640 medium were purchased from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The transfection reagents used in the present study was Lipofectamine[®] 2000 (cat. no. 11668-019), which was purchased from Invitrogen; Thermo Fisher Scientific, Inc. The total RNA extraction kit (cat. no. MK700) was obtained from Takara Biotechnology Co., Ltd. (Dalian, China). The cDNA reverse transcription kit was also obtained from Takara Biotechnology Co., Ltd. (cat. no. 6110). The recombinant β -catenin (cat. no. 11279-H20B-50) was purchased from Sino Biological Inc. (Beijing, China). The specific anti-sense-microRNA oligonucleotides (AMOs) were designed and synthesized by GenePharma Corporation (Shanghai, China).

Cell transfection. Cells (0.5×10^5 cells per well) were plated in a 24-well plate and cultured for 24 h. A mixture of Opti-MEM reduced serum medium (cat. no. 31985-070; Invitrogen; Thermo Fisher Scientific, Inc.) with 8 μ g/ml polybrene was then prepared. Has-mir-214 lentivirus (cat. no. mir-LV171) and mir-control lentivirus (cat. no. mir-LV000) were obtained from Biosettia, Inc. (San Diego, CA, USA). The cells were transfected using 1 μ l lentivirus and were incubated at 37°C overnight with 5% CO₂.

Western blot analysis. The cells stably transfected with the miR-214 lentivirus or control lentivirus were cultured for 6 h. The cells were then washed with ice-cold PBS and centrifuged at 850 x g for 5 min at room temperature. Cell lysates were then prepared for western blot analysis, using RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology, Haimen, China). The membranes were blocked with 5% FBS in TBST buffer (0.1% Tween-20), and the amount of protein per lane was 25 μ g. The antibodies used were as follows: Rabbit polyclonal anti-cyclin D1 (cat. no. PA5-32373; 1:1,000) was purchased from Thermo Fisher Scientific, Inc.; rabbit polyclonal anti-MYC/c-Myc antibody (calculated MW 49 kDa; cat. no. ALS15021; 1:1,000) and rabbit anti-lymphoid enhancer-binding factor-1 (LEF-1; 1:1,000; cat. no. AP1048A) polyclonal antibodies were purchased from Abgent Biotech Co., Ltd. (Suzhou, China); rabbit polyclonal anti-Wnt1 (C-terminal; cat. no. AP6785b; 1:1,000) was immunized with a KLH conjugated synthetic peptide 267-296 amino acids from the C-terminal region of human Wnt1; rabbit polyclonal anti-Wnt2 (C-terminal region; cat. no. A110314; 1:1,000); rabbit polyclonal anti-Wnt4 (center; cat. no. AP6683C; 1:1,000), generated from rabbits immunized with a KLH conjugated synthetic peptide 211-239 amino acids from the central region of human Wnt 4. The Wnt 1, Wnt 2 and Wnt 4 antibodies were purchased from Abgent Biotech Co., Ltd. The secondary antibodies were goat anti-rabbit IgG-HRP (cat. no. sc-2004; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and goat anti-mouse IgG-HRP (cat. no. sc-2005; 1:1,000; Santa Cruz Biotechnology,

Inc.). Rabbit polyclonal anti-axin (cat. no. ab1457; 1:1,000; Abcam, Cambridge, UK); rabbit polyclonal GSK-3 β (H-76; cat. no. sc-9166; 1:1,000; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti- β -catenin (cat. no. H-102; 1:1,000; Santa Cruz Biotechnology, Inc.; 200 μ g/ml). The primary antibodies were incubated overnight at 4°C. Anti- β -actin and the HRP-conjugated goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc and incubated for 40 min at room temperature. The bands were visualized using a gel imaging system (BioRad GelDoc XR; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the Quantity One 1-D analysis software (version 4.6.9) that came with the system.

Cell growth curve. The MG63 and U2OS were infected with the hsa-mir-214 lentivirus or control lentivirus for 48 h, and stable cell lines were screened and selected. Cells in the logarithmic growth phase were trypsinized and adjusted at a concentration of 4×10^4 cells/ml. The cells were then plated in a 96 well-plate in three replicates. The cells were incubated at 37°C in a 5% CO₂ in air atmosphere. Trypan blue staining was used for detect the ratio of living cells. The cells were digested and counted every 24 h, with four fields of view assessed under a light microscope. After 5 days, a cell growth curve was prepared. The culture duration was used as the horizontal axis and the number of cells was used as the vertical axis.

FACS. The cells (1×10^6) were collected by centrifugation at 850 x g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS at room temperature. Subsequently, the cells were fixed in cold 70% ethanol for 30 min at 4°C. The cells were washed in PBS and centrifuged at 850 x g for 10 min at 4°C to avoid cell loss. Subsequently, the cells were treated with ribonuclease by adding 50 μ l of a 100 μ g/ml stock of RNase. Finally, 200 μ l PI (from a 50 μ g/ml stock solution) was added for assessment using FACS.

