Research Paper

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

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A B S T R A C T

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.

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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′-ACGTGACACGTTCGGA-GAATT-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′-TGACGTG-GACATCCGCAAAG-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′-AACTTGT-CACGTTTACGTTCGGAG-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 2×10^5. 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′-GUG-GAUAUUGUUGCCAUCA-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
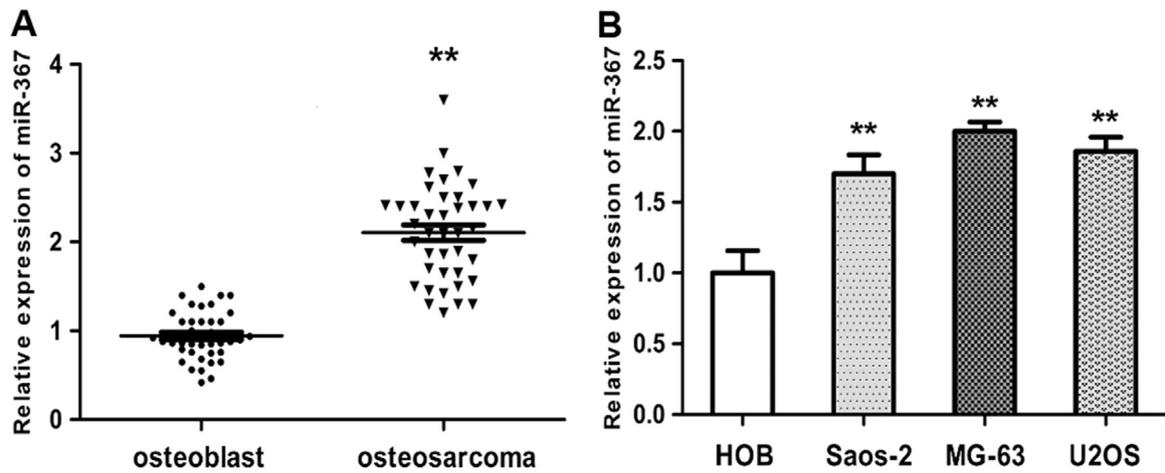
Fig. 1. Expression of miR-367 was increased in osteosarcoma tissues and cell lines. A. Expression of miR-367 increased obviously in osteosarcoma tissues compared to osteoblast (**$P < 