MicroRNA-410 regulates autophagy-related gene ATG16L1 expression and enhances chemosensitivity via autophagy inhibition in osteosarcoma

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Abstract. Osteosarcoma, which is the most common type of primary bone tumor in adolescents, is characterized by complex genetic alterations and frequent resistance to conventional treatments. MicroRNAs (miRs) have emerged as fundamental regulators in gene expression through their ability to silence gene expression at post-transcriptional and translational levels. The present study investigated the role of miR-410 in the progression of osteosarcoma. The results demonstrated that the expression of miR-410 was markedly downregulated in human osteosarcoma tissues, and U2OS and MG-63 osteosarcoma cell lines. Clinicopathological significance suggested that miR-410 may be a potential biomarker for chemotherapy-resistant osteosarcoma. Furthermore, overexpression of miR-410 exhibited a limited effect on cell viability in U2OS and MG-63 cells. Target prediction algorithms (TargetScan and miRanda) indicated that autophagy related 16-like 1 (ATG16L1) was a potential target gene of miR-410. A luciferase reporter assay demonstrated that miR-410 directly decreased ATG16L1 expression by targeting its 3'-untranslated region. In addition, the results revealed that miR-410 was able to markedly inhibit autophagy. Accordingly, autophagy was activated as a protective mechanism when osteosarcoma cells were exposed to three common anticancer drugs, including rapamycin, doxorubicin and cisplatin. Furthermore, the autophagy inhibitor 3-methyladenine and miR-410 expression were able to improve the therapeutic response of the cells to chemotherapy drugs (rapamycin, doxorubicin and cisplatin), thus indicating that miR-410 enhanced chemosensitivity through autophagy inhibition in osteosarcoma cells. In conclusion, studies regarding the function of miR-410 on autophagy provided insight into the biological function of miR-410 in osteosarcoma and may offer a promising approach for the treatment of osteosarcoma.

Introduction

Osteosarcoma is one of the most common malignant bone tumors that affects adolescents and children worldwide. Frequent acquisition of drug resistance is often associated with the chemotherapeutic treatment of osteosarcoma (1). Therefore, there is an urgent requirement to elucidate the underlying molecular mechanisms of chemoresistance in osteosarcoma, in order to facilitate the development of therapeutic strategies for patients.

Previous studies have demonstrated that autophagy is usually activated as a protective mechanism in tumor cells against numerous chemotherapeutics. Limited efficacy of doxorubicin (Dox), cisplatin (DDP) and methotrexate due to the induction of autophagy has been certified in osteosarcoma cells (2-4). Autophagy is a highly conserved process that entails the degradation of intracellular components to regenerate metabolites for energy and growth through the lysosomal machinery (5). However, the molecular mechanisms underlying autophagy-mediated chemotherapy resistance of osteosarcoma cells remain largely unknown.

MicroRNAs (miRNAs; miRs) are single-stranded, small noncoding RNAs ~22 nucleotides in length, which negatively regulate gene expression through base-pairing to the 3'-untranslated region (3'UTR) of target mRNA (6,7). miRNAs are involved in crucial biological processes, and the dysregulation of miRNAs is associated with tumorigenesis and the development of numerous malignancies, including osteosarcoma (8). Previous studies have investigated the role of miR-410 in several types of cancer (9-11); however, the role of miR-410 in osteosarcoma remains unknown.

The present study demonstrated that miR-410 expression was downregulated in osteosarcoma cell lines and tissues. Target prediction algorithms (TargetScan and miRanda) indicated that autophagy related 16-like 1 (ATG16L1) was a potential target gene of miR-410. ATG16L1 is a component of a large protein complex essential for autophagy, and autophagy is a tightly regulated intracellular catabolic pathway involving the lysosomal degradation of cytoplasmic organelles and proteins. Previous studies have demonstrated miRNAs mediate the regulation of proteins in the autophagy pathway,
and autophagy is commonly activated as a protective mechanism when cancer cells were exposed to three commonly used anticancer drugs (12-14). In the current study, it was demonstrated that miR-410 overexpression is able to improve the therapeutic response of cells to chemotherapy [including rapamycin (Rap), Dox and DDP]. Furthermore, miR-410 could enhance the chemosensitivity of those drugs via autophagy inhibition through targeting ATG16L1 in osteosarcoma cells. The present study provided an insight into the biological function of miR-410 in osteosarcoma and offered a promising approach for future osteosarcoma treatment.

