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# MicroRNA-301a modulates doxorubicin resistance in osteosarcoma cells by targeting AMP-activated protein kinase alpha 1

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ABSTRACT

MicroRNAs have been implicated in drug resistance of osteosarcoma (OS). MicroRNA-301a (miR-301a) is up-regulated and functions as an oncogene in various cancers. However, little is known about the role of miR-301a in drug resistance of OS cells. In this study, we found that doxorubicin induced time-dependent expression of miR-301a in OS cells. Meantime, doxorubicin promoted HMGCR expression and inhibited AMPKα1 expression, which was further facilitated by miR-301a overexpression. Luciferase reporter assay identified AMPKα1 as direct target gene of miR-301a. Notably, miR-301a reduced doxorubicin-induced cell apoptosis whereas anti-miR-301a enhanced apoptosis in OS cells, suggesting that up-regulation of miR-301a contributed to chemoresistance of OS cells. Consistently, our data showed that miR-301a and HMGCR were up-regulated in chemotherapy-resistant OS compared to those in control OS. Our findings suggested that miR-301a might be a potential biomarker for chemotherapy-resistant OS and a promising therapeutic target for overcoming drug resistance of OS.



## 1. Introduction

Osteosarcoma (OS) is the most common primary bone tumor in the childhood and adolescent [1]. OS is prone to early metastasis and is poorly respond to drug and radiation therapies, which are the reasons for its high lethality [2]. Doxorubicin and cisplatin are well established to be used as chemotherapeutic drugs in osteosarcoma [3,4]. The patients that are not sensitive to these drugs possess poor prognosis with 5~20% survival rate after surgery [5]. Since multiple chemotherapy was introduced in the 1970s, 5-year survival rate has been increased from 20% to 70% [6]. However, the survival rate remains unimproved in the last 3 decades, despite more drugs or increasing drug dosages [7]. The chemoresistance of osteosarcoma is responsible for standstill, but molecular mechanisms underlying the chemoresistance are largely unclear.

MicroRNAs (miRNAs) are endogenous noncoding RNAs with 18–25 nucleotides. MiRNAs play critical roles in mediating multiple genes at the post-transcriptional level by interacting with the 3′ untranslated region (UTR) of targeted mRNAs [8]. MiRNAs are estimated to regulate over 30% of all human genes and each miRNA can affect more than 100 target genes expression [9,10], for which their biological functions remain complex and mysterious. The differential expression of miRNA between tumors and the normal counterparts have been explored in various cancers, including lung [11], breast [12], prostate [13], gastric [14] and pancreas cancer [15], suggesting that miRNAs play critical roles in the pathogenesis of cancers. A series studies on miRNAs in OS have been performed [8,16]. MicroRNA-33a has been found to be up-regulated in chemotherapy-resistant OS and promotes chemoresistance by suppressing TWIST [17]. Functional analysis of miR-140 demonstrated implication of miR-140 in chemoresistance of OS by targeting Histone deacetylase 4 [18]. Over-expression of miR-215 promotes MTX-chemoresistance in human OS cell lines by inhibiting denticleless protein homolog expression [19]. Multiple studies have demonstrated up-regulation of miR-301a in various cancers, controlling cancer cell proliferation and metastasis [20–22]. However, the role of miR-301a in chemoresistance of OS has never been investigated.

In this study, we found that exposing OS cells to doxorubicin increased the expression of miR-301a and HMGCR whereas decreased AMPKα1 level. Further investigations demonstrated that miR-301a targeted directly AMPKα1, an enzyme that phosphorylates and inactivates HMGCR, leading to elevated HMGCR level. Overexpression of miR-301a promoted resistance of OS cells to

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doxorubicin. Up-regulation of miR-301a and HMGCR were confirmed in chemotherapy-resistant OS compared to those in chemotherapy-sensitive OS. Our results indicated that inhibition of miR-301a might be promising therapeutic strategy for overcoming chemoresistance of OS.