3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. An MTT assay was performed to determine cell viability. Briefly, the stable cell lines were plated into a 96-well plate at a density of 5×10^3 cells/well. After 6 h, the cells were treated with miR-214-specific AMOs for 24, 48 and 72 h at 37°C. At 4 h prior to assessment, 10 μ l of MTT (5 mg/ml) was added to the medium. The cells were incubated at 37°C in 5% CO₂. The absorbance of each well was read at 490 nm using a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA samples were extracted with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) Reverse transcription was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. SYBR-green was purchased from Thermo Fisher Scientific, Inc. (cat. no. 4364346). The primers used for mir-214 were as follows: reverse transcription, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACACTGCC-3'; PCR forward, 5'-TGCGGGTGTCTCCGCTTCGGCAGC-3' and reverse 5'-CAGTGCAGGGTCCGAGGT-3'. The thermocycler conditions used were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 5 sec, annealing at 60°C for

34 sec and detection at 74°C for 3 sec. Three replicates were performed for each group. The $\Delta\Delta C_q$ method was used for quantification (19).

Statistical analysis. The data were analyzed using SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). A Student's t-test was performed to analyze data. $P < 0.05$ was considered to indicate a statistically different difference. Images of the bands were captured and analyzed by using Image J software (National Institutes of Health, Bethesda, MD, USA).

Results

Higher expression levels of miR-214 are observed in patients with osteosarcoma and osteosarcoma cell lines. In the present study, tissues were collected from a total of 36 patients at the Second Affiliated Hospital of Bengbu Medical College, with the median age of 16.87 years (range 6-21 years). In order to determine the role of miR-214 in the progression of osteosarcoma, the expression levels of miR-214 were detected in the osteosarcoma specimens and the paired adjacent normal tissues. The total RNA was extracted from the specimens of osteosarcoma and paired adjacent normal tissues. As shown in Fig. 1A, the RNA purity and integrity were detected using formaldehyde denaturing agarose gel electrophoresis. Subsequently, the expression levels of miR-214 were detected in the osteosarcoma and paired peritumoral tissues. The results showed that increased expression levels of miR-214 were observed in the osteosarcoma tissues ($P < 0.01$), compared with the peritumoral tissues (Fig. 1B). The levels of miR-214 were also assessed in various osteosarcoma tumor cell lines, including the MG63, Saos-2 and U2OS cell lines, and were compared with the levels in the nontumor HSkMC cell. As shown in Fig. 1C, the miR-214 levels were significantly upregulated in the osteosarcoma cancer cells. The HSkMCs were used as normal controls, which were isolated from the skeletal muscle of limbs from adult or fetal donors. These data showed that the expression levels of miR-214 were significantly increased in the patients with osteosarcoma and the osteosarcoma cell lines.

Overexpression of miR-214 promotes osteosarcoma cell proliferation. In order to assess the role of miR-214 (Fig. 2A) in regulating cell proliferation, the hsa-mir-214 lentivirus was used to infect the osteosarcoma cancer cells, and cell proliferation was determined using an MTT assay. The MG63 cells, Saos-2 cells and U2OS cells were infected with hsa-mir-214 lentivirus for 48 h, as shown in Fig. 2B, and the levels of miR-214 were significantly upregulated in the osteosarcoma cancer cells.

The MTT assay was used to detect the cell proliferation of osteosarcoma cells. The MG-63 cells and Saos-2 cells were infected with the hsa-mir-214 lentivirus and cultured for 1, 2, 3, 4 and 5 days, respectively. As shown in Fig. 3A, the cells infected with the has-miR-214 lentivirus grew at a significantly higher rate, compared with those infected with the negative control lentivirus ($*P < 0.05$; $**P < 0.01$).

Subsequently, specific AMOs were used to reverse the effect of the hsa-mir-214 lentivirus. The miR-214 lentivirus-infected cells grew at a faster rate, compared with