0.01$). B. Expression of miR-367 increased in OS cell lines Saos-2, MG-63 and U2OS compared to osteoblasts (HOB) (**$P < 0.01$, respectively). Expression of miR-367 was calculated and normalized to U6 small RNA.
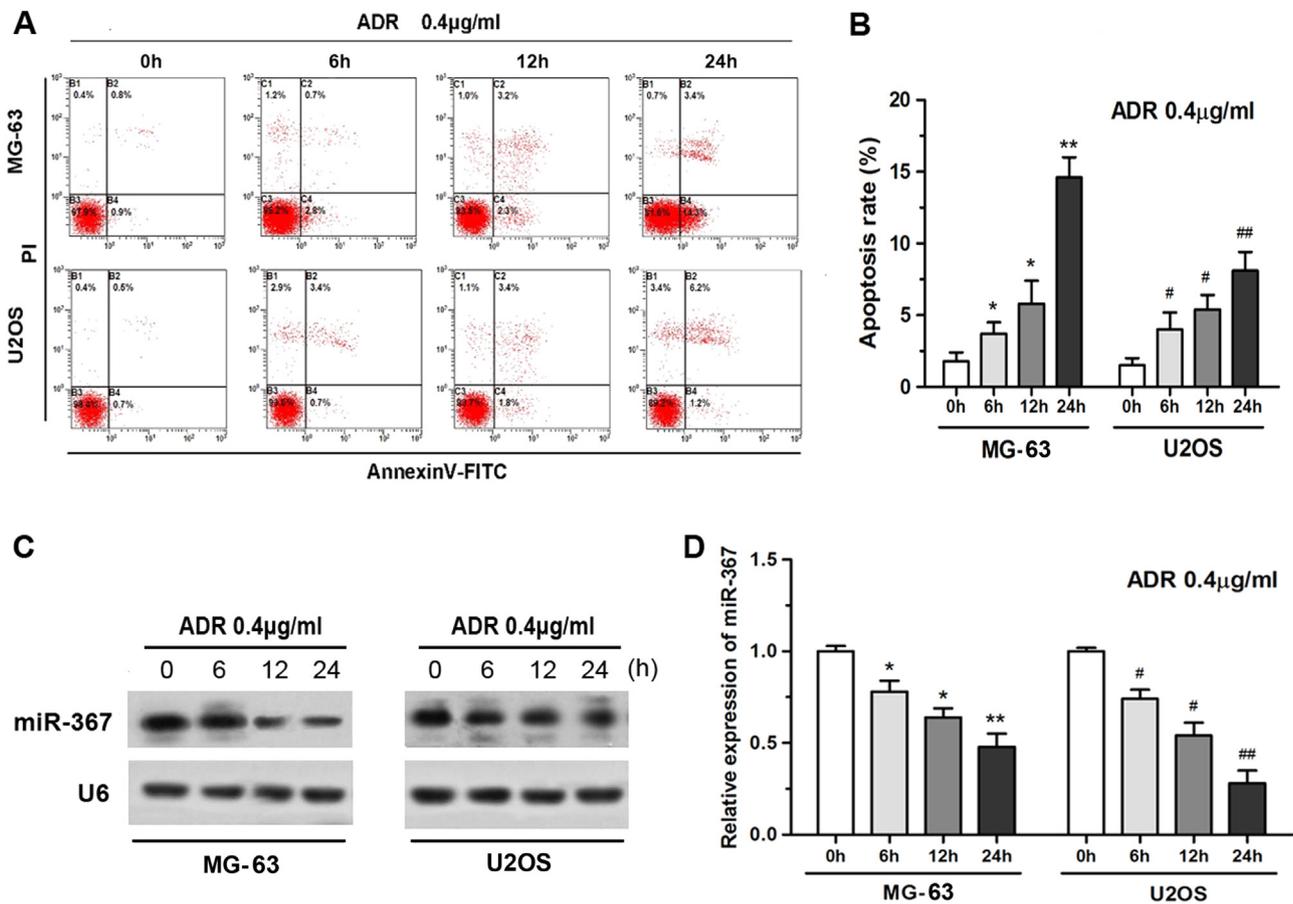
Fig. 2. ADR induced apoptosis in osteosarcoma cells and suppressed miR-367. A–B. Apoptotic rate was detected with flow cytometry analysis of the Annexin V staining in MG-63 and U2OS cells treated with 0.4 μg/ml ADR for indicated time (MG-63: *$P < 0.05$, **$P < 0.01$; U2OS: #$P < 0.05$, ##$P < 0.01$, respectively). ADR induced apoptosis in a time-dependent manner. C. Expression of miR-367 were detected by northern blot analysis in MG-63 and U2OS cells with ADR treatment for indicated time. D. RT-PCR were performed to determine expression of miR-367 in MG-63 and U2OS cells (MG-63: *$P < 0.05$, **$P < 0.01$; U2OS: #$P < 0.05$, ##$P < 0.01$, respectively).