## 2. Materials and methods

### 2.1. Antibodies and reagents

The antibodies for ERK, phosphor-ERK and caspase3 were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies for AMPKα1 and HMGCR were from Abcam (Cambridge, MA). The antibody for beta-actin (pAb) was from Anbo Biotechnology Company (San Francisco, CA, USA). RIPA lysis buffer was purchased from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Carlsbad, CA). Anti-miR-301a and miR-301a were obtained from Biosune biotechnology (Shanghai, China).

### 2.2. Study population

From August 2008 to May 2014, twenty-six human OS specimens were obtained from OS patients with poor chemoresponse by the surgeon. Twenty-six age- and sex-matched OS patients with a good chemoresponse were recruited as controls. All tissues were washed with saline and were directly stored in liquid nitrogen until use. The histologic responses were evaluated by the expert pathologists. When the percentage of tumor necrosis was ≥90%, the patients were defined as good responders. When the percentage of tumor necrosis was lower than 90%, the patients were defined as poor responders. This study was approved by the Medical Ethics Committee of Tianjin Medical University, and written informed consents were obtained from all the participants.

### 2.3. Cell culture

The human OS cell lines U2OS and MG-63 (ATCC, Manassas, VA) were cultured in DMEM medium (Hyclone, Austrilian) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in 5% $CO_2$. When grown to approximately 80% confluence, the cells were treated with 1.0 μM doxorubicin for indicated time.

### 2.4. Cell viability

Cells were seeded in 96-well plates at densities of $2 \times 10^4$. After transfecting with miR-301a, anti-miR-301a or negative control (NC), the cells were treated with 1.0 μM doxorubicin for 24h, and then incubated with MTT solution (0.5 mg/ml) at 37 °C for 4 h. The generated formazan product was dissolved in DMSO and measured at 570 nm using a SpectraMax M2.

### 2.5. Apoptosis analysis

DNA fragmentation assay was performed as previously described [23]. Briefly, the cells were seeded at a density of $1 \times 10^6$ in 6-well plates. After transfecting with anti-miR-301a or NC, the cells were treated with 1.0 μM doxorubicin for 24h, followed by incubation in the buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 0.5% TritonX-100 and RNase A for 30 min at 37 °C. Subsequently, the lysates were treated with proteinase K for 1h at 50 °C. The supernatants were collected by centrifugation. The fragmented DNA was further extracted from the supernatant using phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and separated by agarose electrophoresis.

Apoptosis was further determined by flow cytometer analysis. The cells were digested using trypsin without EDTA and phenol red at 37 °C. After washing with PBS, cells were stained with FITC-conjugated annexin-V reagent (2.5 mg $mL^{-1}$) and PI (5 mg $mL^{-1}$) in binding buffer and were analyzed by flow cytometer.

### 2.6. Quantitative RT-PCR

The isolation of total RNA from clinic tissues was performed using Trizol reagent (Invitrogen). The miR-301a expression was detected using the TeqMan MicroRNA assay Kit (ABI, Foster City, CA, USA) on the LightCycler 480 instrument (Roche). The small nuclear RNA U6 was used for normalization. The relative amount of miR-301awas calculated using the CT (cycle threshold) value as the relative miRNA level. Each sample was performed in triplicate. For HMGCR expression, the cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The following primers were used to amplify HMGCR and GADPH: HMGCR Forward 5′ CTCCAGTACCTACCTTACAGGGATT 3′ and Reverse 5′ GCTGCTGGCACCTCCA 3′) [24]; GADPH Forward 5′-GGTGAAGGTCGGAGTCAACGG-3′ and Reverse 5′-GGTCATGAGTCCTTCCACGATACC-3′) [25].

