Restoration of microRNA-130b expression suppresses osteosarcoma cell malignant behavior in vitro

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Abstract. The alteration of microRNA (miR)-130b expression has been associated with promoting or suppressing numerous types of human cancer. A previous study evaluated the expression level of miR-130b in osteosarcoma tissues, and subsequently investigated the effects of miR-130b on the regulation of osteosarcoma cells malignant behavior in vitro. The study revealed that miR-130b expression levels were significantly reduced in osteosarcoma tissues and cell lines, compared with in adjacent tissues or normal cell lines. The expression of miR-130b inhibited the proliferation of osteosarcoma U-2OS and Saos-2 cells and impaired their ability to migrate, invade and form colonies. Furthermore, analysis using TargetScan and a dual-luciferase reporter assay demonstrated that miR-130b directly interacted with the 3'-untranslated region of transforming growth factor α (TGFA) and suppressed TGFA expression. TGFA and miR-130b were also inversely expressed in osteosarcoma tissues. In addition, expression of TGFA was able to alter miR-130-regulated osteosarcoma cell proliferation, migration and invasion. Thus, the present study demonstrated that miR-130b was downregulated in osteosarcoma tissues and cell lines, whereas the expression of miR-130b suppressed osteosarcoma cell malignant behavior. At the gene level, miR-130 directly targets and inhibits TGFA expression, in addition to phosphorylated protein kinase B and epidermal growth factor receptor expression levels. Further study is required to evaluate miR-130b antitumor activity in osteosarcoma.

Introduction

Osteosarcoma is a commonly occurring primary malignant bone tumor in children and young adolescents, with a secondary incidence peak in individuals who are ≥50 years old (1). Clinically, the incidence of osteosarcoma is ~1-3 cases/million individuals (2). Osteosarcoma most commonly occurs in the long tubular bones, including the femur, tibia and humerus and, less commonly, in the skull, jaw or pelvis; ≤1% of osteosarcoma cases are localized in the hands or feet (3). Similar to the majority of cancer types, osteosarcoma development is a multistep process and the precise etiology and pathogenesis remain to be determined, although a previous study indicated that ionizing radiation, with or without chemotherapy, was associated with a higher risk of developing osteosarcoma (4). Despite amputation having been frequently utilized over previous years in order to treat patients with osteosarcoma, therapeutic methods or alternative methods to treat osteosarcoma remained limited (1). This did not change until the early 1970s, and with the introduction of adjuvant chemotherapy, in particular the use of high-dose methotrexate, facilitating the surgical resection of patients with osteosarcoma has become possible (5). Advancement in the early detection and treatment of osteosarcoma has been made in previous years; however, the survival rate of patients with osteosarcoma has remained virtually unchanged over the last 30 years (5). Previous studies revealed that the five-year survival rate remains at ~20% (5,6). Thus, more specific biomarkers for the early diagnosis and targeted therapy of osteosarcoma are required.