Materials and methods

Osteosarcoma tissues, cell lines and transfection. U2OS and MG-63 human osteosarcoma cell lines, the hFOB 1.19 human osteoblast cell line, and human embryonic kidney (HEK)293T cells were purchased from the Cell Resource Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The cells were cultured in Dulbecco's modified Eagle's medium (GlutaMAX™; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (12483-020; Thermo Fisher Scientific, Inc.), 100 µg/ml streptomycin and 100 IU/ml penicillin (Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO₂. Transfection was performed when the cells had grown to 80% confluence, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 40 paired tumor tissues and matched non-tumor tissues were obtained from patients with osteosarcoma at Renmin Hospital of Wuhan University (Wuhan, China) between 2013 and 2015. All tissues were immediately stored in liquid nitrogen until further use. Histological diagnosis of the tumors was made and agreed upon by two senior pathologists at the Department of Pathology (Renmin Hospital of Wuhan University) based on World Health Organization criteria. The present study was approved by the Institutional Review Board and Human Ethics Committee of Renmin Hospital of Wuhan University.

Reagents and antibodies. Reagents used were as follows: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M5655; Sigma-Aldrich, St. Louis, MO, USA) and Hoechst 3342 (B2261; Sigma-Aldrich), both of which were dissolved in phosphate-buffered saline (PBS). The following antibodies were obtained: Anti-microtubule-associated protein 1A/1B-light chain 3 (LC3)I/II (1:1,000; ab58610; Abcam, Cambridge, MA, USA), anti-caspase 3 (1:1,000; ab2171; Abcam), anti-ATG16L1 (1:1,000; 8089S; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-β-actin (1:2,000; ab6276; Abcam). 3-methyladenine (3-MA; M9281; Sigma-Aldrich); Rap (37094; Sigma-Aldrich); Dox (D1515; Sigma-Aldrich). DDP (Chengdu Organic chemicals Co., Ltd., Wuhou, China). All drugs were dissolved in dimethyl sulfoxide and were stored at -20˚C until the cells were treated with the drugs for 48 h (Rap, 20 nM; Dox, 0.1 µg/ml; DDP, 10 µM).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Tissues were homogenized prior to RNA extraction. Total RNA was extracted from the cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and was reverse transcribed using Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using the Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific, Inc.) on the Real-Time PCR Detection system (iQ5; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cDNA templates were diluted to 1:10 and 20 µl per reaction (SYBR Green Mix 10 µl, forward primer 2 µl, reverse primer 2 µl, RNase Free dH₂O 4.5 µl) was analyzed. The PCR conditions were as follows: DNA denaturation at 94˚C for 4 min, followed by 40 cycles of amplification at 94˚C for 40 sec, 52˚C for 40 sec and 72˚C for 40 sec for data collection. The Bulge-Loop™ hsa-miR-410 qRT-PCR primer set (miRQ0002171-1-2) was purchased from Guangzhou Ribobio Co., Ltd (Guangzhou, China). The qPCR experiments were run three times on separate days, each with independent biological samples (n=4 per group); within each experiment run, relative expression values were normalized to the standard deviations from the mean. The relative expression levels of miR-410 were normalized to those of the internal control gene U6 using the 2^ΔΔCq cycle quantification method (15).

Cell transfection. The sequences of miR-410 were retrieved from the miRNA database miRBase (www.mirbase.org) (AAUAUAACACAGAUGGCCGUGU). miR-410 (miR-410) and negative control (NC) mimics were purchased from Guangzhou Ribobio Co., Ltd. and were transfected into the U2OS and MG-63 cells using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) or 24 h or 48 h in different experiments. qPCR assays were used to detect the expression levels of miR-410 subsequent to each transfection.

Cell proliferation and apoptosis assays. Cells were seeded in 96-well culture plates at 30% confluence 1 day prior to transfection. Post-transfection with miR-410 or NC the cells were evaluated using an MTT assay. The MTT assay was used to determine relative cell growth, according to the manufacturer's protocol. Subsequently, medium was replaced with 0.1 ml dimethyl sulfoxide (Sigma-Aldrich) and the 96-well culture plates were agitated at room temperature for 1 min. The absorbance was subsequently measured at 490 nm. In addition, a Hoechst assay was used to detect cell apoptosis. Cells were seeded in 6-well culture plates at 40% confluence 1 day prior to transfection. After 24 h, the cells were washed with PBS and were fixed in 4% paraformaldehyde for 15 min. Subsequently, Hoechst 33342 diluted in PBS (final concentration, 5%) was added to each well for 15 min, the cells were washed twice with PBS (10 min/wash), the blue-stained nuclei were observed and the apoptotic rate was calculated under fluorescence microscopy (Olympus Corporation, Hamburg, Germany).