### 2.7. Northern blot analysis

The total RNA was isolated from clinic tissues using Trizol reagent (Invitrogen). 20 μg RNA was loaded on a 15% urea–polyacrylamide gel and was electrotransferred to nylon membrane, followed by hybridization with oligonucleotide probes against miR-301a and U6. The probe sequences were used as follows: 5′-GCTTTGACAATACTATTGCACTG-3′ (miR-301a) and 5′- TGTGCTGCCGAAGCGAGCAC-3′ (U6). The signals of blots were detected by the Fujifilm LAS-4000 imaging system.

### 2.8. Luciferase reporter assay

The wide type (WT) or mutant (MT) 3′UTR of AMPKα1 was cloned into pHSA-MIR-REPORT (Ambion). The U2OS cells were co-transfected with the vectors carrying WT 3′UTR or MT 3′UTR and miR-301a or negative control using Lipofectamine 2000 reagent (Invitrogen). Transfection of pRL-SV40 plasmid (Promega) was used as a normalizing control. After 24h incubation, cells were harvested to detect the activies of luciferase using the Dual-Luciferase Assay (Promega).

### 2.9. Gene transfection

The OS cell lines HOS and U2OS were seeded in 6 well-plates. After the cell density reached to about 80% confluency, the cells were transfected with 100 nM miR-301a or anti-miR-301a using Lipofectamine 2000 (Invitrogen). After 48h incubation, the cells were harvested and rinsed with ice-cold PBS for subsequent analysis.

### 2.10. Western blot analysis

The harvested cells were lysed in RIPA lysis buffer. The supernatants were obtained by centrifugation, protein concentration of which was measured using the bicinchoninic acid method. Equal amounts of protein samples were resolved on 10% SDS polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. After blocking with 5% skim milk, the membranes were probed with anti-AMPKα1 (1:1000), anti-HMGCR (1:1000), anti-phospho-ERK1/2 (1:2000), anti-caspase 3 (1:1000) and anti-

actin (1:2000) antibodies overnight at 4 °C. After rinsing for three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using the Fujifilm LAS-4000 imaging system.

### 2.11. Immunofluorescence

The cells were grown on glass coverslips and transfected with miR-301a or negative control. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 5% BSA for 1 h at room temperature. After rinsing with TBST, the cells were incubated with AMPKα1 antibodies overnight at 4 °C followed by incubation with FITC-conjugated secondary antibody for 2 h. The images were acquired with LEICA DMIRE2 fluorescence microscope.

### 2.12. Cholesterol assay

Free cholesterol contents in OS tissues were measured using free cholesterol Assay kits according to the manufacturer's instructions (Applygen Technologies, Beijing, China).

### 2.13. Statistical analysis

All data were represented as mean ± SEM of at least three independent experiments. The comparison between two groups was analyzed by Student's t-test. The data among multiple groups were analyzed by one-way ANOVA. *P* values <0.05 were considered to be statistically significant. Statistical analysis was performed using GraphPad Software.