One approach for the development of targeted therapy and specific biomarkers is to focus on microRNAs (miRNA/miR), which are a class of small non-coding RNAs ~17-24 nucleotides in length (7). miRNAs are able to regulate the expression level of target genes by base-pairing to the 3'-untranslated region (3'-UTR) of mRNA, thereby repressing protein translation or inducing mRNA degradation. For example, Friedman et al (8) demonstrated that ~60% of human genes are regulated by manifold miRNAs in a cooperative manner. Accumulating evidence has suggested that dysregulated miRNA expression may contribute to cancer development and progression (9). The elucidation of miRNA expression and function in human cancer is important for the understanding of cancer pathogenesis, as miRNAs may affect cell proliferation, differentiation, metastasis, apoptosis and sensitivity to chemotherapy and radiotherapy (10). Given their pleiotropic actions to repress multiple target genes simultaneously (11), miRNAs may be ideal candidates to provide a comprehensive understanding of osteosarcoma pathogenesis. Thus, the
present study investigated a particular miRNA, miR-130b, in paired osteosarcoma and adjacent normal tissues and cell lines. The miR-130b gene is localized at chromosome 22q11, and has been proposed as a novel tumor-associated miRNA (12). Previous studies have demonstrated altered miR-130b expression in promoting or suppressing tumorigenesis; for example, in papillary thyroid carcinomas (13), endometrial cancer (14) and pancreatic cancer (15), miR-130b was down-regulated. However, miR-130b expression was upregulated in melanoma (16), bladder cancer (17) and metastatic renal carcinoma (18). A previous study revealed an association between miR-130 expression levels and osteosarcoma cell lines using microarray profiling (19); however, the molecular pathways by which miR-130b modulates osteosarcoma development and progression have not been elucidated. The present study also investigated the effects of miR-130b expression in the regulation of osteosarcoma cell proliferation, colony formation, migration and invasion in vitro. Bioinformatic analysis and dual-luciferase reporter assays were subsequently performed in order to identify the target gene. The present study aimed to provide an improved understanding of the effects and function of miR-130b in osteosarcoma, which may help to develop a novel therapeutic strategy for managing osteosarcoma.

Materials and methods

Tissues. Osteosarcoma and paired normal tissues from 13 patients were collected between January 2013 and February 2015 from the Department of Surgery, The Second Xiangya Hospital, Central South University (Changsha, China). The average of age was 13 (range, 9-19); 6 patients were female and 7 were male. All patients were histologically diagnosed and verified according to the 2013 World Health Organization classification guidelines (20). None of the patients had received chemotherapy or radiotherapy prior to surgery. All tissues were obtained via the surgical resection, snap-frozen in liquid nitrogen and stored at -80°C. The present study was approved by the Human Ethics Committee of the Xiangya Medical School of the Central South University. Written informed consent was obtained from all patients prior to enrollment in the present study.

Cell lines and culture. U-2OS, Saos-2 and MG-63 human osteosarcoma cell lines, hFOB normal human osteoblast cells and HEK293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Oligonucleotides, plasmids and cell transfection. A total of four oligonucleotides (miRNA-130b mimics, miRNA-130b mimics negative control (mimics NC), miRNA-130b inhibitor, miRNA-130b inhibitor negative control (inhibitor NC) were chemically synthesized by Genepharm, Inc. (Sunnyvale, CA, USA). The open reading frame of human transforming growth factor α (TGFα; Gen-Bank Accession no., KJ892270.1) was cloned into pcDNA3.1 (+; Invitrogen; Thermo Fisher Scientific, Inc.) and the DNA sequence was confirmed by PCR kit (PCR master mix; cat. no. K0171; Invitrogen; Thermo Fisher Scientific, Inc.). The primers for TGFα were: Forward, 5’-AGCAGTGGTGTCGCCATTTTAA-3' and reverse, 5’-AGC GTTCTCCCTTCAGG-3'. The thermocycling conditions were as follows: Initial denaturation 95°C 1 min, 30 cycles of denaturation 95°C 30 sec, annealing 55°C 30 sec and elongation 72°C 1 min; and final extension 72°C 10 min. For gene transfection, the oligonucleotides and plasmids were mixed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, and then added to the cell culture and 37°C incubated for 6 h prior to the growth medium being changed, and subsequently 37°C incubated for an additional 42 h.

RNA isolation and reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from the tissues and cultured cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's instructions, and quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). In order to detect the expression level of miRNA-130b in tissues and cells, cDNA was reverse transcribed using a TaqMan® miRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and subsequently subjected to RT-qPCR using a SYBR® Premix Ex Taq™ kit (Takara Biotecnology, Co., Ltd., Dalian, China). The U6 transcript was used as an internal control. The level of TGFA mRNA was determined using an ABI Prism® 7900 Sequence Detector system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and GAPDH was used as an internal control. miR-103b and TGFA mRNA expression levels were determined using the 2-ΔΔCq method and compared as the relative fold changes of internal controls (21).