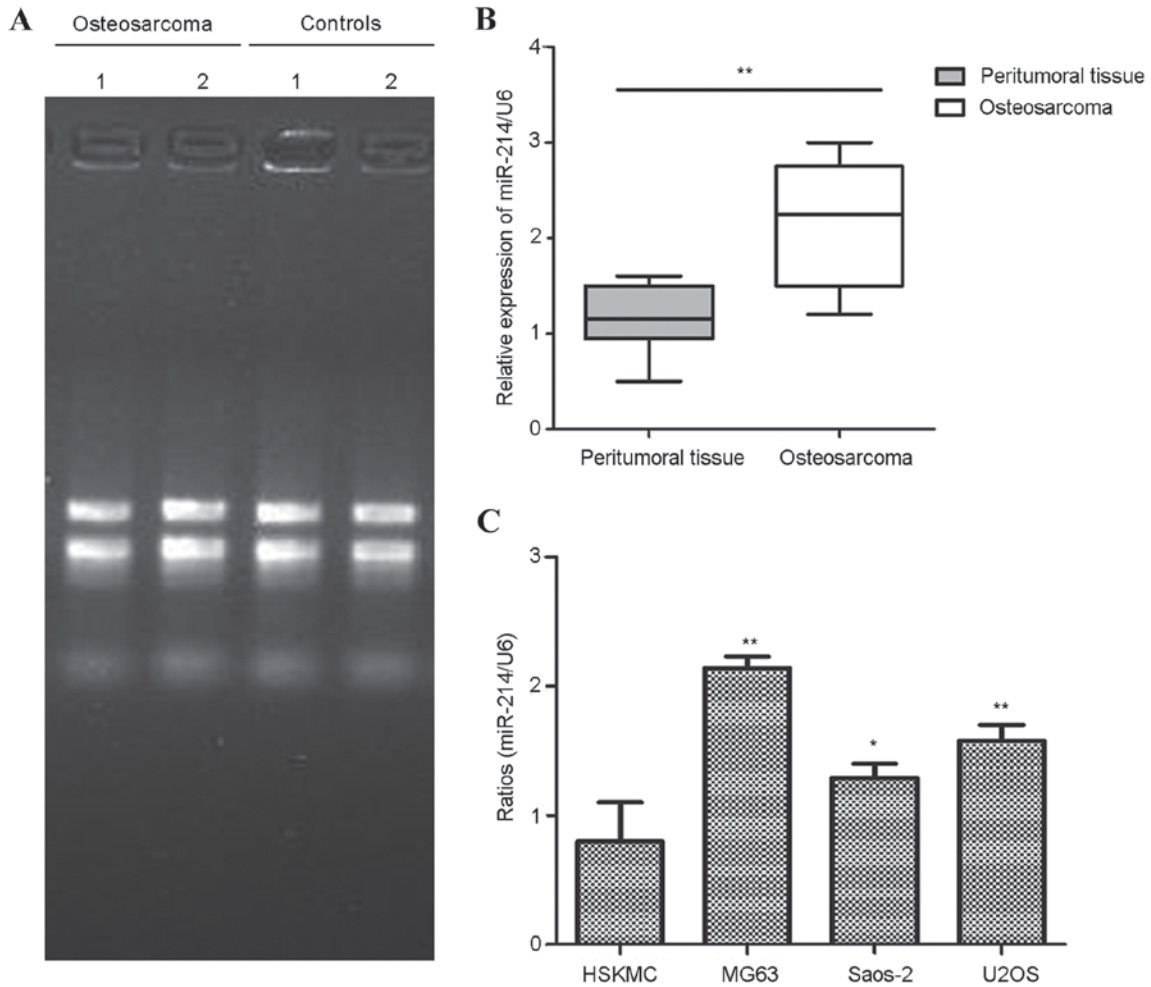


Figure 1. Expression of miR-214 is higher in patients with osteosarcoma and osteosarcoma cell lines. (A) Specimens from osteosarcoma tissues and paired peritumoral tissues were collected, and two of the independent samples are shown. Total RNA was extracted from the tissues, and the RNA purity and integrity were determined using formaldehyde denaturing agarose gel electrophoresis. Visible bands are shown at 28S and 18S, and a faint band is shown at 5S. (B) Relative expression of miR-214 was detected in osteosarcoma tissues and paired peritumoral tissues using RT-qPCR analysis. ***P*<0.01, compared with peritumoral tissues. (C) Analysis of expression levels of miR-214 in osteosarcoma cell lines (MG63, Saos-2 and U2OS), compared with normal HSKMCs using RT-qPCR analysis. **P*<0.05; ***P*<0.01, compared with the HSKMCs. All experiments were performed in duplicate with three technical replicates. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HSkMCs, human skeletal muscle cells.