induced by ADR in osteosarcoma, we detected expression of miR-367 after the treatment of ADR in a time-dependent manner. As shown in Fig. 2A, ADR induced significant apoptosis in MG-63 (6 h, $P < 0.05$; 12 h, $P < 0.01$; 24 h, $P < 0.01$, vs. 0 h group, Fig. 2B) and U2OS (6 h, $P < 0.01$; 12 h, $P < 0.01$; 24 h, $P < 0.01$, vs. 0 h group, Fig. 2B) cells by flow cytometer analysis. Together, Northern blots (Fig. 2C) and quantitive analysis (Fig. 2D) showed the decreased expression of miR-367 in a time-dependent manner in MG-63

(6 h, $P < 0.05$; 12 h, $P < 0.01$; 24 h, $P < 0.01$, vs. 0 h group) and U2OS (6 h, $P < 0.05$; 12 h, $P < 0.05$; 24 h, $P < 0.01$, vs. 0 h group) cells. RT-PCR analysis further supported the results (Fig. 2E) (MG63: 6 h, $P < 0.05$; 12 h, $P < 0.01$; 24 h, $P < 0.01$, vs. 0 h group; U2OS: 6 h, $P < 0.05$; 12 h, $P < 0.05$; 24 h, $P < 0.01$, vs. 0 h group). The data demonstrated that miR-367 was involved with the treatment of ADR and suppressed by ADR.
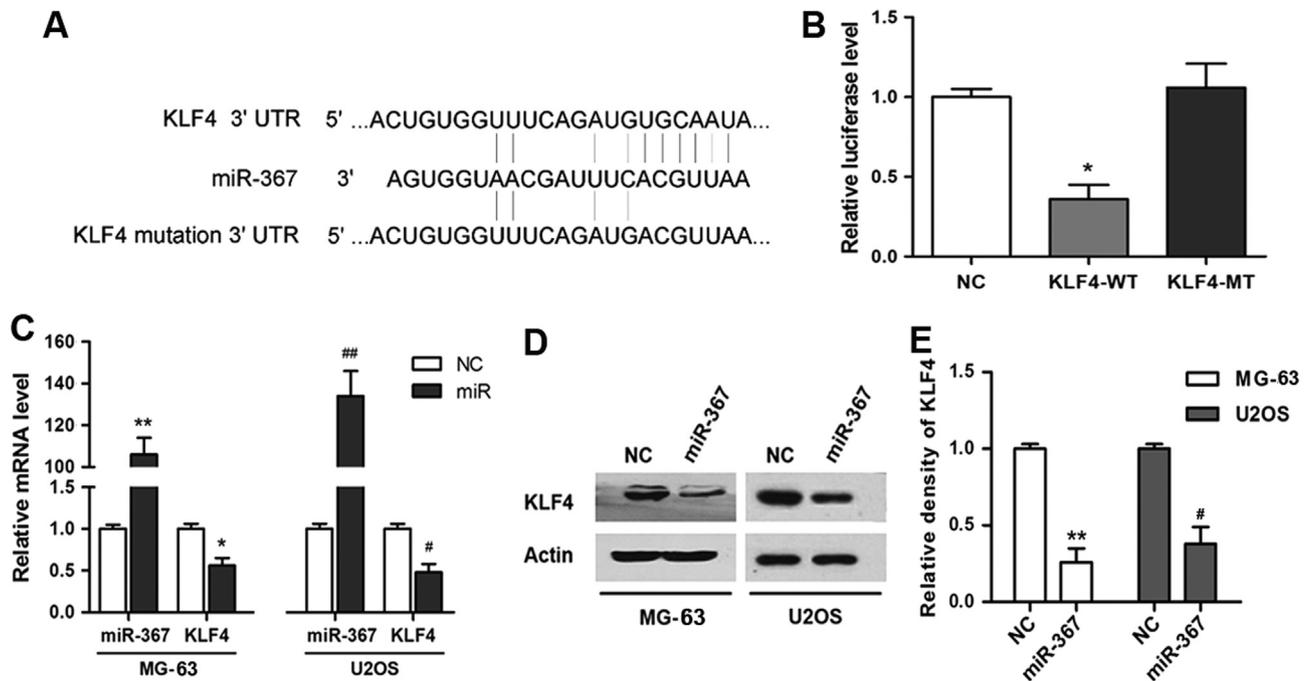
Fig. 3. KLF4 was a direct target of miR-367. A. Sequence of KLF4 3′UTR carrying wild type and mutant miR-367 binding site. B. Luciferase reporter assay in MG-63 cells. The cells was co-transfected with miR-367 and wild type (WT) or mutant type (MT) KLF4 3′UTR. The luciferase activities were measured after 24 hours ($P < 0.05$, KLF-WT vs. NC group). C. Expression of miR-367 and KLF4 mRNA level. MG-63 and U2OS cells were transfected with miR-367 and RT-PCR were performed after the transfection for 24 h. Expression of KLF4 decreased due to miR-367 (KLF4: *$P < 0.05$ in MG-63 cells, #$P < 0.05$ in U2OS cells, miR-367 vs. NC group, respectively). D. Expression of KLF4 in MG-63 and U2OS cells. Overexpression of miR-367 suppressed the expression of KLF4. E. Relative density analysis of western blots in D (MG-63: **$P < 0.01$; U2OS: #$P < 0.05$, miR-367 vs. NC group, respectively).

