miR-542-3p overexpression is associated with enhanced osteosarcoma cell proliferation and migration ability by targeting Van Gogh-like 2

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Abstract. Osteosarcoma is the most common histological form of primary bone cancer, which arises from osteoid tissue. It occurs predominantly in infants and adolescents, with an incidence of 4-5 cases/100,000,000. The 5-year survival rate of patients with osteosarcoma has significantly improved over time; however, there remains a significant proportion of patients that respond poorly to chemotherapy. An improved understanding of the pathology of osteosarcoma is required to provide more effective treatment strategies, identify biomarkers and develop novel chemotherapeutic agents. Disturbance in microRNA (miRNA) expression has been identified in osteosarcoma tissues and cell lines; however, the roles of miRNA during osteosarcoma pathogenesis remain to be elucidated. In the present study, the expression levels of eight selected miRNAs were investigated in osteosarcoma tissues and the results revealed that the expression levels of miR-542-3p and miR-542-5p were significantly upregulated and the expression of miR-199-3p was significantly downregulated. Using a dual luciferase assay and western blot analysis, the present study confirmed that Van Gogh-like 2, which is a non-canonical Wnt pathway suppressor, was a target gene of miR-542-3p. Subsequently, the biological function of miR-542-3p in U2OS cells was examined, which revealed that overexpression of miR-542-3p can enhance the cell proliferation and migration ability of U2OS cells. This indicated that miR-542-3p may act as an oncogene in osteosarcoma pathogenesis. The findings of the present study may provide assistance in understanding the development of osteosarcoma and aid in the development of strategies for the diagnosis and treatment of osteosarcoma.

Introduction

Osteosarcoma is the most common type of primary bone malignancy, which arises from osteoid tissue and produces immature bone. Osteosarcoma occurs mainly in infants and adolescents and has an incidence of 4-5 cases/100,000,000 (1,2). There has been a significant improvement in the 5 year survival rate of patients with osteosarcoma to ~60-70% since combinational chemotherapy was introduced (3). However, the response to chemotherapy is poor in a significant proportion of these patients, with the possibility of local relapse or distant metastasis following curative resection of the primary tumor and intensive chemotherapy (1-4). An increased understanding of the pathogenesis of osteosarcoma is required to improve treatment strategies, identify novel biomarkers and develop chemotherapeutic agents.

MicroRNA (miRNA) is a type of short non-coding RNA, which suppresses the expression of protein coding genes by partial complementary binding, particularly to the 3’untranslated regions (UTRs) of messenger RNAs (5,6). Alterations to the expression of miRNAs are involved in the initiation, progression and metastasis of human cancer and it is hypothesized that miRNAs function as tumor suppressors and as oncogenes in cancer development (4,5). Several studies have investigated the expression profile of osteosarcoma tissues and a variety of miRNA expression has been identified. Taullli et al (6) reported that, in mice xenografts, myogenic differentiation is promoted by the miRNAs, miR-1 and miR-206 to regulate skeletal muscle development and inhibit rhabdomyosarcoma tumor growth. Subramanian et al (7) examined the miRNA expression profiles in histological soft tissue samples, including 27 from synovial sarcoma, liposarcoma, rhabdomyosarcoma, leiomyosarcoma and gastrointestinal stromal tumor and seven from normal tissues. In addition, analyses of the miRNA expression profile of 19 human osteosarcoma cell lines by Namlos et al revealed 177 miRNAs that were differentially expressed in osteosarcoma cell lines compared with normal bone cells (8).
In order to contribute to the clarification of the roles of miRNA during osteosarcoma pathogenesis, the expression of eight candidate miRNAs was detected in a total of 13 paired soft tissue sarcoma and normal tissue samples in the present study. Following identification of significantly altered miRNAs in a screen, one of their target genes, which was predicted by bioinformatics tools, was selected for studying its function in the migration and invasion ability of U2OS cells.