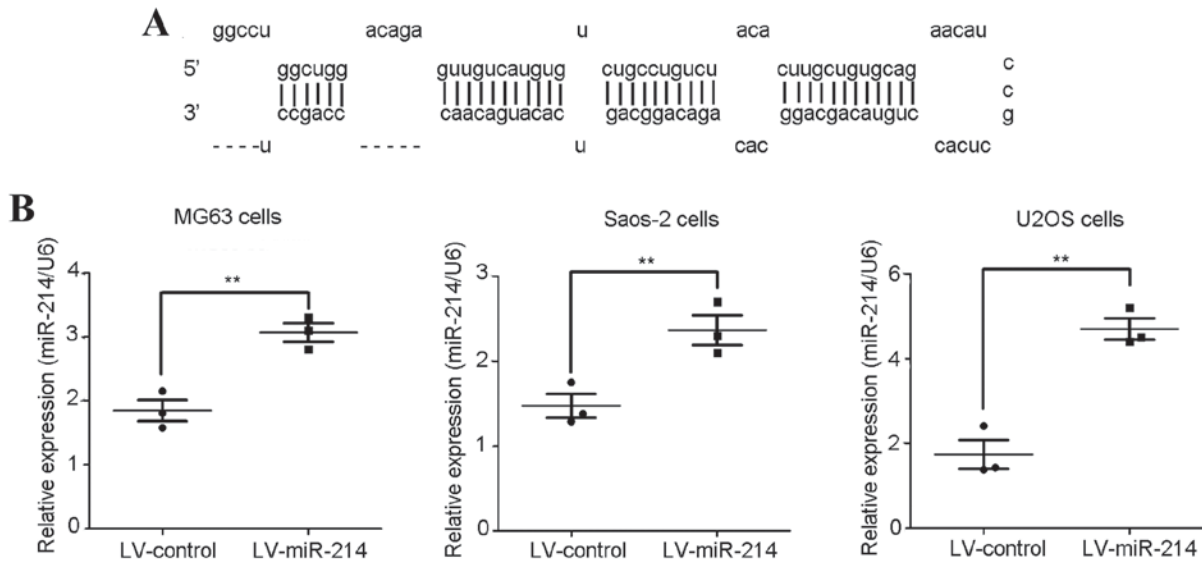


Figure 2. miR-214 is upregulated by infection with has-miR-214 lentivirus. (A) Sequence of miR-214. (B) MG63, Saos-2 and U2OS osteosarcoma cell lines were infected with has-miR-214 lentivirus. The expression of miR-214 was detected using reverse transcription-quantitative polymerase chain reaction analysis. ***P*<0.01. miR, microRNA.