3.3. KLF4 was a direct target of miR-367

To further investigate the function of miR-367 in ADR-induced apoptosis, we found that KLF4 was probably a direct target of miR-367 by TargetScan (Fig. 2A), which could facilitate apoptosis via regulating Bax and Bcl-2. To demonstrate the prediction, we constructed the luciferase activity of vector carrying the wild type or the mutant 3′-UTR of KLF4, as shown in Fig. 2B, miR-367 obviously reduced the luciferase activities of vector carrying wild type 3′UTR of KLF4 ($P < 0.05$), while it had almost no influence on the mutant 3′UTR of KLF4 ($P > 0.05$), thus suggested that miR-367 could interact with the 3′UTR of KLF4. Furthermore, overexpression of miR-367 in MG-63 and U2OS cells distinctly reduced the expression of KLF4 (MG-63: $P < 0.05$, vs. NC group; U2OS: $P < 0.05$, vs. NC group) (Fig. 2C). Western blots also showed miR-367 decreased expression of KLF4 (Fig. 2D) in MG-63 and U2OS cells. The results demonstrated that miR-367 suppressed KLF4 by interacting with 3′UTR, and suggested that miR-367 might be involved in OS apoptosis via regulating KLF4 (Fig. 3).

3.4. miR-367 negatively modulated the apoptosis induced by ADR via regulating KLF4

miR-367 directly targeted on KLF4, which was a definite anti-oncogene according to a variety of previous researches. To clarify the function of miR-367 and KLF4 in ADR induced apoptosis in OS, MG-63 and U2OS cells were transfected with miR-367 or anti-miR-367, then the cells were treated with ADR for 24 h. As showed in Fig. 4A and B, ADR induced significant apoptosis in MG-63 and U2OS cells ($P < 0.01$, ADR/NC vs. Con/NC group, respectively); anti-miR-367 also induced significant increase of apoptotic rate in Con/miR-367 group compared to Con/NC group in OS cells ($P < 0.01$, respectively). Moreover, anti-miR-367 obviously enhanced the apoptosis induced by ADR ($P < 0.01$, ADR/miR-367 vs. ADR/NC groups, respectively), suggesting that miR-367 acted as an oncogene which could inhibit the apoptosis.

Bax was a direct target of KLF4, which significantly induced apoptosis in mammalian cells through activating caspase-3 [23,24]. Western blots analysis showed (Fig. 4B) that ADR treatment increased the expression of KLF4, caspase-3, Cleaved caspase-3 and Bax expression in MG-63 and U2OS cells. Overexpression of miR-367 not only inhibited KLF4, Bax and Cleaved caspase-3, but also blocked the effective treatment of ADR in OS cells (Fig. 4C and E). The results suggested that miR-367 promoted the resistance of MG-63 and U2OS cells to ADR. In contrast, anti-miR-367 increased the expression of KLF4, Bax and Cleaved caspase-3, moreover, which were enhanced by combining with ADR in OS cells (Fig. 4D and F). Thus indicated that anti-miR-367 enhanced the sensibility of MG-63 and U2OS cells to ADR. In summary, these data showed that miR-367 suppressed the apoptosis induced by ADR via the regulation of KLF4, and suggested that miR-367 enhanced the resistance of OS cells to ADR.