**Materials and methods**

**Patients and tissue samples.** The present study had been permitted by the Yidu Central Hospital (Weifang, China) and Yantai Yuhuangding Hospital (Yantai, China). Written informed consent had been obtained from all patients prior to participation in the study. According to the ethical and legal standards [NO. (2011)03 ethical and legal standards of Yantai Yuhuangding Hospital], all specimens were made anonymous. Thirteen pediatric patients who were diagnosed with osteosarcoma were 10-16 (median 13) years old. Prior to neoadjuvant therapy, the tumor biopsies were obtained, freshly frozen and stored at -80°C, and histologically confirmed by pathologists. Osteosarcoma tissue and the corresponding normal bone tissue samples from the same specimens were successively obtained in Yidu Central Hospital or Yantai Yuhuangding Hospital in 2011 and 2012.

**Quantitative reverse-transcription polymerase chain reaction (RT-qPCR).** RT-qPCR analysis was used to determine the relative expression level of eight candidate microRNAs (13). TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA; 1 ml TRIzol to 50-100 mg of tissue), was used to extract total RNA from the osteosarcoma or normal bone tissues, according to the manufacturer’s instructions. The expression levels of eight candidate miRNAs were measured by TaqMan miRNA RT-qPCR. Single-stranded cDNA for each miRNA was synthesized with TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) referring to the manufacturer’s instructions. TaqMan Universal PCR Master mix with miRNA-specific TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA) was used to amplify the cDNA. U6 snRNA served as a normalizer. Primer sequences were as follows: miR-542-3p forward, 5'-TGT GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CGT AGC ACC TGC GGT CTC GTG-3'; common reverse, 5'-GTG CAG AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TGC GGG AGC TAC-3'; miR-199-3p forward, 5'-CCG GTA CCG TGC AGT ACT A-3' and stem-loop RT primer, 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CGT AGC ACC TGC GGT CTC GTG-3'; common reverse, 5'-GTG CAG AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TGC GGG AGC TAC-3'; miR-21 forward, 5'-TAG CTT ATC GTA CCG TGC AGT ACT A-3' and stem-loop RT primer, 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TGC GGT CTC GTG-3'; miR-143-5p forward, 5'-TGA GAT GAA GCA CTG T-3' and stem-loop RT primer, 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CGT AGC ACC TGC GGG AGC TAC-3'; miR-542-5p forward, 5'-TGA GAT GAA GCA CTG T-3' and stem-loop RT primer, 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CGT AGC ACC TGC GGT CTC GTG-3'; common reverse, 5'-GTG CAG AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TGC GGG AGC TAC-3'; miR-143 forward, 5'-TGA GAT GAA GCA CTG T-3' and stem-loop RT primer, 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CGT AGC ACC TGC GGG AGC TAC-3'. The scramble control was the result of the random rearrangement of miR-542-3P CAAG UAG UGA GCU AGU and the sequence was GAC UAG UGA UGA UUA. Total RNA (1 µg) was used for RT-qPCR. PCR reaction conditions were as follows: Stage 1, 95°C for 15 sec; stage 2 (40 circles), 95°C for 5 sec, 64°C for 34 sec; stage 3, melting curve. All the above experiments were performed in triplicate and repeated three times. Data analysis was performed using Microsoft Excel (Microsoft Corp., Redmont, WA, USA).

**Cell culture.** The U2OS cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/ml streptomycin (HyClone, Logan, UT, USA). All cells were maintained at 37°C under an atmosphere of 5% CO₂.

**3'-UTR luciferase reporter assays.** Luciferase reporter assays were conducted according to a published procedure (14). The target sequence was amplified by PCR using the following primers: Forward, 5'-CCG GTA CCG CTG AAT AGA TCC CTG AGG T3'- and reverse, 5'-CGC TCG AGG GGC CAG CAA ATT TTG CTC A-3' and cloned into pGL3-control vector through KpnI and Xhol sites. Mimic was synthesized according to the sequence of miR-542-3p A VNNAGL2 3'-UTR region (516 bp) containing the predicted miR-542-3p binding site was cloned into the pGL3-control vector (Promega Corporation, Madison, WI, USA) downstream of the firefly luciferase gene, after which the 3'-UTR luciferase reporter was obtained. The control was a mutant target site of miR-542-3p in 3'-UTR of VANGL2. The miR-542-3p mimic and miR-542-3p inhibitor used in this study were customized and synthesized by GenePharma Co., Ltd. (Shanghai, China). Thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK; Promega) was co-transfected into the U2OS cells. The cells were seeded in 48-well plates and cultivated at 37°C. Together with the miR-542-3p mimic or the miR-542-3p inhibitor, the luciferase reporter vectors (100 ng) were co-transfected in the presence of 0.5 µl Lipofectamine 2000 (1 µg/ml; Invitrogen Life Technologies) and then incubated for 48 h in 5% CO₂ at 37°C. Two days following transfection, the cells were collected and evaluated by Dual-Luciferase assay (Promega). Every treatment was performed in triplicate, and independent experiments were performed. The results were presented as the relative luciferase activity, and Renilla luciferase was used to normalize the expression of firefly luciferase.