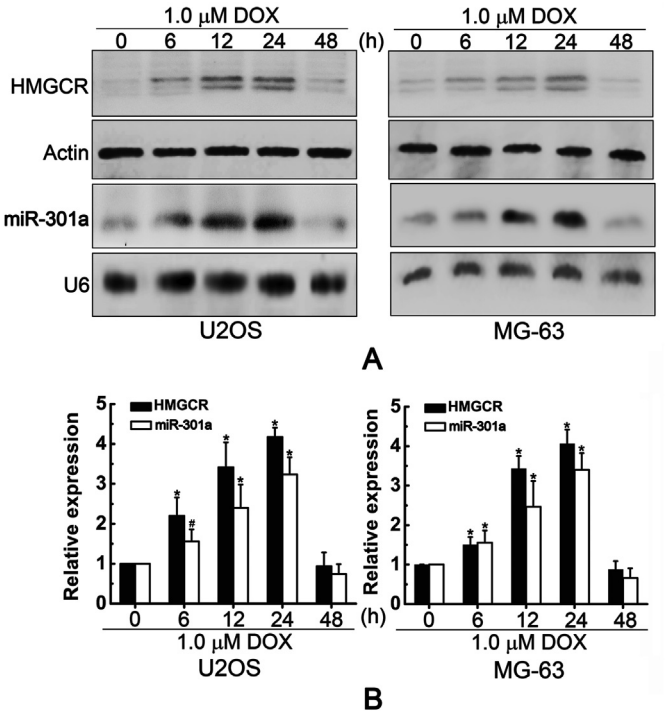


**Fig. 1.** The effect of doxorubicin on the expression of miR-301a and HMGCR in cells. A. U2OS and MG-63 cells were exposed to 1.0 μM doxorubicin for indicated time. HMGCR expression was determined by western blot analysis and miR-301a was determined by northern blot analysis. B. RT-PCR was used to determine miR-301a level and HMGCR mRNA level.

## 3. Results

### 3.1. Doxorubicin treatment promoted the expression of HMGCR and miR-301a in a time-dependent manner

We first assessed the effects of doxorubicin on the expression of miR-301a. As shown in Fig. 1A, exposing U2OS and MG-63 cells to 1.0 μM doxorubicin significantly promoted time-dependent expression of miR-301a. After treated with 1.0 μM doxorubicin for 48h, the expression of miR-301a were declined to baseline. Interestingly, doxorubicin induced HMGCR expression mimicking its effect on miR-301a. Consistently, qRT-PCR further confirmed the effect of doxorubicin on expression of HMGCR and miR-301a (Fig. 1B). These data suggested the key roles of HMGCR and miR-301a on chemoresistance of OS cells.

### 3.2. AMPKα1 was a direct target of miR-301a

To clarify the relation between HMGCR and miR-301a, we further assayed the effect of doxorubicin on the expression of AMP-activated protein kinase alpha 1 (AMPKα1), a protein phosphorylating and inactivating HMGCR [26]. As expected, doxorubicin inhibited AMPKα1 expression in a time-dependent manner (Fig. 2A). Intriguingly, AMPKα1 was predicted to be target gene of miR-301a by TargetScan (Fig. 2B). To verify the prediction, we constructed the luciferase reporter vector containing wild type or mutant 3′UTR of AMPKα1. As shown in Fig. 2C, miR-301a distinctly reduced the luciferase activity of vector carrying wild type 3′UTR of AMPKα1 compared to that carrying mutant 3′UTR of AMPKα1, suggesting the interaction between miR-301a and the 3′UTR of AMPKα1. Overexpression of miR-301a led to a dramatic decrease in AMPKα1 level, whereas anti-miR-301a promoted AMPKα1 expression in U2OS cells (Fig. 2D). Immunofluorescence analysis also validated the inhibitory effect of miR-301a on AMPKα1 expression. Taken together, these results suggested that miR-301a targeted directly AMPKα1 in U2OS cells.

### 3.3. miR-301a promoted resistance of OS cells to doxorubicin by mediating AMPKα1/HMGCR

As shown in Fig. 3A, the expression of AMPKα1 was reduced after exposing U2OS and MG-63 cells to doxorubicin, which was further attenuated by miR-301a overexpression. Accompany with decrease in AMPKα1 level, HMGCR level was increased, which was further enhanced by miR-301a. Doxorubicin treatment also promoted phosphorylation of ERK1/2, which was enhanced by miR-301a overexpression. Importantly, doxorubicin treatment promoted the cleavage of caspase 3, which was blocked by miR-301a overexpression. These results suggested that miR-301a strengthened the resistance of U2OS and MG-63 cells to doxorubicin. In contrast, anti-miR-301a increased AMPKα1 expression despite doxorubicin treatment (Fig. 3B). Correspondingly, anti-miR-301a reduced HMGCR expression, especially it reversed the HMGCR expression elevated by doxorubicin. Remarkably, anti-miR-301a promoted the cleaved caspase 3, especially it enhanced the cleaved caspase 3 induced by doxorubicin. These results indicated that inhibition of miR-301a sensitized U2OS and MG-63 cells to doxorubicin. In view of the result that miR-301a targeted AMPKα1, our data suggested that miR-301a increased HMGCR expression by suppressing AMPKα1, promoted ERK1/2 phosphorylation and blocked caspase 3 activation induced by doxorubicin, suggesting contribution of miR-301a to resistance of OS cells to doxorubicin.