Dual-luciferase reporter assay. A fragment of the 3’-UTR of miR-130b containing the putative binding sites (3344-3351) to TGFA was subcloned into a firefly luciferase pGL3-control vector (Invitrogen; Thermo Fisher Scientific, Inc.). All constructs were verified by by PCR kit (PCR master mix; cat. no. K0171; Invitrogen; ThermoFisherScientific,Inc.)TheRVprimer3(5’-CTA GCAAATAGGCTGTCCC-3’) and GLprimer2 (5’-CTTTAT GTTTTGGCCTCTTCCA-3’) were used according to the manufacturer's procol. The thermocycling conditions were as follows: Initial denaturation 95°C 1 min, 28 cycles of denaturation 95°C 30 sec, annealing 55°C 30 sec and elongation 72°C 1 min; and final extension 72°C 10 min. HEK293T cells (in the logarithmic growth phase) were co-transfected with wild type TGFA (WT-TGFA) or mutated TGFA (mut-TGFA) in combination with control or miR-130b-mimic, using Lipofectamine® 2000 according to the manufacturer's instructions. The cells were harvested 24 h following transfection and the relative luciferase activities were determined using the dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA) in a GLOMAX20/20 luminometer (Promega Corporation) and normalized to the Renilla luciferase activity.

Protein extraction and western blot analysis. Osteosarcoma cells were harvested and lysed using 1X radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, Inc.,

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were cultured for 24 h at 37˚C. Subsequently, the non‑migrated cells were seeded into the upper wells on a filter coated with or without Matrigel in DMEM without serum, and were incubated overnight at 4°C with a primary antibody against human TGFA (dilution, 1:1,000; cat. no., sc-36; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), protein kinase B (Akt) (dilution, 1:1,000; cat. no., sc-135829; Santa Cruz Biotechnology, Inc.), phosphorylated (p)Akt (dilution, 1:1,000; cat. no., sc-16646-R; Santa Cruz Biotechnology, Inc.), epidermal growth factor receptor (EGFR; dilution, 1:1,000; cat. no., sc-80543; Santa Cruz Biotechnology, Inc.), p-EGFR (dilution, 1:1,000; cat. no., sc-71776; Santa Cruz Biotechnology, Inc.) or β-actin (dilution, 1:2,000; cat. no., sc-517582 Santa Cruz Biotechnology, Inc.), and subsequently with a horseradish peroxidase‑conjugated anti-mouse IgG secondary antibody (dilution, 1:10,000; cat. no., sc-516102; Santa Cruz Biotechnology, Inc.). The signals were visualized using a gel‑imaging analyzer (Bio‑Rad Laboratories Inc., Hercules, CA, USA) and the protein band intensity was quantified using Quantity One software (Version 4.5.0, Bio‑Rad Laboratories, Inc.) and normalized to the β-actin level.

**Cell proliferation assay.** The transfected cells were seeded into 96-well plates, in DMEM medium supplemented with 10% FBS, at a density of 2,000 cells/well for Saos-2 and 5,000 cells/well for U-2OS, and cultured at 37°C for ≤5 days. At the end of each experiment, a 10-μl Cell Counting kit-8 (CCK-8) solution was added to each well and subsequently incubated for 2 h at 37°C. The relative optical density (OD) level was evaluated at an absorbance of 450 nm and normalized to that of the controls using a standard microplate reader (Thermo Fisher Scientific, Inc.).

**Colony-formation assay.** Following transfection, 300 Saos-2 or U-2OS cells were seeded into each well of 6-well plates and incubated at 37°C for 14 days. The culture medium was replaced twice a week and on day 14, cells were washed with PBS, fixed with methanol for approximately 30 min, subsequently stained with 1% crystal violet solution (Beyotime Institute of Biotechnology, Haimen, China) for 30 min, washed three times with PBS and then air-dried for 2 h. Colonies containing ≥50 cells were identified as a clone. Data are expressed as the mean ± standard error of the mean (SEM) and compared with the control cells.