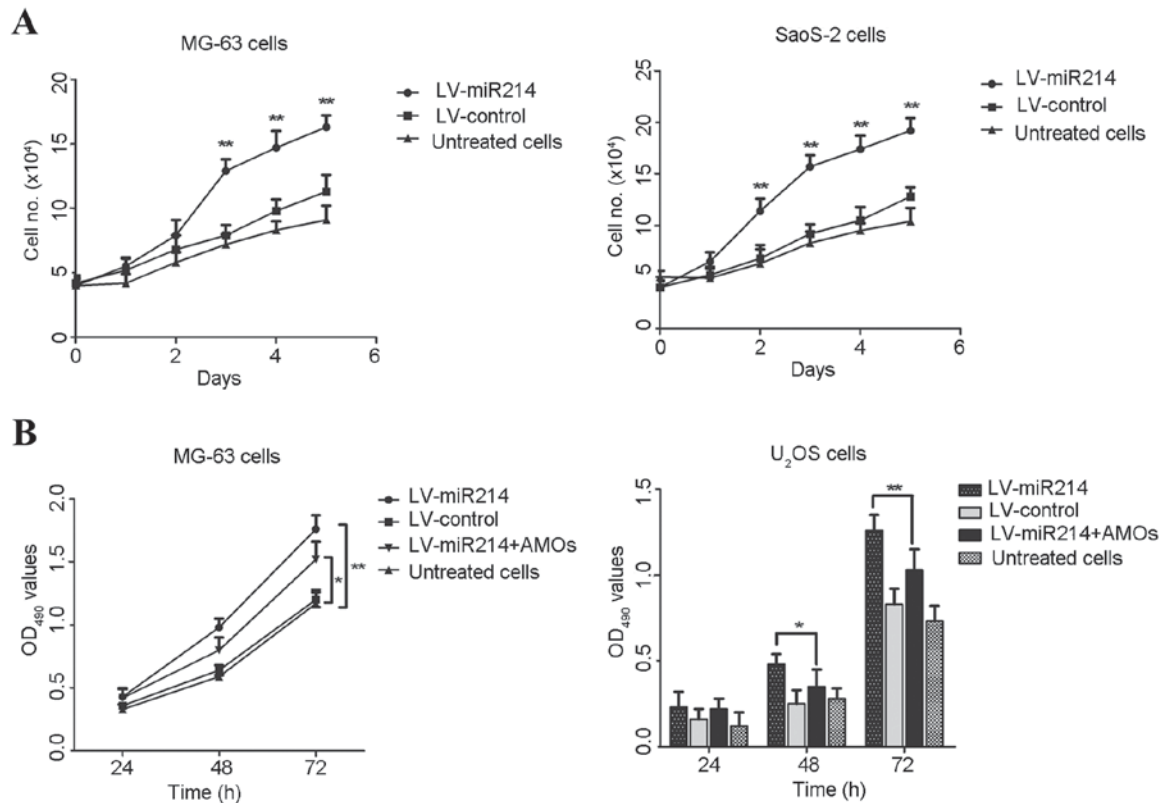


Figure 3. Overexpression of miR-214 inhibits osteosarcoma cell proliferation. Cell lines were infected with miR-214 lentivirus or control lentivirus, and stable cell lines in the logarithmic growth phase were collected. The concentration of the cell suspension was adjusted into 4×10^4 /ml. (A) Stable cells were plated into 96-well plate and cultured for 5 days, with cells digested and counted every 24 h. Each group contained three replicates. (B) Stable cell lines were treated with specific AMOs for 24, 48 and 72 h, respectively. The proliferation of MG63 cells and U2OS cells were determined using a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. * $P < 0.05$ and ** $P < 0.01$, compared with the AMO-treated group. miR, microRNA; AMOs, antisense-microRNA oligonucleotides; OD, optical density.

the control lentivirus-infected cells. However, following treatment of the miR-214-infected osteosarcoma cells with miR-214-specific AMOs for 48 and 72 h, respectively, cell proliferation was significantly decreased, compared with that in the miR-214-infected osteosarcoma cells (Fig. 3B). These data showed that higher levels of miR214 promoted the proliferation of osteosarcoma cancer cells, and miR-214 specific-AMOs reversed its effect on the osteosarcoma cells.

miR-214-specific AMOs regulate cell cycle progression and induce cell cycle arrest at the G0/G1 phase. To further examine the effect of miR-214 on cell cycle progression, a FACS assay was used to detect cell distribution in the miR-214 lentivirus-infected cancer cells and control lentivirus-infected cells. As shown in Fig. 4A and B, MG63 cells infected with miR-214 lentivirus were arrested in the G0/G1 phase; the percentage of cells in the G0/G1 phase was significantly increased from 52.90 to 67.21% ($P < 0.01$), compared with the cells infected with control lentivirus. These data suggested that miR-214 was important in regulating the G1/S transition.

Overexpression of miR-214 upregulates the Wnt/ β -catenin signaling pathway. Studies have shown that aberrant activation of the Wnt/ β -catenin pathway is common in the progression of osteosarcoma. In order to determine whether miR-214 affects the activation of the Wnt/ β -catenin signaling pathway, western blot analysis was performed to detect the protein expression

of downstream target genes of the Wnt/ β -catenin pathway. As shown in Fig. 5A and B, in the miR-214 lentivirus-infected cells, the levels of cyclin D1, c-myc and LEF-1 were significantly increased, compared with those in the control lentivirus-infected cancer cells (LEF, $P < 0.05$; cyclin D1, $P < 0.01$; c-myc, $P < 0.01$). These results revealed that miR-214 may be involved in regulating the Wnt/ β -catenin pathway.

LV-miR-214 infection upregulates the protein level of β -catenin. It is known that the expression of miR-214 regulates the Wnt/ β -catenin signaling pathway. The present study aimed to further examine the further mechanism underlying the effect of miR-214, western blot analysis was performed to detect the levels of relative proteins in the Wnt/ β -catenin signaling pathway. As shown in Fig. 6A, no significant variations were found in the levels of Wnt1, Wnt2, Wnt4, Axin and Gsk-3 β in the miR-214 lentivirus-infected U2OS cells, compared with those in the control lentivirus-infected U2OS cancer cells. Variation in the levels of β -catenin in the MG63 cells and Saos-2 cells were also examined. Consistent with the data in Fig. 6A, the levels of β -catenin were increased in the LV-miR-214-infected MG63 cells and Saos-2 cells (Fig. 6B). However, the mRNA levels of β -catenin were not altered in the LV-miR-214-infected MG63 or Saos-2 cells (Fig. 6C), and there were no statistical differences between the LV-control and LV-miR-214-infected cells. These data suggested that β -catenin may be a target of miR-214 in osteosarcoma cells.