**Western blot analysis.** The protein extracts obtained from the cells of osteosarcoma tissue of normal bone tissue samples were boiled in SDS/β-mercaptoethanol sample buffer [containing Tris–HCl, SDS, β-mercaptoethanol, bromophenol blue and glycerol (Owl Scientific, Inc., Woburn, MA, USA)] at 55°C for 15 min, and 20 µg of each extract was loaded into each lane.
of 8% polyacrylamide gels. The proteins were separated by electrophoresis and the proteins in the gels were blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, St. Albans, UK) by electrophoretic transfer. The membrane was incubated with goat anti-VANGL2 polyclonal antibody (Abcam, Cambridge, MA, USA) and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at 37˚C. The specific protein-antibody complex was then detected using horseradish peroxidase-conjugated rabbit anti-goat or rabbit anti-mouse immunoglobulin (Ig)G (Cell Signaling Technology, Inc., Danvers, MA, USA). After the membrane was washed using PBS containing 0.1% Triton-X 100), the determinands were developed using an enhanced chemiluminescence kit (ECL; Pierce Biotechnology, Inc., Danvers, MA, USA). The film was quantified using ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MD, USA).

**Cell proliferation assay.** The U2OS cells were seeded into 96-well plates at low density (5x10^3 cells/well) in DMEM culture overnight for attachment to occur. The cells were then transfected with either the miR-542-3p mimic or the scrambled miRNA (miRNA control). MTT (5 mg/ml; 20 µl; Sigma, St. Louis, MO, USA) were added into each well 48 h after transfection and the cells were incubated for a further 4 h. The absorbance was recorded at A570 nm using a 96-well plate reader (BioTek Instruments, Inc., Burlington, VT, USA) following the addition of 150 µl dimethyl sulfoxide (Sigma).

**In vitro migration assays.** The U2OS cells transfected with the miR-542-3p mimic, scramble miRNA, miR-542-3p inhibitor or anti-miR control were harvested 48 h after transfection and subjected to the following assays. For migration assays, the transfected cells (0.5x10^6 cells/ml) were seeded into the top of an 8.0-mm pore membrane chamber (Corning Costar, Cambridge, MA, USA). Following 12 h of incubation, the cells that had passed through the membrane to attach to the bottom of membrane were fixed with methanol and stained with hematoxylin and eosin (Sigma). The cells were scraped and removed from the top of chamber, the membranes were mounted on cover slides and the cell migration was quantified by counting the number of cells that had passed through the pores in five randomly selected fields per sample at magnification of x100 under a microscope (Nikon Eclipse TE2000-U; Nikon, Tokyo, Japan).

**Statistical analysis.** Data were analyzed using SPSS statistical software, version 16 (SPSS, Inc., Chicago, IL, USA) followed by analysis with the independent two-sample t-test. P<0.05 was considered to indicate a statistically significant difference.
Results

Disturbance of the microRNA expression profile in osteosarcoma tissues. There is evidence that altered patterns of microRNA expression correlate with various human diseases and particularly several types of cancer (7,8). The behavior of microRNAs is complex due to their regulation of hundreds of targets, which can result in the downregulation of numerous target genes, including oncogenes and tumor suppressor genes. Therefore, examining their clinical potential is particularly worthwhile.

Several studies have reported that the microRNA expression profile is altered significantly in the progression of osteosarcoma (9,10), however, further clarification is required. In the present study, the expression profiles of eight microRNAs, which were identified by another study as differentially expressed in osteosarcoma tissues or osteosarcoma cell lines (11), were determined by RT-qPCR. As shown in Fig. 1, microRNA-542-3p and microRNA-542-5p were significantly upregulated and microRNA-199-3p was significantly downregulated in the osteosarcoma tissues.