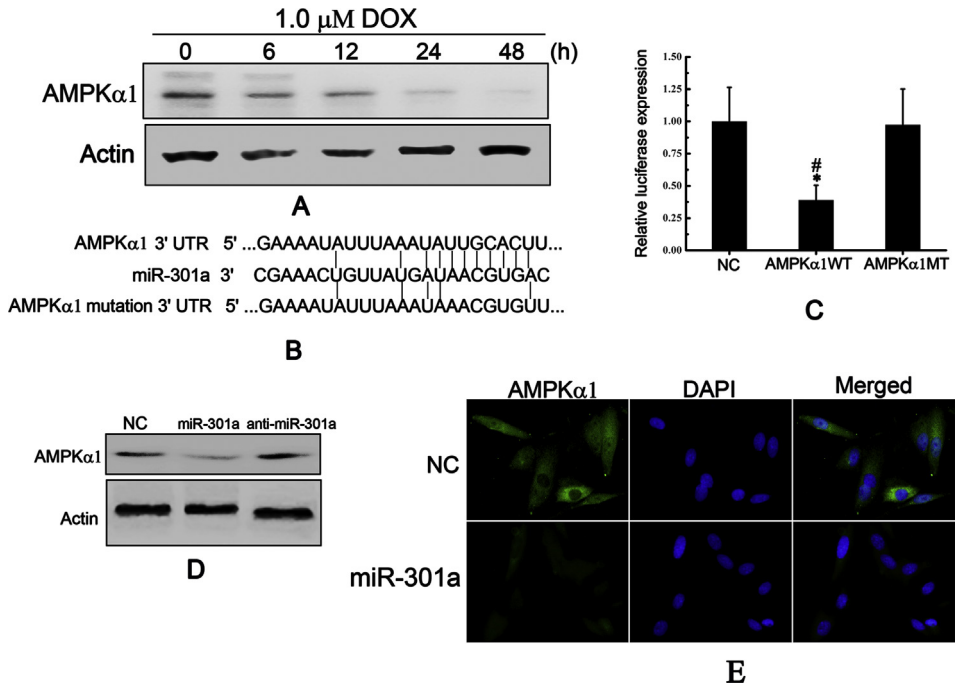


**Fig. 2.** AMPKα1 is a direct target of miR-301a in osteosarcoma cells. A. Exposing U2OS cells to 1.0 μM doxorubicin led to a time-dependent decrease in AMPKα1 level. B. Sequence of AMPKα1 3′UTR containing wide-type or mutated miR-301a binding site. C. Luciferase reporter assay in U2OS cells co-transfected with miR-301a or NC and wide-type (WT) or mutant-type (MT) AMPKα1 3′UTR. D. The effects of miR-301a and anti-miR-301a on AMPKα1 expression. E. Immunofluorescence was used to determine the effect of miR-301a on AMPKα1 expression in U2OS cells.

MTT assay showed that miR-301a overexpression significantly inhibited the cell death induced by doxorubicin, whereas anti-miR-301a facilitated doxorubicin-induced cell death (Fig. 3C). miR-301a had little effects on cell viabilities of U2OS and MG-63, even if doxorubicin was present. In contrast, anti-miR-301a led to cell death in approximately 30% and 40% in U2OS and MG-63 cells, which were raised to about 70% and 75% by combination with doxorubicin, respectively (Fig. 3C). DNA fragmentation assay validated that anti-miR-301a actuated doxorubicin-induced degradation of the genome DNA (Fig. 3D), suggesting inhibition of miR-301a promoted doxorubicin-induced cell apoptosis, which was confirmed by flow cytometry analysis (Fig. 3E). Taken together, these data suggested that miR-301a overexpression promoted resistance of OS cells to doxorubicin by inhibiting AMPKα1 and increasing HMGCR.