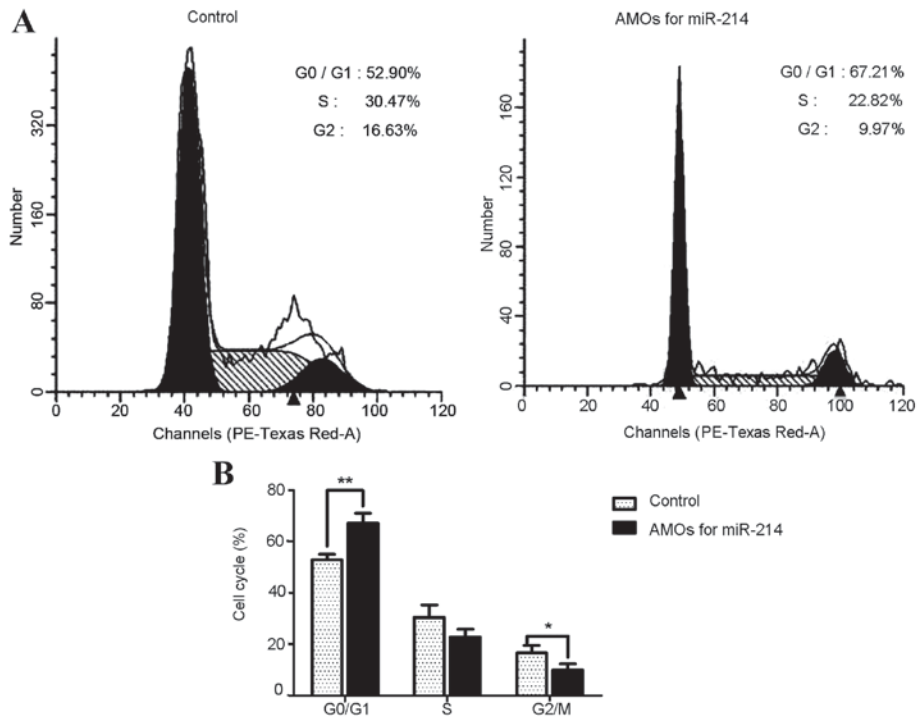


Figure 4. miR-214 regulates cell cycle progression and induces cell cycle arrest at the G0/G1 phase. (A) MG63 cells were stained with propidium iodide and cell distribution was determined using flow cytometry. (B) Histogram of cell cycle distribution. *P<0.05 and **P<0.01, compared with MG63 cells infected with control lentivirus. miR, microRNA; AMOs, antisense-microRNA oligonucleotides.

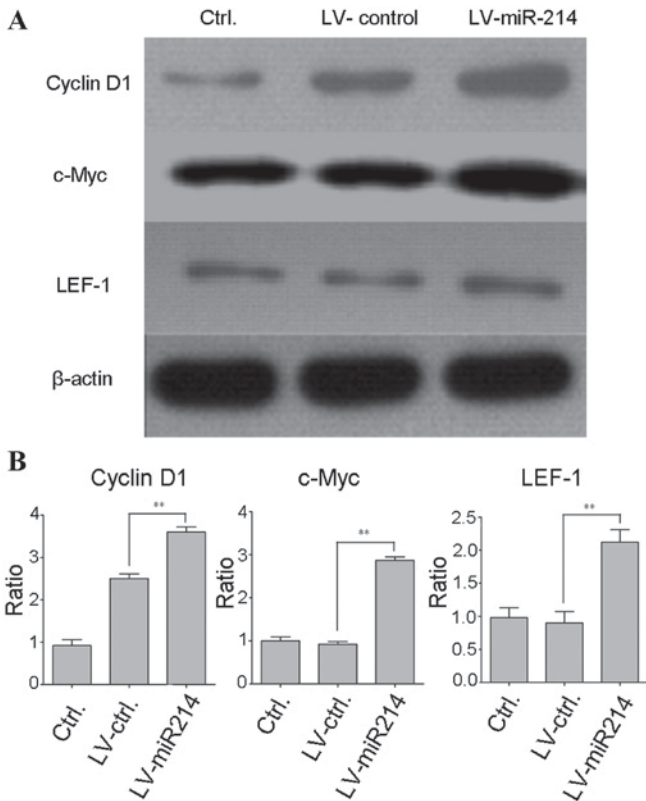


Figure 5. Overexpression of miR-214 downregulates the Wnt/β-catenin signaling pathway. (A) Downstream proteins in the Wnt/β-catenin signaling pathway were determined using western blot analysis. MG63 cells stably transfected with miR-214 lentivirus or control lentivirus were lysed. The levels of cyclin D, c-myc and LEF-1 were determined using western blot analysis. (B) Histogram of relative levels of cyclin D1, c-myc and LEF-1. **P<0.01. miR, microRNA; LEF-1, lymphoid enhancer-binding factor 1; Ctrl, control.

Exogenous addition of β-catenin effectively reverses the efficiency of miR-214-specific AMOs. In order to assess the specificity of β-catenin in the LV-miR-214-infected MG63 cells, a rescue experiment was performed by adding β-catenin protein to serum-starved MG63 cells. As shown in Fig. 7, the LV-miR-214-infected MG63 cells were treated with 100 ng/ml β-catenin for 24, 48 and 72 h, respectively. The results showed that treatment with exogenously added β-catenin significantly reversed the efficiency of miR-214-specific AMOs. This reverse experiment can be used to assess the specificity of β-catenin in miR-214 lentivirus-infected cells.