VANGL2 expression is repressed by microRNA-542-3p. The function of an microRNA is reflected mainly in its repressive effects on the expression of its target genes (12,13). microRNA-542-3p and microRNA-542-5p are products of the same molecule, pre-microRNA-542, and microRNA-542-5p was considered to be the passenger strand, therefore the present study investigated the target gene of microRNA-542-3p using online bioinformatics tools. Using miRanda (http://www.microrna.org/microrna/home.do) and TargetScan (http://www.targetscan.org/; and Pictar, http://pictar.mdc-berlin.de/), VANGL2 was identified as a possible target gene of microRNA-542-3p, the downregulation of which is associated with enhanced cancer cell migration and invasion ability.

To confirm whether VANGL2 was the target gene of microRNA-542-3p, a 516 bp segment of the VANGL2 3’-UTR, containing the interaction sites of microRNA-542-3p, was cloned into the pGL3 control vector (pGL3-VANGL2) downstream of the firefly luciferase reporter gene (Fig. 2A) to perform a dual luciferase assay. The U2OS cells were cotransfected with the pGL3-VANGL2 and either the microRNA-542-3p mimic or inhibitor (Fig. 2B). Compared with the microRNA control, the luciferase activity of microRNA-542-3p mimic was significantly decreased in the U2OS cells cotransfected with the microRNA-542-3p mimic and pGL3-VANGL2 compared with those cotransfected with pGL3-VANGL2 or pGL3. (D) VANGL2 protein levels in the microRNA-542-3p mimic and inhibitor-treated U2OS cells were detected by western blot analysis. miR, microRNA; VANGL2, Van Gogh-like 2; UTR, untranslated region; Mu, mutant; PRL-TK, thymidine kinase promoter-Renilla luciferase reporter plasmid.
luciferase activity was significantly decreased by miR-542-3p by ~47.8% (P<0.05). Furthermore, luciferase activity was significantly increased by the miR-542-3p inhibitor compared with the anti-miR control by ~23.5% (P<0.05). These results indicated that miR-542-3p targets the 3'–UTR of VANGL2, leading to a change in firefly luciferase translation.

A seed sequence mutation clone was also used to confirm the binding site for miR-542-3p (Fig. 2A). A vector, containing putative miR-542-3p binding regions in the 3′-UTR of VANGL2 with five mutant nucleotides (pGL3-VANGL2-Mu) was used and a wild type VANGL2 vector was used as a control. As the histogram in Fig. 2B (right) shows, there were no differences in enzyme activity between the cells cotransfected with miR-542-3p mimics or pGL3-VANGL2-Mu compared with pGL3-VANGL2. These data indicated that miR-542-3p may suppress the expression of VANGL2 by binding to the seed sequence at the 3′-UTR of VANGL2.

miR-542-3p regulates the endogenous expression of VANGL2 in U2OS cells. Although VANGL2 was identified as a target gene for miR-542-3p, whether miR-542-3p was able to regulate the endogenous expression of VANGL2 remained unclear. The U2OS cells were transfected with the miR-542-3p mimic or inhibitor to determine whether dysregulation of the expression of miR-542-3p affected the endogenous expression of VANGL2. Compared with the corresponding control, the protein expression level of VANGL2 in the U20S cells was increased in the cells transfected with the miR-542-3p mimic compared with the pre-miR control (P<0.01) and was decreased in the cells transfected with the miR-542-3p inhibitor (Fig. 2C).

miR-542-3p overexpression enhances U2OS cell migration. U2OS cells were transfected with either the pre-miR control, miR-542-3p mimic, anti-miR control or miR-542-3p inhibitor. The cells were harvested 48 h after transfection, recounted to 0.5x10⁶ cells/ml in every group and seeded into Transwells for a cell migration assay. Subsequently, the cells on the top of the membranes were removed and the cells on the bottom of the membranes were stained with hematoxylin and eosin. Cell migration was quantified by counting the number of cells passing through the membrane from five randomly selected fields in each sample 12 h after incubation (magnification, x100). Representative photomicrographs of the cells passing through the membrane (magnification, x100) are shown. Data are expressed as the mean of independent triplicate experiments. *P<0.05; **P<0.01. miR, microRNA.
transfected with the miR-542-3p inhibitor compared with the anti-miR control (P<0.05) as shown in Fig. 3A.