**Tumor cell Transwell migration and invasion assays.** Migration and invasion assays were determined using a BD Transwell assay (pore size 8 μm) with or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), in accordance with the manufacturer's protocol. Following transfection, Saos-2 or U-2OS cells (5x10^4) were seeded into the upper wells on a filter coated with or without Matrigel in DMEM without serum, and the lower chambers were filled with DMEM supplemented with 10% FBS, which acted as a chemoattractant. The cells were cultured for 24 h at 37°C. Subsequently, the non-migrated or invaded cells on the upper surface of the filter were removed using cotton swabs. Cells that had migrated or invaded into the bottom surface were fixed with 75% ethanol and stained with 1% crystal violet solution at room temperature for 20 min. The relative degree of cell migration or invasion was quantified using a light microscope (magnification, x100) and normalized to the control cells in 5 fields of view.

**Statistical analysis.** All the data are expressed as the mean ± SEM. Data were statistically analyzed using a paired-samples t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Spearman's correlation analysis was performed using Graphpad prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

The expression of miR-130b is downregulated in osteosarcoma tissues and cell lines. In order to investigate the role of miR-130b in osteosarcoma, the present study determined the expression levels of miR-130b in osteosarcoma and paired adjacent normal tissues from 13 patients, using RT-qPCR. It was revealed that miR-130b expression levels were significantly lower in osteosarcoma tissues compared with in normal tissues (P<0.0001; Fig. 1A). Furthermore, the expression level of miR-130b was also low in three osteosarcoma cell lines, Saos-2, MG-63 and U-2OS, as compared with hFOB normal human osteoblast cells (Fig. 1B).

Restoration of miR-130b expression levels inhibits osteosarcoma cell proliferation, colony formation, migration and invasion. The present study restored and suppressed miR-130b expression levels in U-2OS and Saos-2 cells via transfection of miRNA-130b mimics and an miRNA-130b inhibitor, respectively. The results revealed that miRNA-130b mimics and miRNA-130b inhibitors induced or suppressed miR-130b expression levels respectively, in U-2OS and Saos-2 cells compared with the negative control transfection (Fig. 2A). Following alteration of the miR-130b expression level, osteosarcoma cells were subjected to cell proliferation and colony formation assays. The cell proliferation CCK-8 assay demonstrated that miR-130b-mimics reduced tumor cell proliferation, compared with the negative control, from 1-5 days (Fig. 2B). Furthermore, the colony formation assay revealed that the miR-130b-mimics reduced tumor cell colony formation (Fig. 2C); the tumor cell Transwell migration and invasion assays also demonstrated that the miR-130b-mimic reduced migration and invasion capacity of osteosarcoma cells (Fig. 2D and E). These results indicate that the upregulation of miR-130b expression suppressed osteosarcoma cell malignant behavior.

**TGFA is the target gene of miR-130b in osteosarcoma cells.** As miRNA serves a role in the inhibition of targeting gene transcription and translation, a TargetScan search was performed in order to screen for potential target genes of miR-130b. It was revealed that miR-130b may suppress TGFA mRNA translation. Therefore, the present study constructed luciferase reporter plasmids and conducted a dual-luciferase reporter assay. The results demonstrated that miR-130b mimics...
were able to directly target the 3'-UTR of TGFA mRNA (Fig. 3A and B); however, there was no significant difference in luciferase activity detected between the miR-130b mimics and negative control in the mut-TGFA-transfected U-2OS cells.
Thus, this study determined that TGFA is the downstream target of miR-130b. Furthermore, it was verified that miR‑130b mimics induced a significant reduction of TGFA expression in osteosarcoma cells (Fig. 3G); subsequently, the expression levels of TGFA were evaluated when compared to normal tissues (P=0.0009). Spearman's correlation analysis demonstrated that TGFA expression levels in osteosarcoma tissues were inversely associated with miR-130b expression levels (Spearman's correlation, r=‑0.8309; P<0.0001; Fig. 3F). Following this, the relation of miR-130b and TGFA expression in osteosarcoma cells was further confirmed by transfecting the miR-130b mimics, miR-130b inhibitor and matched negative control into osteosarcoma cells. The western blot analysis demonstrated that miR-130b mimics inhibited TGFA expression, whereas the miR-130b inhibitor upregulated TGFA expression in osteosarcoma cells (P<0.01; Fig. 3G and H). Thus, miR‑130b may inhibit TGFA expression by targeting TGFA 3'‑UTR in osteosarcoma cells.