### *3.4. Up-regulation of miR-301a and HMGCR in chemotherapy-resistant OS*

Given that miR-301a and HMGCR played important roles in chemoresistance of OS cells, we further tested the level of miR-301a and HMGCR in chemotherapy-resistant OS. Our data showed that miR-301a was significantly elevated in specimens from OS patients with a poor chemoresponse compared to that with a good chemoresponse (Fig. 4A), which was verified by northern blot analysis (Fig. 4B). The up-regulation of HMGCR in chemotherapy-resistant OS was validated by qRT-PCR and western blot analysis (Fig. 4C and D). HMGCR has been well known as the key enzyme in cholesterol synthesis. Therefore, free cholesterol contents in OS tissues were determined using free cholesterol assay kits. The result demonstrated the higher cholesterol level in chemotherapy-resistant OS (Fig. 4E), suggesting that miR-301a might affect the cholesterol synthesis to control the sensitivity to chemotherapy agents by mediating HMGCR expression.

## 4. Discussion

Chemoresistance of osteosarcoma (OS) is the burning question, and facilitates the relapse and poor prognosis. In this study, we evidenced that doxorubicin induced time-dependent expression of miR-301a in OS cells, suggesting that miR-301a might participate in OS chemoresistance. Up-regulation of miR-301a has been found in diverse cancers, and is involved in cells proliferation and metastasis [20–22]. However, little is known about contribution of miR-301a to chemoresistance of cancers. In this investigation, our data tested the differential expression of miR-301a in chemotherapy-resistant OS and chemotherapy-sensitive OS. Our data showed up-regulation of miR-301a in chemotherapy-resistant OS, which indicated that miR-301a should be critical for chemoresistance of OS.

To explore the role of miR-301a in chemoresistance of OS, the luciferase reporter assay was performed to identify the potential target genes of miR-301a. The results showed that miR-301a directly targeted AMP-activated protein kinase alpha 1 (AMPKα1) in U2OS cells. AMPKα1 is crucial for cell metabolism by regulating cellular energy homeostasis. Because tumor cells consume numbers of energy to maintain their ongoing proliferation, AMPKα1 has been implicated in cancer development [27,28]. However, the role of AMPKα1 in cancer progression is controversial. On one hand, AMPKα1 activation inhibited the proliferation and migration of colon cancer cells and ovarian cancer cells by suppressing mTOR pathway [29,30]. On the other hand, AMPKα1 promoted the migration of chondrosarcoma cancer cells [31]. Our data showed that AMPKα1 was suppressed by doxorubicin in OS cells, consistent with the result in mouse embryonic fibroblasts [32]. Then, we hypothesized that elevated miR-301a targeted AMPKα1 expression to withstand toxicity of doxorubicin.

Our results also showed that doxorubicin stimulated HMG-CoA reductase (HMGCR) expression in a time-dependent manner.