Discussion

Osteosarcoma is the most common type of bone cancer, and is most often found in teenagers (20). The incidence rate of osteosarcoma in teenagers is high, suggesting that rapid bone growth induces osteosarcoma (3). Investigations on the molecular mechanisms of osteosarcoma have gradually increased in number, however, a comprehensive understanding of the mechanisms underlying osteosarcoma remains to be fully elucidated. Following substantial progression in understanding of the roles of miRNAs, their role in cancer has received significant attention (21-23). In the present study, the role of miR-214 in the progression of human osteosarcoma was established and the specific molecular mechanisms of miRNA-214 in human osteosarcoma were clarified.

The present study detected the expression of miRNA-214 in human osteosarcoma specimens and paired peritumoral tissues. The results demonstrated that increased levels of miR-214 were observed in the osteosarcoma tissues. This was in contrast to data by Wen *et al* (24) on cervical cancer.

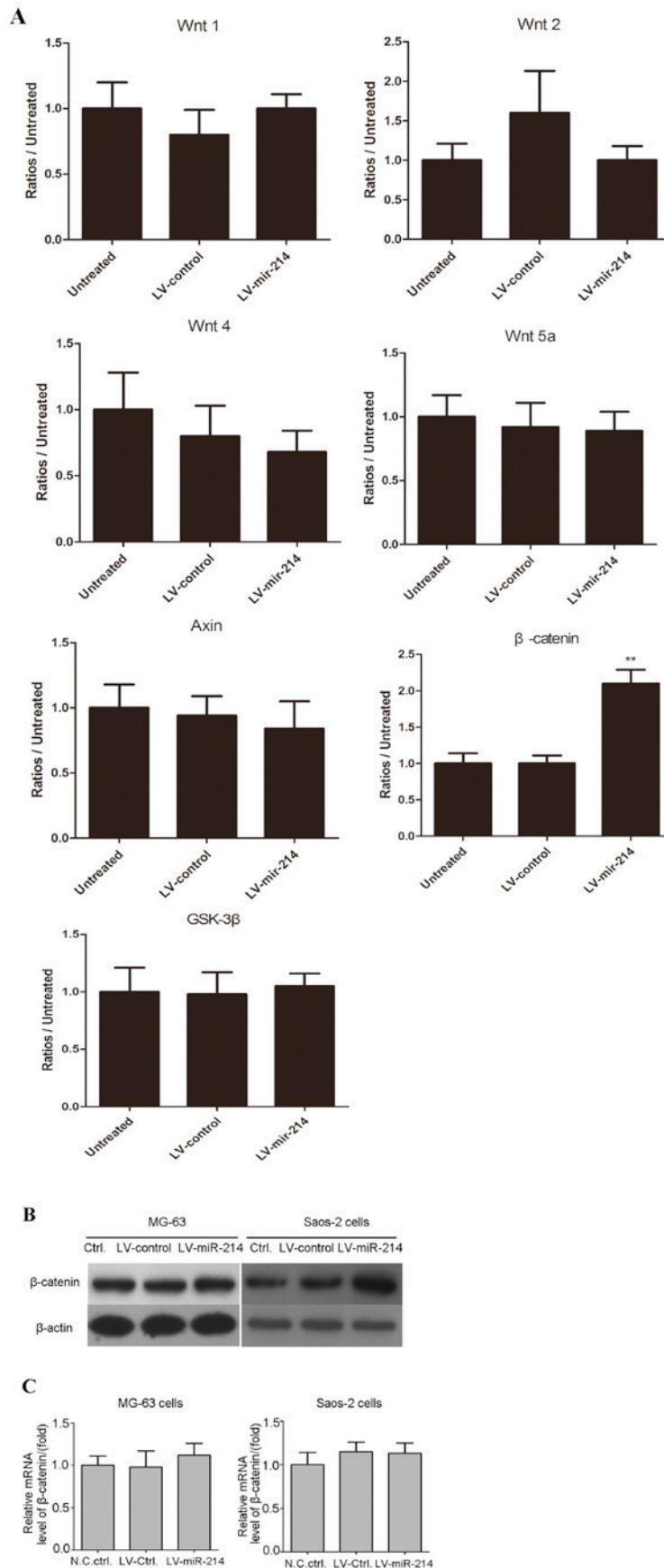


Figure 6. LV-miR-214 infection downregulates the protein level of β -catenin. (A) Stable cells (LV-miR-214-U2OS and LV-control-U2OS; 3×10^5 cells/per well) were plated into a 24-well plate, cultured for 24 h and cell lysate was prepared using 1X SDS lysis buffer. The levels of Wnt1, Wnt2, Wnt4, Wnt5a, Axin, β -catenin and Gsk-3 β were detected using western blot analysis. ** $P < 0.01$, compared with untreated cells. (B) Levels of β -catenin were detected in MG-63 cells and Saos-2 cells. β -actin was used as internal reference. (C) Histogram of relative mRNA levels of β -catenin in MG-132 and Saos-2 cells infected with the miR-214 lentivirus and control lentivirus. Expression of β -catenin was detected using reverse transcription-quantitative polymerase chain reaction analysis. β -actin was used as internal reference. No statistical differences were found between groups. miR, microRNA; Gsk-3 β , glycogen synthase kinase β ; Ctrl, control; N.C, negative control.