 Luciferase reporter assay. The 3’UTR of the human ATG16L1 gene was predicted to interact with miR-410. A 360 bp-fragment of the ATG16L1 3’-UTR, which contained a putative binding site of miR-410, was subcloned into the 3’-UTR region of the pMIR-REPORT firefly luciferase
miR-410 is downregulated in osteosarcoma tissues and cell lines. qPCR was used to detect the expression levels of miR-410 in U2OS and MG-63 human osteosarcoma cell lines. The expression levels of miR-410 were significantly downregulated in U2OS and MG-63 cells compared with...
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Table I. Association between clinicopathological features and miR-410 expression levels.

| Factor                        | Cases (n) | miR-410 expression | P-value |
|-------------------------------|-----------|--------------------|---------|
|                               |           | High (6)           | Low (34) |         |
| Age (years)                   |           |                    |         |         |
| ≥25                           | 15        | 2                  | 13      | NS      |
| <25                           | 25        | 4                  | 21      |         |
| Gender                        |           |                    |         |         |
| Male                          | 24        | 3                  | 21      |         |
| Female                        | 16        | 3                  | 13      |         |
| Tumor size (cm)               |           |                    |         |         |
| ≥8                            | 19        | 4                  | 15      | NS      |
| <8                            | 21        | 2                  | 19      |         |
| Serum level of lactate dehydrogenase | 26    | 4                  | 22      | NS |
| Elevated                      | 14        | 2                  | 12      |         |
| Normal                        |           |                    |         |         |
| Serum level of alkaline dehydrogenase | 28    | 5                  | 23      | NS |
| Elevated                      | 12        | 1                  | 11      |         |
| Normal                        |           |                    |         |         |
| Clinical stage                |           |                    |         |         |
| IIA                           | 23        | 3                  | 20      | NS      |
| IIB/III                       | 17        | 3                  | 14      |         |
| Response to chemotherapy      |           |                    |         | <0.05   |
| Good                          | 14        | 6                  | 9       |         |
| Poor                          | 26        | 0                  | 26      |         |

miR-410, microRNA-410; NS, not significant.

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Figure 2. miR-410 has limited influence on osteosarcoma cell proliferation. MTT assays were performed to detect the cell viability of U2OS and MG-63 cells post-transfection with miR-410 or NC mimics for 48 h. Data are presented as the mean ± standard deviation. Blank control, untransfected group; miR-410, microRNA-410; NC, negative control; OD, optical density.

miR-410 exerts a limited influence on the proliferation of osteosarcoma cells. To determine whether miR-410 was able to affect the proliferation of osteosarcoma cells, proliferation assays were conducted in U2OS and MG-63 cells transfected with miR-410 or NC mimics. As shown in Fig. 2 overexpression of miR-410 had almost no affect on the viability of U2OS and MG-63 cells. Therefore, further studies were conducted to examine whether other mechanisms associated with antitumor function were affected by miR-410.

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were used to predict the targets of miR-410 in osteosarcoma cells. ATG16L1 was predicted to be a target gene of miR-410, and putative miR-410 binding sites were predicted to be present in the 3'UTR of ATG16L1 mRNA. ATG16L1 is a component of a large protein complex, which is essential for autophagosome formation. Autophagy is generally considered to be a pro-survival mechanism that preserves cell viability in response to cancer therapy (16). To validate the prediction that ATG16L1 is a direct target of miR-410, luciferase reporter vectors containing wild-type (WT) or mutant ATG16L1 3'UTR were generated (Fig. 3A). The luciferase assay indicated that miR-410 inhibited luciferase activity in the ATG16L1 WT 3'UTR group; however, the inhibitory effects were abolished when the ATG16L1 3'UTR binding sites were mutated (Fig. 3B). Furthermore, an immunofluorescence assay demonstrated that overexpression of miR-410 suppressed ATG16L1 expression in U2OS and MG-63 cells transfected with miR-410 or NC for 48 h. (D) Western blotting was conducted to detect the effects of miR-410 on ATG16L1 expression post-transfection with miR-410 or NC for 48 h. Rapamycin (20 nM), which is an activator of autophagy, increased ATG16L1 expression. Data are presented as the mean ± standard deviation; *P<0.05. ATG16L1, autophagy related 16-like 1; miR-410, microRNA-410; NC, negative control; WT, wild-type; mut/MT, mutant; Rap, rapamycin; UTR, untranslated region; DAPI, 4',6-diamidino-2-phenylindole.