4. Discussion

Discovery and developing novel therapeutic target and strategy against osteosarcoma remains less optimistic. Recently, miRNAs have been investigated to regulate diverse physiological and pathological progression, even the tumorigenesis [13,25]. Though accumulating evidences show that miRNAs participate in tumor initiation and progression by regulating their target genes, however, their functions have not been fully understood. Previous studies showed that miR-367 acted multiple functions in different kinds of cancer by targeting different genes through inhibiting or promoting cellular invasion and metastasis [20,21,26]. However, the expression of miR-367 and its function in OS is little known. In our study, we confirmed that miR-367 increased in osteosarcoma and OS cell lines, indicating that miR-367 could be an oncogene in OS.

Our data also demonstrated that miR-367 was involved in ADR-
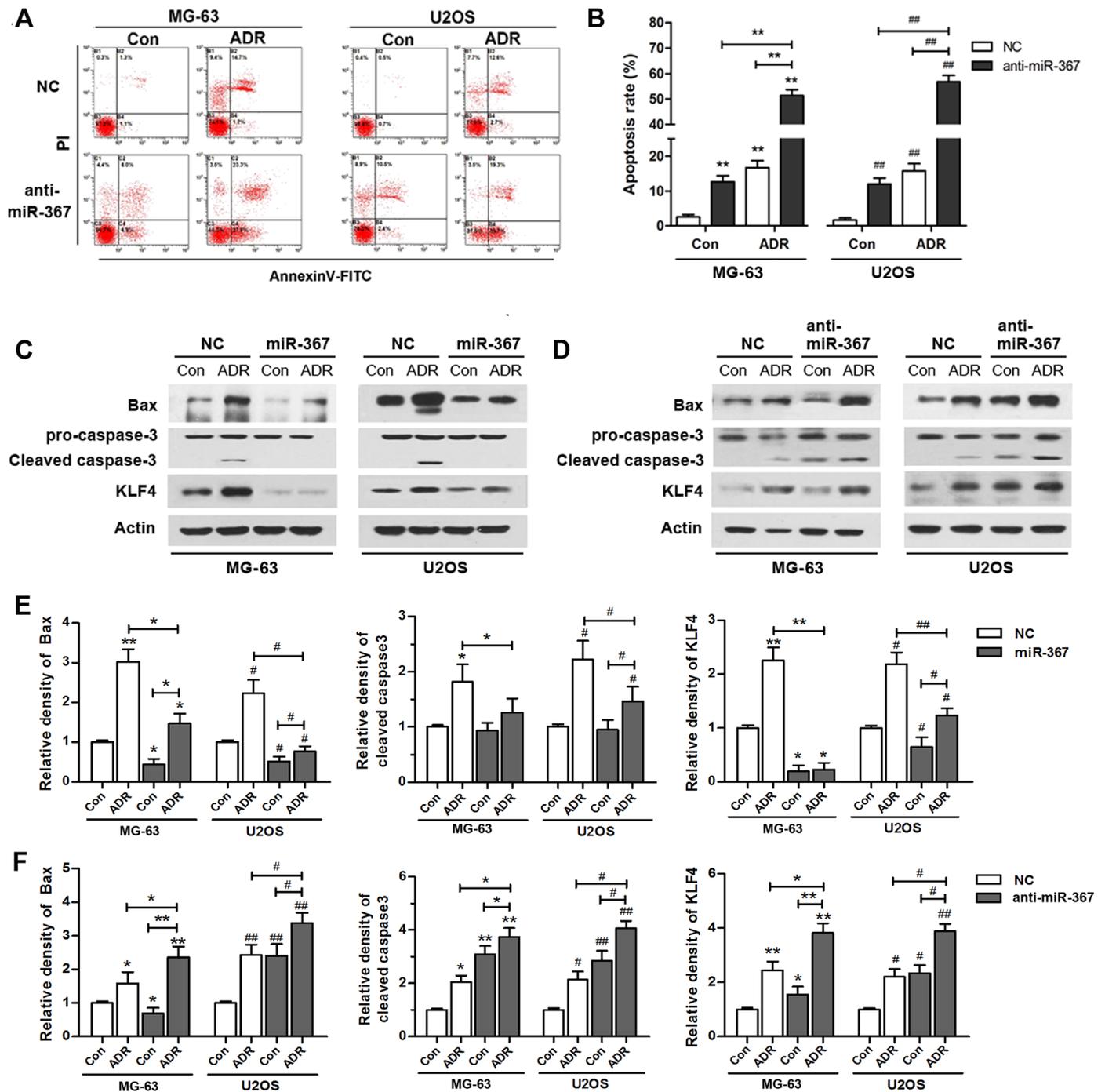
Fig. 4. miR-367 modulates resistance of osteosarcoma cells to ADR. A and B. Flow cytometer analysis was used to determine the effect of anti-miR-367 on apoptosis of MG-63 and U2OS cells with or without treatment of ADR. Anti-miR-367 enhanced the apoptosis induced by ADR (MG-63: **$P < 0.01$; U2OS: ##$P < 0.01$, vs. Con/NC group or the indicated group, respectively). C and D. Western blots detected the effect of miR-367 or anti-miR-367 overexpression on the expression of KLF4, Bax and caspase3 in MG-63 and U2OS cells with or without treatment of ADR. E and F. Relative density analysis of western blots in C and D, respectively (MG-63: *$P < 0.05$, **$P < 0.01$; U2OS: #$P < 0.05$,##$P < 0.05$, vs. Con group or indicated group, respectively).