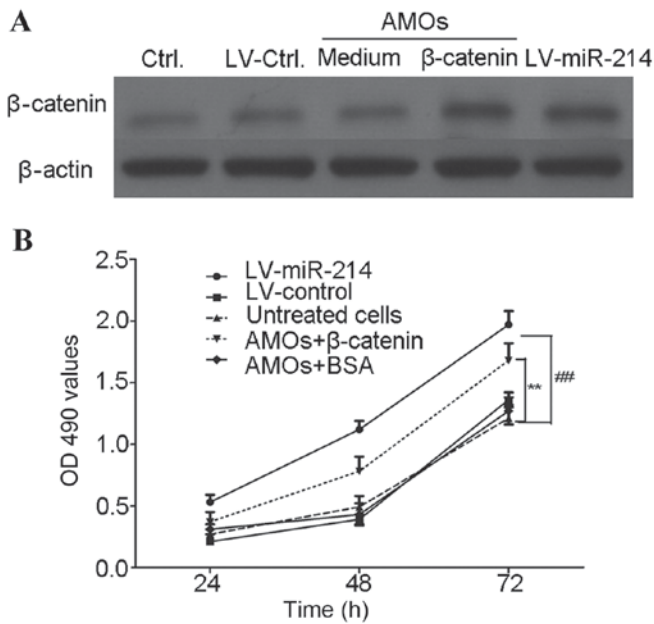


Figure 7. Exogenous addition of β -catenin effectively reverses the efficiency of the miR-214 lentivirus. The stable cells (LV-miR-214-MG63 and LV-control-MG63; 3×10^5 cells/per well) were plated into a 24-well plate for 6 h, (A) LV-miR-214-MG63 cells were treated with 100 ng/ml of β -catenin for 48 h and the expression of β -catenin was determined using western blot analysis. (B) Cells were treated with β -catenin for 24, 48 and 72, respectively. The proliferation of MG63 cells was determined using a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. ** $P < 0.01$, compared with LV-miR-214+ β -catenin group; ### $P < 0.01$, compared with LV-control group. miR, microRNA; AMOs, antisense-microRNA oligonucleotides; Ctrl, control; OD, optical density.

However, Yang *et al* (15) found that miR-214 was overexpressed in gastric cancer tissues and cell lines, which was consistent with the results of the present study. Thus, the emerging data demonstrated that miR-214 has a controversial role in different types of tumor, which may be due to organ-specific actions and the possibility that miR-214 has different target genes to exert opposing functions.

It has been reported that abnormal expression of the Wnt/ β -catenin signaling pathway has been detected in osteosarcoma tissues, which significantly contributes to the promotion of cell survival and proliferation in multiple types of malignancy (25-27). Thus, the Wnt/ β -catenin signaling pathway has received increased attention in targeted therapy for osteosarcoma. The present study aimed to detect the association between the expression of miR-214 and the activity of the Wnt/ β -catenin signaling pathway. The results of the present study showed no significant variation in the levels of Wnt1, Wnt2, Wnt4, Axin or Gsk-3 β in the miR-214 lentivirus-infected cells, compared with those in the control lentivirus-infected U2OS cancer cells, however, the levels of β -catenin in the MG63 cells and Saos-2 cells were upregulated. As expected, miR-214 may be involved in regulating and activating the Wnt/ β -catenin pathway by directly regulating β -catenin.

The results of the FACS assay showed that miR-214-specific AMOs inhibited the cell cycle and induced cell cycle arrest at the G0/G1 phase, which also demonstrated that miR-214 regulated cell cycle progression and promoted the progression of cells through the check point of the G0/G1 phase. In addition,

a reverse experiment was designed in which the MG63 cells were treated with exogenously added β -catenin, which significantly reversed the efficiency of the miR-214-specific AMOs. It was shown that this reversal experiment was able to assess the specificity of β -catenin in miR-214-specific AMOs treated cells. Taken together, the results of the present study showed that miR-214 in osteosarcoma cells had an important oncogenic role to regulate multiple targets in the Wnt/ β -catenin pathway. Therefore, miR-214 offers potential as a novel target for the clinical therapy of human osteosarcoma.

Acknowledgements

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