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Research Paper

# MiR-367 negatively regulates apoptosis induced by adriamycin in osteosarcoma cells by targeting KLF4

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ABSTRACT

Diverse functions of microRNAs have been investigated in tumorigenesis in osteosarcoma (OS), involving the regulation of proliferation, invasion, migration, apoptosis and drug resistance. MiR-367 was found to be an oncogene and increased in OS. However, the function of miR-367 in drug resistance in OS cells is still unknown. In this study, we found that miR-367 was up-regulated in OS tissues and OS cell cultures. Meanwhile, treatment with adriamycin (ADR) induced apoptosis of OS cells with upregulation of miR-367. Notably, KLF4 was demonstrated to be a direct target of miR-367 by gene reporter assay, and miR-367 significantly blocked both mRNA and protein level of KLF4. In addition, overexpression of miR-367 markedly suppressed the increase of KLF4 induced by ADR in OS cells, as well as Bax and cleaved caspase-3, which were significantly reversed by anti-miR-367 transfection. Taken together, our data demonstrates that miR-367 and KLF4 play important roles in OS treatment and ADR resistance, suggesting that miR-367 is a potential biomarker of chemotherapy resistance in OS and also probably a novel therapeutic target against OS.



## 1. Introduction

Osteosarcoma (OS) is the most common type of bone tumor occurred in adolescents and children [1]. OS is highly malignant and accounts for about half of bone sarcomas with high potential of metastasis [2]. Treatment for OS is mainly depended on surgical operation, assisted with chemotherapy [3]. However, even with multiple chemotherapies, it is still less optimistic due to the poor 5-year survival rates of OS, regardless of increasing kinds or dosages of chemotherapeutic drugs [4]. Recently, drug resistance in OS has draw more attention in understanding the molecular mechanism in OS treatement [5]. However, it is still an ongoing progress in discovering and developing novel therapeutic target.

microRNAs (miRNAs) are non-coding RNAs with 17–25 nucleotides [6,7]. As is known, miRNAs have diverse functions in multiple physiological progression [8–10]. Since the initiation, more than 1000 miRNAs have been found to regulate the development and progression in human diseases and tumorigenesis [11–14]. miRNAs regulate the target genes at the post-transcriptional level by binding with 3′ untranslated region. Recently, growing evidence showed that the dysfunction and dysregulation of miRNAs resulted in the occurrence and development of tumor [15–17]. In OS, multiple miRNAs had showed differential expression between tumor tissues and normal counterparts, and some had been verified to be involved with drug resistance [18,19].

miR-367 has been recognized to be a tumor suppressor gene in gastric cancer by inhibiting invasion and metastasis *via* regulating Rab23 [20]. On other hand, miR-367 also functioned as an oncogene in pancreatic ductal adenocarcinoma by promoting epithelial-to-mesenchymal transition through the Smad7-TGF-beta signaling pathway [21]. Thus studies showed the diverse functions of miR-367 in different tumorigenesis. However, little is known about the functions of miR-367 in OS and in drug resistance.

In this study, we focused on the function of miR-367 in drug resistance in OS. We found that the expression of miR-367 increased in human OS tissues and OS cell lines. Meantime, down-regulation of miR-367 participated in apoptosis induced by adriamycin (ADR), a classical drug in OS chemotherapy. Further investigations demonstrated that KLF4 was a direct target of miR-367, which was involved in the ADR induced apoptosis. Furthermore, overexpression of miR-367 promote the resistance to ADR in OS cells, by decreasing the expression of KLF4, Bax and Cleaved Caspase-3. Our study suggested that miR-367 could be a biomarker of the chemoresistance in OS treatment, and miR-367 could be a potential target in clinical OS therapies.

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## 2. Materials and methods

### 2.1. Patients and tissues

The human osteosarcoma tissues were obtained from 40 patients underwent standard surgery within 2009–2014 at department of Orthopedics, Changhai hospital (the Second Military Medical University). The counterparts were obtained 5 cm away from the tumor tissues at the same time. All of the tumor tissues and counterparts were put into liquid nitrogen immediately post-operation for the following examinations. None of the patients had received thermotherapy or radiotherapy before the surgery. The histologic responses were evaluated by professional pathologists in our hospital. The research progress was conducted according to the Declaration of Helsinki of the World Medical Association.

### 2.2. Cell cultures and drug administration

The human OS cell lines MG-63, U2OS, Saos-2 and normal osteoblastic cell line HOB (ATCC) were cultured in dilbecco modified eagle medium (DMEM) (Hyclone) with 10% fetal bovine serum (Gbico), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in 5% $CO_2$ incubators. 0.4 μg/ml of ADR was administrated in cells for 6, 12, 24 h, respectively. The cells were harvested and store at −80 °C before analysis after drug treatment for further analysis.