induced apoptosis in OS cells. ADR is a kind of chemotherapeutic drug applied in kinds of malignant cancers [27,28]. ADR induced OS cells apoptosis, meanwhile, decreased the expression of miR-367. To further investigate the role of miR-367 on regulating of the apoptosis, we found KLF4, a tumor suppressor gene which could facilitate apoptosis [29,30], could be a direct target of miR-367 in OS cells. Luciferase reporter system was constructed to verify the interaction of miR-367 and the 3'UTR of KLF4. The results showed that miR-367 binded to the 3'UTR of KLF4, indicating that KLF4 was regulated at the translational level by miR-367. We also found

that overexpression of miR-367 suppressed the expression of KLF4 at mRNA and protein level. Definitive evidences in Fig. 4 showed transfection of anti-miR367 induced apoptosis, increased KLF4 expression, and also enhanced the sensitivity to ADR in OS cells, all which were reversed by overexpression of miR-367. Thus strongly demonstrated miR-367 acted as an oncogene in OS, and could be involved with the chemotherapy resistance of OS. These findings also suggested KLF4 might play the key role in miR-367-involved cellular proliferation and anti-apoptosis in OS.

Depending on the target gene, KLF4 may function as both a

repressor and activator of transcription [31,32]. Previous studies demonstrated Bax could be a tumor-suppressor target of KLF4 functioning as an anti-oncogene [33,34]. When transfected with anti-miR-367, MG-63 and U2OS cells showed enhanced sensitivity to ADR, with up-regulation of KLF4, Bax and activation of caspase-3, which were also negatively regulated by miR-367 over-expression. These data suggested that ADR induced apoptosis in OS cells through the regulation of KLF4 *via* miR-367, then the pro-apoptosis factor Bax and cleavage of caspase-3 could lead to final apoptosis.

In summary, our data demonstrates that miR-367 functions as an oncogene in OS targeting the tumor suppressor KLF4; ADR induces apoptosis in MG-63 and U2OS *via* the regulation of miR-367/KLF4/Bax signaling pathway; miR-367 enhances the chemotherapy resistance of ADR to OS cells through suppressing KLF4. Our data shows that miR-367 could be a potential biomarker of chemotherapy resistance and therapeutic target against OS.

Acknowledgments

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