TGFA upregulation reverses the effects of miR‑130b on osteosarcoma cells. As aforementioned, miR-130b is able to target TGFA and inhibit TGFA expression; therefore, the present study determined whether the upregulation of TGFA expression may reserve the effects of miR‑130b on osteosarcoma cells via transfection of the TGFA-expression vector with or without co-transfection of miR‑130b mimics or miR‑130b mimics NC, into U‑2OS and Saos-2 osteosarcoma cells. Tumor cell proliferation and clone formation assays revealed that osteosarcoma cell proliferation was reduced

Figure 3. miR‑130b binding and targeting of TGFA in osteosarcoma cells. (A) TargetScan prediction of the miR-130 targeting gene. Putative binding sites of miR‑130b within TGFA 3'‑UTR are presented. (B) Dual‑luciferase reporter assay. Data confirmed the interaction of miR‑130b with TGFA. It was performed using HEK 293T cells co‑transfected with firefly luciferase constructs supplemented with WT‑TGFA or mut‑TGFA, and miR‑137‑mimic or NC. Renilla luciferase plasmid was used to normalize luciferase activity. (C) Levels of TGFA mRNA expression in osteosarcoma tissues evaluated by RT‑qPCR. (D and E) Levels of TGFA protein expression in osteosarcoma cell lines evaluated by western blotting. (F) Spearman's correlation test to determine the association between expression of TGFA and miR‑130b. (G and H) Western blot analysis was performed 48 h following transfection in order to detect expression levels of TGFA. miR, microRNA; TGFA, transforming growth factor α; WT, wild type; mut, mutated; NC, negative control; RT‑qPCR, reverse transcription‑quantitative polymerase chain reaction; LUC, luciferase; hsa, homo sapiens.
following miR-130b mimic transfection, but was reversed by co-transfection with the pcDNA3.1-TGFA plasmid (Fig. 4A). Subsequently, clone formation assays demonstrated that TGFA upregulation significantly restored the clone formation capability of osteosarcoma cells (P<0.01; Fig. 4C). Overexpression of TGFA restored miR-130b-inhibited expression levels of pAkt and EGFR (Fig. 4B), which suggested that miR-130b may suppress the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway by targeting TGFA.

Discussion

Over the previous decades, the knowledge and understanding of the criteria and classification of osteosarcoma have progressed; however, certain aspects of this disease remain controversial (19). Developments in the study of miRNAs have provided a more complete understanding of how miRNAs contribute to cancer development and progression (22). It is estimated that miRNAs are able to regulate the expression of 30-60% of all human genes (8) via inhibiting the expression of their target genes by binding to the 3'-UTR of mRNA (23). A previous study revealed that miR-130b expression is significantly reduced in endometrial cancer tissues, and that mutant tumor protein 53 accelerates tumor progression and metastasis via the regulation of miR-130b expression (14). miR-130b is downregulated in multidrug resistant ovarian cancer cells, and the restoration of miR-130b expression is concomitant with increased sensitivity of tumors to anticancer drugs (24). Conversely, miR-130b is significantly overexpressed in esophageal squamous cell carcinoma cells, which increases the viability of tumor cells and their ability to migrate and invade in vitro by targeting phosphatase and tensin homolog (25). Therefore, miR-130b may serve disparate tumor-associated roles depending on the tumor type and targeted genes. Furthermore, the present study demonstrated that miR-130b expression is downregulated in osteosarcoma tissues and cell lines, whereas restoration of miR-130b expression inhibits osteosarcoma cell proliferation, colony formation, migration and invasion in vitro. miR-130b is able to directly target TGFA mRNA and inhibit TGFA expression in osteosarcoma cells. TGFA expression also reverses the effect of miR-130b on osteosarcoma cells.