Figure 3. miR-410 directly targets ATG16L1 in osteosarcoma cell lines. (A) miR-410/ATG16L1 alignment by miRanda analysis, and schematic diagram of the pMIR-ATG16L1/pMIR-ATG16L1mut paired sequences for miR-410. (B) Normalized luciferase activity of pMIR-ATG16L1/pMIR-ATG16L1mut reporter in human embryonic kidney 293T cells transfected with miR-410 or NC mimics. *P<0.05. (C) Immunofluorescent detection of ATG16L1 expression in U2OS and MG-63 cells transfected with miR-410 or NC for 48 h. (D) Western blotting was conducted to detect the effects of miR-410 on ATG16L1 expression post-transfection with miR-410 or NC for 48 h. Rapamycin (20 nM), which is an activator of autophagy, increased ATG16L1 expression. Data are presented as the mean ± standard deviation; *P<0.05. ATG16L1, autophagy related 16-like 1; miR-410, microRNA-410; NC, negative control; WT, wild-type; mut/MT, mutant; Rap, rapamycin; UTR, untranslated region; DAPI, 4',6-diamidino-2-phenylindole.
miR-410 inhibits autophagy in osteosarcoma cell lines. In relation to tumorigenesis, the role of autophagy is complex and depends on the environmental cues that cells are exposed to. Accordingly, intrinsic resistance to Dox and DDP through autophagic activity has been certified in osteosarcoma cells (17). Therefore, since miR-410 directly targeted ATG16L1 in osteosarcoma cancer cells, it was hypothesized that miR-410 may be able to reverse chemoresistance via autophagy inhibition. In order to verify this hypothesis, three common chemotherapy drugs, Rap, Dox and DDP were used to treat the cells in subsequent experiments. Initially, cell strains that stably expressed GFP-LC3 were generated (U2OS-GFP-LC3 and MG-63-GFP-LC3) by transfection of U2OS and MG-63 cells with NC or miR-410 for 48 h. β-actin was used as an internal control. Data are presented as the mean ± standard deviation and are representative of three independent experiments (*P<0.05). ATG16L1, autophagy related 16-like 1; miR-410, microRNA-410; NC, negative control; Rap, rapamycin; Dox, doxorubicin; DDP, cisplatin; GFP, green fluorescent protein; LC3I/II, microtubule-associated protein 1A/1B-light chain 3; OD, optical density; 3-MA, 3-methyladenine; DMSO, dimethyl sulfoxide.
(fluorescent dots) were accumulated due to the GFP-LC3 translocation to the structural components of the double-membrane autophagosome. As shown in Fig. 4A and B, the percentage of GFP-LC3 puncta-positive cells was significantly increased in the drug treatment groups. Furthermore, 3-MA significantly enhanced the sensitivity of cells to chemotherapy drugs in the drug treatment groups (Fig. 4C). In order to determine the effects of miR-410 on autophagy inhibition, U2OS and MG-63 cells, which were transfected with miR-410 or NC, were treated with Rap, Dox and DDP. The number of fluorescent dots and GFP-LC3 puncta-positive cells was significantly reduced in the cells transfected with miR-410 compared with in the NC group (Fig. 4D and E). These results suggest that miR-410 may function as a potential autophagy inhibitor in U2OS and MG-63 cells.

miR-410 enhances chemosensitivity of cells to Rap, Dox and DDP via autophagy inhibition. To further determine the
influence of miR-410-induced autophagy inhibition on chemoresistance in osteosarcoma cell lines, an MTT assay was used to detect cell viability in U2OS and MG-63 cells. As shown in Fig. 5A, miR-410 sensitized U2OS and MG-63 cells to Rap, Dox and DDP, thus suggesting that the presence of miR-410 could improve the therapeutic response to those agents. Subsequently, cell apoptosis was detected by Hoechst assay. Cells treated with miR-410 and chemotherapy agents exhibited enhanced cell apoptosis compared with the cells treated with chemotherapy agents alone (Fig. 5B). Notably, Rap-, Dox- and DDP-treated groups all activated the cleavage of caspase 3 (early molecular marker of apoptosis), whereas miR-410 enhanced the apoptotic-inducing ability of these agents (Fig. 5C). The cell apoptotic rate of the aforementioned groups is presented in Fig. 5D. These results suggest that miR-410 may reverse the resistance of U2OS and MG-63 cells to Rap, Dox and DDP through autophagy inhibition. In conclusion, miR-410 regulates ATG16L1 expression and enhances chemosensitivity of cells via autophagy inhibition in osteosarcoma (Fig. 6).