Cell proliferation and viability were determined using an MTT assay 48 h after transfection. As shown in Fig. 3B, the relative proliferation rates in the U2OS cells transfected with miR-542-3p mimics were increased by ~52.3% compared with the pre-miR control (P<0.05). The relative proliferation rates in the U2OS cells transfected with the miR-542-3p inhibitor decreased ~55.2% compared with the anti-miR control (P<0.05). These results indicated that overexpression of miR-542-3p significantly increased osteosarcoma cell viability, while downregulation of miR-542-3p repressed osteosarcoma cell proliferation.

miR-542-3p modulates the migration capacity of osteosarcoma cells in vitro. In order to investigate the role of miR-542-3p in the metastasis of osteosarcoma cells, the present study then analyzed the effects of miR-542-3p on the migratory behavior of osteosarcoma cells (Fig. 4). The results revealed that the migration capacity of the U2OS cells transfected with the miR-542-3p mimic were significantly higher compared with those transfected with the miR control (P<0.05). Conversely, the migration capacity was significantly suppressed in the U2OS cells transfected with the miR-542-3p inhibitor compared with the anti-miR control (P<0.01). These findings suggested that the level of miR-542-3p may be closely associated with the metastasis of osteosarcoma cells.

Discussion

Previous evidence has demonstrated that altered patterns of miRNA expression are associated with various human diseases and, in particular, several types of cancer. The behavior of miRNAs is complex as they regulate hundreds of targets, which can result in the downregulation of numerous genes, including oncogenes and tumor suppressors. Therefore, examining the clinical potential of miRNAs is particularly useful.

In the present study, the expression of eight candidate miRNAs was detected in 13 soft tissue sarcoma samples and 13 normal tissue samples by RT-qPCR. The miRNAs miR-542-3p and miR-542-5p were significantly upregulated and miR-199-3p was significantly downregulated. As miR-542-3p and miR-542-5p are products of pre-miR-542, of which miR-542-3p is the main product, the present study subsequently investigated the biological function of upregulated miR-542-3p. The possible target genes of miR-542-3p were predicted using online bioinformatics tools. Of the thousands of predicted miR-542-3p target genes, the expression of VANGL2 was found to be repressed by miR-542-3p. VANGL2 belongs to the non-canonical WNT pathway, whose activation inhibits canonical WNT signaling. Previous studies have demonstrated that the expression of VANGL2 is significantly downregulated in several cancer cell lines and primary tumors, and a low expression of VANGL2 is associated with a significantly worse clinical outcome in neuroblastoma (9-11). Additionally, siRNA experiments have revealed that knock down of VANGL2 increases cell proliferation of neuroblastoma cells (10). Wnt transduction pathways function to modulate cell fate and proliferation, and regulate cell growth and differentiation in a variety of organ systems (16). In osteosarcoma cells, as opposed to normal cells, several Wnt ligands, receptors and coreceptors are highly expressed, while Wnt inhibitors are downregulated; therefore, Wnt signaling is aberrantly activated (17). VANGL2 has been proved to be structurally associated with the Wnt pathway, and to be implicated in the development of osteosarcomas (18). However, as a target gene of miR-542-3p, whether the osteosarcoma cell proliferation and migration mediated by VANGL2 are regulated by miR-542-3p has remained elusive. Therefore, the present study investigated the impact of miR-542-3p overexpression on U2OS cell proliferation and migration ability. As expected, miR-542-3p enhanced U2OS cell proliferation and migration, which indicated that miR-542-3p may act as an oncogene by repressing VANGL2 and, thus, preventing the inhibition of the non-canonical WNT pathway.

In conclusion, the results of the present study indicated that miR-542-3p may function as an oncogene by targeting VANGL2 during osteosarcoma pathogenesis. These findings may provide insight into the development of osteosarcoma and aid in the development of novel diagnostic and therapeutic strategies for osteosarcoma.

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