The association between miR-130b expression and osteosarcoma has been demonstrated in a previous study by global microarray analyses of a panel of 19 human osteosarcoma
cell lines and normal bone tissues (19). To the best of our knowledge, there have been no further studies investigating the role or the molecular mechanisms underlying miR-130b in osteosarcoma. In the present study, the expression level of miR-130b was determined in osteosarcoma tissues and three osteosarcoma cell lines, and compared with adjacent tissues and normal osteoblast cells, respectively. In contrast to a previous study by Wu et al. (18), the present study revealed that miR-130b expression is downregulated in osteosarcoma tissues and cell lines, which is consistent with that in ovarian cancer, papillary thyroid carcinoma and hepatocellular carcinoma (13,24,26). Furthermore, miR-130b expression in osteosarcoma cells suppresses tumor cell proliferation, colony formation, migration and invasion in vitro. The present study further confirmed that miR-130b may function as a tumor suppressor in osteosarcoma development and progression.

TGFA encodes a growth factor that is a ligand for the EGFR (27). The latter regulates cell growth signaling and mediates cell proliferation in a number of types of cancer, including lung, breast and prostate cancer (28). Accumulating evidence has demonstrated that EGFR activation via amphiregulin, epiregulin and TGFA, triggers numerous biological responses, including cell survival, proliferation, invasion, differentiation and migration (28,29). It has previously been suggested that there is an association between EGFR activation and other signaling pathways, including the RAS-activated Akt signaling pathway (30). PI3K/Akt is a major signaling pathway that serves a role in the regulation of cell division in malignant cells (31). Previous studies have suggested that this signaling pathway may also have a role in tumorigenesis and cancer progression by affecting the activation state of a range of downstream effector molecules (32,33). In the present study, it was demonstrated that miR-130b is able to bind directly to the TGFA 3'-UTR and inhibit TGFA expression in osteosarcoma cells. Furthermore, it was also revealed that the overexpression of TGFA restored miR-130b-inhibited levels of pAkt and EGFR, which suggests that miR-130b suppress the PI3K/Akt signaling pathway by targeting TGFA. The data of the current study indicate that targeting the miR-130b-mediated gene pathway may be a strategy for the development of osteosarcoma treatments. In addition, the tumor-suppressive effects of miR-130b upregulation on the proliferation and migration of osteosarcoma cells were reversed following transfection with TGFA. Further studies evaluating the downstream genes of miR-130b, including Akt, may improve understanding of the involvement of the PI3K/Akt signaling pathway in osteosarcoma development or progression.

The present study is a proof-of-principle investigation. Further research is required in order to confirm the downregulation of miR-130b expression and its association with TGFA expression in a larger number of tissues from patients with osteosarcoma. In conclusion, the data from the current study demonstrate that miR-130b is a novel regulator in osteosarcoma development and progression, and that miR-130b-regulated TGFA expression contributes to osteosarcoma cell malignant behavior. Investigation of the underlying mechanisms of miR-130b and its associated signaling pathways may assist the development of novel therapeutic strategies to treat patients with osteosarcoma.

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Availability of data and materials
The datasets used/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
YW was responsible for the study design and the acquisition of data, WS undertook data analysis, YK and BL performed the functional experiments. MZ helped to design the experiments and interpret the data. WW undertook project design and manuscript revisions.

Ethics approval and consent to participate
The present study was approved by the Human Ethics Committee of the Xiangya Medical School of the Central South University.

Consent for publication
Written informed consent was obtained from all patients prior to enrollment in the present study.

Competing interests
The authors declare that they no financial conflicts of interest.

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