### 2.3. Quantitative PR-PCR

The isolation of RNA from tumor tissues and the counterparts was conducted using Trizol reagent (Takara, Tokyo, Japan). The reverse transcription with oligo (dT) priming (Toyobo) was performed to generate complementary DNAs (cDNAs). Level of miRNA was performed using quantitative RT-PCR analysis with SYBR RT-PCR kit (Takara). Analysis of miR-367 and KLF4 was calculated using CT (cycle threshold) value. RT primers for miR-367 were following: 5′-TTCTCCGAACTTGTCACGTTT-3′ (forward), 5′-ACGTGACACGTTCGGAGAATT-3′ (reverse). The small nuclear RNA U6 was used as control. RT primers for KLF4 were following: 5′-ATCTTTCTCCACGTTCGCGTCTG-3′ (forward) and 5′-AAGCACTGGGGGAAGTCGCTTC-3′ (reverse) [22]. Actin was used as control, and the primers were following: 5′-TGACGTGGACATCCGCAAAG-3′ (forward), 5′-CTGGAAGGTGGACAGCGAGG-3′ (reverse). Each sample was detected in triplicate.

### 2.4. Apoptosis anaylsis

Cell apoptosis was detected by flow cytometer analysis. When the cells reached 80% of confluence, 0.4 μg/ml of ADR was added for 24 h. Then the cells were digested using trypsin without EDTA at 37 °C. Wash these cells with cold PBS for three times. Then the cells were stained with FITC-conjugated annexinV reagent (2.0 mg/ml) and PI (5 mg/ml) in binding buffer and then were analyzed by flow cytometer.

### 2.5. Northern blot assay

The RNA is isolated using Trizol reagent (Takara) followed by the standard protocol. 20 μg RNA was separated with 15% urea–polyacrylamide gel, then electrotransferred to nylon membrane. Hybridization with oligonucleotide probes was performed to detect miR-367 and U6. The probe sequences were following: 5′-AACTTGTCACGTTTACGTTCGGAG-3′ (miR-367), 5′-TGTGGTGCCGGAGCGAGCAG-3′ (U6). The blots analysis were performed by Fujifilm LAS-4000 system.

### 2.6. Cell transfection

The OS cells were seeded in 6 well-plates at $2 \times 10^5$. When the confluence attained 80%, refreshed the medium without FBS and transfected 100 nM miR-367 or anti-miR-367 using Lipofectamine 2000 (Invitrogen) for 6 h. Anti-miR-367 (an inhibitor of miR-367), 5′ -UCAACAUCAGUCUGAUAAGCUA-3′; Anti-miR-C (used as a NC for anti-miR-367 in the antagonism experiment), 5′-GUGGAUAUUGUUGCCAUCA-3′. Then refresh the medium with 10% FBS. After incubation for 24 h, treated cells with ADR and harvested the cells with cold PBS, stored at −80 °C for the following analysis.

### 2.7. Western blot analysis

Cells were lysed in RIPA buffer with appropriate concentration of protease inhibitor cocktails (Roche). The supernatants were obtained and measured by BCA Protein Assay Kit (Pierce). 20 μg of protein samples were subjected to SDS-PAGE, then transferred onto PVDF membranes. After incubation with 5% of skim milk, the membranes were incubated with anti-β-actin (1:2000, Cell Signaling Technology), anti-KLF4 (1:1000, Cell Signaling Technology), anti-Bax (1:1000, Cell Signaling Technology), anti-caspase-3 (1:1000, Cell Signaling Technology), anti-cleaved caspase-3 (1:1000, Cell Signaling Technology) antibodies at 4 °C over night. The HRP-labeled secondary antibodies were incubated for 1 h at RT. The results were detected using Fujifilm LAS-4000 system.

### 2.8. Luciferase reporter assay

The wild type and mutant 3′UTR of KLF4 were cloned into PGL3-basic vector. The plasmids were transfected into MG-63 cells using lipofectamine 2000 reagent (Invitrogen). The miR-367 was co-transfected at the same time. The normalizing control was pRL-SV40 plasmid (Promega). After incubation for 24 h, cells were harvested to detect the activities of luciferase using Dual-Luciferase Reporter Assay System (Promega).

### 2.9. Statistical analysis

All of the data were represented as mean ± SEM for at least three repeated experiments for each group. Data were analyzed using SPSS 17.0 software (SPSS). The comparison between two groups were conducted by Student's non-paired t-test, and the comparison of multiple groups were conducted by one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. Expression of miR-367 was increased in osteosarcoma and cell cultures

The expression of miR-367 was analyzed in osteosarcoma tissues and OS cells by RT-PCR. As shown in Fig. 1A, expression of miR-367 was significantly increased in osteosarcoma tissues compared with osteoblast from 40 patients ($P < 0.01$). Meanwhile, miR-367 was also increased in OS cells MG-63, U2OS and Saos-2 compared with normal osteoblastic cell line HOB ($P < 0.01$, *vs.* HOB group, respectively) (Fig. 1B). These data demonstrated that miR-367 was upregulated in osteosarcoma tissues and OS cell lines.

### 3.2. ADR induced apoptosis in osteosarcoma cells via suppressing miR-367

To investigate whether miR-367 was involved in the apoptosis