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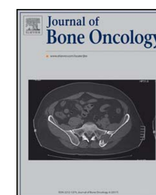

Research Paper

Long non-coding RNA ANRIL is associated with a poor prognosis of osteosarcoma and promotes tumorigenesis via PI3K/Akt pathway

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A R T I C L E I N F O

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A B S T R A C T

Aim: Increasing evidence has shown that long noncoding RNAs (lncRNAs) ANRIL may function as oncogenes in various types of malignancies. However, there is still a lack of knowledge concerning its role in osteosarcoma (OS). In this study, we aimed to investigate the influence of ANRIL on cell proliferation and invasion of OS and to determine its association with clinicopathological features of the patients.
Methods: The tumor specimens and the adjacent normal tissues were collected from 57 OS patients and the expression level of ANRIL was quantified by RT-qPCR. High expression of ANRIL was defined as a relative mRNA expression of > 1.5 fold (tumor/normal). Knockdown of ANRIL was performed in human OS cell lines to investigate its influence on cell proliferation, apoptosis and invasion. In addition, expression of downstream genes in the transfected cells were determined by Western blot.
Results: The expression level of ANRIL was significantly increased in OS tissues than in the adjacent normal tissues. 33 patients were included in the high expression group and the other 24 patients were included in the normal expression group. ANRIL expression was significantly associated with tumor size (5.7 cm ± 2.4 cm vs. 4.3 cm ± 1.7 cm, $p = 0.02$) and the 5-year survival rate (51.5% vs. 79.1%, $p = 0.03$). Knockdown of ANRIL could significantly induce cell apoptosis and inhibit cell proliferation and invasion. Moreover, knockdown of ANRIL could significantly decrease the expression level of phosphorylated PI3K and AKT in OS cells.
Conclusions: Upregulated expression of ANRIL is associated with the tumor development and prognosis of OS. ANRIL may regulate the function of OS cells through the AKT pathway.

1. Introduction

Osteosarcoma (OS) is one of the most common primary bone tumors that occur during the childhood [1,2]. Currently therapeutic modalities of OS include surgical resection of the tumor combined with preoperative and postoperative chemotherapy, with the overall 5-year disease-free survival rate ranging from 60% to 70% [3,4]. Although the survival rate has improved dramatically with the advance of chemotherapy, distant metastases may still develop in some patients whose survival rate has not improved much. Therefore, identification of biomarkers involved in the development of OS could facilitate personalized treatment strategy and contribute to better outcomes.

In recent years, increasing evidence has shown that long noncoding RNAs (lncRNAs) may function as oncogenes or tumor suppressors in various types of malignancies [5–8]. Initially identified from familial melanoma patient, lncRNA ANRIL has been reported to be a risk factor in several human cancers such as breast, colorectal and bladder cancer [9–11]. Zou et al. [12] found that ANRIL could promote progression of nasopharyngeal via reprograming cell glucose metabolism. Sun et al. [13] reported that ANRIL is upregulated in colorectal cancer tissues, and is associated with cancer cell pathogenesis. Zhang et al. [14] found that ANRIL could promote the progression of cervical cancer via PI3K/AKT pathway and act as an indicator of poor prognosis. Chen et al. [15] reported that over-expression of ANRIL can promote pancreatic cancer by activating the ATM-E2F1 pathway.

Although ANRIL functions as a vital oncogene in many cancers, to the best of our knowledge, there is still a lack of knowledge concerning its role in OS. Wei et al. [16] reported that ANRIL is involved in hypoxia-induced aggressive phenotype in OS. In the present study, we analyzed the expression of ANRIL in OS tissues to determine its association with clinicopathological features and prognosis of the patients. In addition, loss-of-function experiments were performed to investigate the influence of ANRIL on cell proliferation and invasion of OS [16].

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2. Methods

2.1. Patients and tissue samples

Under the approval of the Ethics Committee, 57 OS patients who underwent resection surgery from June 2008 to May 2015 were included in this study. All the patients had a minimum follow-up of 2 years. No chemotherapy was prescribed to the patient before the surgery. The tumor specimens and the corresponding adjacent normal tissues were collected for each patient. All the samples were immediately frozen and stored in liquid nitrogen for RNA extraction. Baseline characteristics of the patients were recorded, including age, gender, Enneking stage, tumor size, distant metastasis and survival rate.

2.2. Cell culture

Human OS cell lines MNNG/HOS and U2 OS were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Both cell lines were cytogenetically tested and authenticated before they were frozen. Cells were maintained in a humidified incubator at 37 °C with 5% CO2.