Discussion

Osteosarcoma is the most common type of primary bone tumor in children and adolescents, which accounts for 8% of the incidence rate and is a leading cause of cancer-associated mortality among young adults (18). Resistance to chemotherapy agents remains a major clinical obstacle to effective therapy; however, the mechanisms underlying osteosarcoma chemoresistance remain largely unknown.

Previous studies have confirmed that autophagy, a chief mechanism for bulk degradation of superfluous or aberrant cytoplasmic components, functions as a protective mechanism that degrades and enables reuse of abnormal proteins and organelles as energy sources to promote cancer cell survival in response to cancer treatment (5,19,20). Mammalian target of rapamycin (mTOR) has generally been regarded as a negative regulator of autophagy, and autophagy-related genes are regulated by proteins upstream of mTOR signaling, including phosphatase and tensin homolog, phosphoinositide-dependent kinase-1, Akt and tuberous sclerosis 1/2 (20). In addition, upregulated autophagy has been identified in various cancer cells following therapeutic stress. Therefore, suppressing cancer cell autophagy is emerging as a novel approach to enhance the efficiency of chemotherapy in cancer treatment (21-23).

miRNAs have emerged as fundamental regulators of gene expression, and are able to silence gene expression at the post-transcriptional and translational levels (8). Dysregulation of miRNAs has been demonstrated to be associated with the tumorigenesis and progression of various types of tumor, including osteosarcoma (24). Downregulation of miR-410 has been detected in several malignant tumors and overexpression of miR-410 inhibits tumor growth and promotes cell apoptosis (11,25,26). In the present study, the role of miR-410 in the progression of osteosarcoma was investigated. The results demonstrated that miR-410 expression was markedly downregulated in human osteosarcoma tissues, and U2OS and MG-63 osteosarcoma cell lines; however, the overexpression of miR-410 exhibited a limited effect on the viability of U2OS and MG-63 cells. Furthermore, ATG16L1 was identified as a direct target of miR-410, and overexpression of miR-410 inhibited the expression of ATG16L1 at the mRNA and protein level in osteosarcoma cell lines. It was also demonstrated that miR-410 was able to markedly inhibit autophagy. Therefore, miR-410 may have the potential to reverse chemoresistance via autophagy inhibition.

The regulatory mechanisms between miRNAs and autophagy remain largely unknown. Chen et al reported that increased miR-155 expression levels upregulated autophagy in osteosarcoma cells, and ameliorated Dox-induced decreases in cell viability (27). Li et al demonstrated that overexpression of miR-22 targeted the 3'UTR of high mobility group box 1 (HMGBI) and inhibited HMGBI-induced autophagy, which contributed to chemotherapy resistance in osteosarcoma in vitro (28). The present study revealed that autophagy was activated after cells were treated with Rap, Dox and DDP. Conversely, the presence of autophagy inhibitor 3-MA or transfection with miR-410 was able to effectively reduce autophagic activation. Further investigations indicated that, compared with the Rap-, Dox- and DDP-treated groups, cell viability was significantly decreased and apoptosis was significantly increased in the drug-treated groups transfected with miR-410.

Taken together, the results of the present study provided novel evidence suggesting that ATG16L1 is a direct target of miR-410. Overexpression of miR-410 significantly sensitized osteosarcoma cells to Rap, Dox and DDP. Understanding the miR-410-mediated tumor suppressor pathways and the potential ability to suppress the autophagic process in human osteosarcoma may provide invaluable therapeutic targets for the treatment of osteosarcoma.

The present study demonstrated that miR-410 expression was downregulated in osteosarcoma cell lines and tissues; however, miR-410 overexpression exhibited limited effects on the viability of U2OS and MG-63 cells. Furthermore, target prediction algorithms identified ATG16L1 as a potential target gene of miR-410, and luciferase reporter assays indicated that miR-410 directly suppressed ATG16L1 expression by targeting its 3'UTR. Furthermore, miR-410 was shown to effectively inhibit autophagy. Accordingly, autophagy was activated as a protective mechanism when osteosarcoma cells were exposed to anticancer drugs, including Rap, Dox and DDP (29,30). The present study demonstrated that the autophagy inhibitor 3-MA and miR-410 expression were able to improve the therapeutic response of cells to chemotherapy drugs (Rap, Dox and DDP), thus indicating that miR-410 may reverse chemoresistance via autophagy inhibition in osteosarcoma cells. The studies conducted regarding the function of miR-410 on autophagy provide insight into the biological function of miR-410 in osteosarcoma, and offer a promising approach for osteosarcoma treatment.

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