2.3. RNA extraction and qRT-PCR analysis

Total RNA was extracted from tissue samples with Trizol reagent (Invitrogen) following the instruction of the manufacture. The expression level of ANRIL was quantified using SYBR Master Mixture (TAKARA, Tokyo, Japan) on the LightCycler 480 (Roche Applied Science, Mannheim, Germany). The primers were as follows: forward 5′- TGCTCTATCCGCCAATCAGG -3′, reverse 5′- GGGCCTCAGTGGCAC ATACC -3′ for ANRIL, and forward 5′- GAGTCAACGGATTTGGTCGT -3′, reverse 5′ -TTGATTTTGGAGGGATCTCG- 3′ for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that was used as the internal control. Relative mRNA expression was analyzed using the ΔΔCt method.

2.4. siRNA transfection

The siRNA targeting ANRIL (siANRIL) was purchased from Shanghai GenePharma Co., Ltd. The sequence of siANRIL was as follows: GGUC AUCUCAUUGCUCUAU. The negative control denoted as siCtrl was nonhomologous to any human genome sequence. The MNNG/HOS and U2 OS cells were plated in 6-well plates for 18 h and then transfected with 20 nM of the RNA duplex and 5 µL of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The knockdown efficiency was evaluated with qRT-PCR as mentioned above.

2.5. Cell proliferation assays

Transfected cells were plated in 96-well plates and cultured for 5 days. For each day, the well was added with MTT (20 µL of 5 mg/mL; Genview) and incubated for 4 h at 37 °C. After the incubation, 200 µL DMSO was added to the cultured cells to dissolve the crystals. To test the cell proliferation, 2×10^3 cells were plated in 96-well plates at 72 h after transfection. The absorbance at 490 nm was read with the Spectramax M5 (Molecular Devices, Sunnyvale, USA) to count the cells for 5 consecutive days.

2.6. Cell apoptosis assay

Flow cytometry (FCM) analysis was used to determine the cell apoptosis. Briefly, after the incubation of transfected cell for 4 days, cell suspensions were generated and plated in 6-cm dishes for further culturing. Subsequently, cells were collected and fixed with pre-cold 70% alcohol for at least 1 h, then washed with PBS and stained with PI buffer. Cell apoptosis was assayed by staining with Annexin V-APC (eBioscience, San Diego, CA, USA) following manufacturer's instructions and the signal was detected by FACS Calibur (Becton-Dickinson, USA). Each experiment was performed in triplicates.

2.7. Cell invasion assay

Assay of cell invasion was performed using a Transwell chamber with an 8-µm pore size (Corning, Corning, NY, USA) in a 24-well plate. 200 µl of the cell suspension medium containing 1×10^5 cells were seeded into the upper chamber coated with Matrigel. DMEM supplemented with 10% FBS (800 µL) was used as the chemoattractant in lower chamber. After 48 h, the cells on the top surface of the membrane were mechanically removed. The cells that had moved to the basal side of the membrane were fixed in 95% ethanol and stained with 0.2% crystal violet solution, which was then imaged using an IX71 inverted microscope (Olympus, Tokyo, Japan).

2.8. Western blot analysis

Expression of 4 target proteins including p-PI3K, PI3K, p-AKT and AKT were determined by Western blot. Total cell proteins were prepared using cell lysis buffer. Equal amounts of cell lysates were separated on a 10–12% SDS polyacrylamide gel and electro-transferred to polyvinylidene fluoride membranes (Immobile P; Millipore). The membranes were then blocked with 5% nonfat dry milk in TBST for 1 h and incubated with the primary antibody (Abcam) overnight at 4 °C, followed by incubation with second antibody (Cell Signaling Technology) for 1 h at 37 4 °C. The signals were detected by enhanced chemiluminescence (Thermo). GAPDH was used as a loading control.

2.9. In vivo tumor growth

6-week-old male BALB/c nude mice were anesthetized with a 1:1 mixture of isoflurane gas and oxygen. MNNG/HOS cells transfected with siANRIL or siCtrl were injected into 20 nude mice subcutaneously. After 4 weeks of inoculation, the tumors were harvested and weighed for each mouse. Animals were purchased from the Shanghai Institute of Biological Sciences (Shanghai, China). Under the approval of the local Institutional Animal Care and Use Committee, all procedures were performed in compliance with the guidelines for the use of laboratory animals.

2.10. Statistical analysis

The statistical analysis was carried out with the SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). High expression of ANRIL was defined as a relative mRNA expression of > 1.5 fold (tumor/normal). Patients were classified into high expression group and normal expression group. The inter-group comparison of clinical parameters of the patient was analyzed using the Student's *t*-test or the Chi-square test. Survival analysis was performed using the Kaplan-Meier method and the log-rank test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Overexpression of ANRIL was correlated with the prognosis of the patients

The expression level of ANRIL was significantly increased in OS tissues than in the adjacent normal tissues (Fig. 1a). 33 patients were included in the high expression group and the other 24 patients were included in the normal expression group. As shown in Table 1, we found that ANRIL expression was significantly associated with tumor size (5.7 cm ± 2.4 cm vs. 4.3 cm ± 1.7 cm, p = 0.02) and the 5-year

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