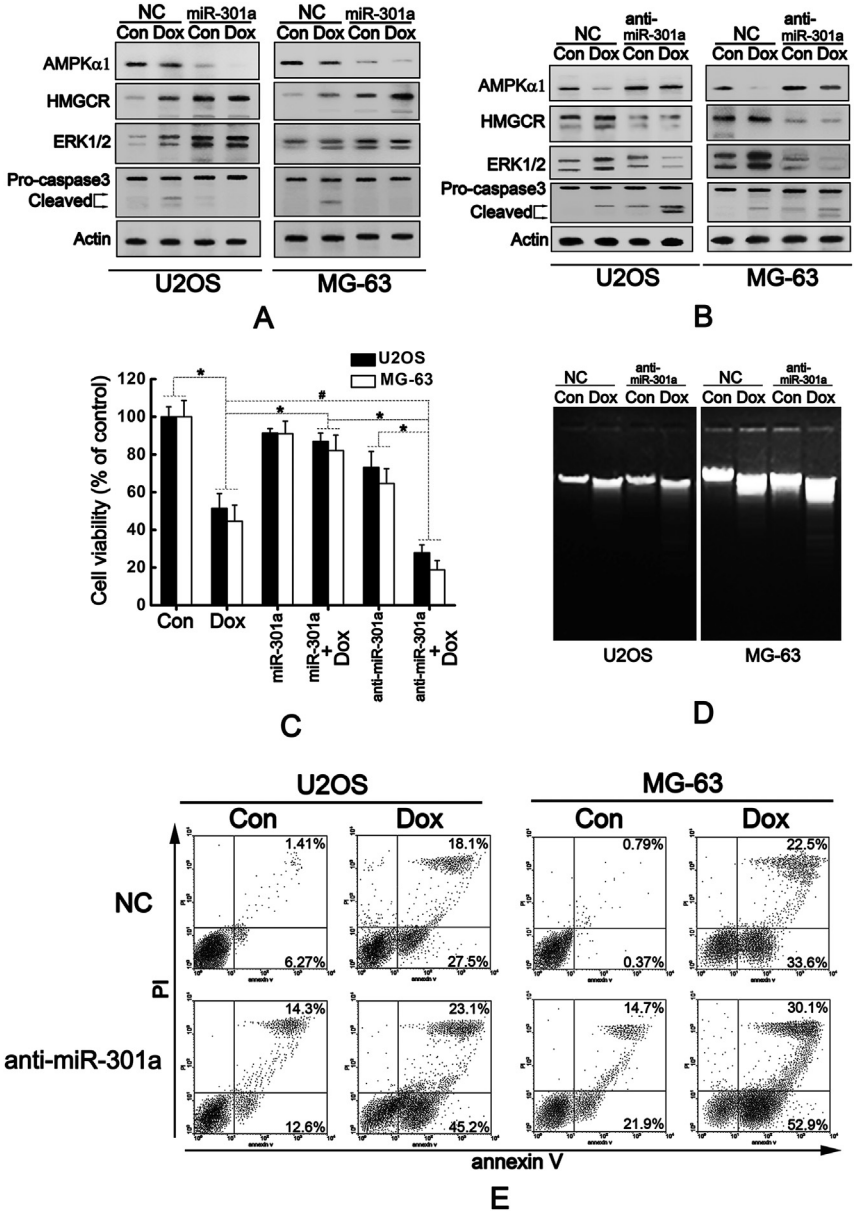


**Fig. 3.** miR-301a contributes to the resistance of OS cells to doxorubicin. A. The effect of miR-301a overexpression on the expression of AMPKα1, HMGCR, and activation of ERK1/2 and caspase 3 in U2OS and MG-63 cells treated with or without doxorubicin. B. The effect of anti-miR-301a overexpression on the expression of AMPKα1, HMGCR, and activation of ERK1/2 and caspase 3 in U2OS and MG-63 cells treated with or without doxorubicin. C. The effect of miR-301a or anti-miR-301a overexpression on cell viability of U2OS and MG-63 cells treated with or without doxorubicin. D. The profile of DNA fragmentation from U2OS and MG-63 cells transfected with anti-miR-301a in the presence or absence of doxorubicin. E. Flow cytometer analysis was used to determine the effect of anti-miR-301a overexpression on apoptosis of U2OS and MG-63 cells treated with or without doxorubicin.

HMGCR acts as a key enzyme in cholesterol synthesis. Cholesterol accumulation has been found in various cancers, and depletion of membrane cholesterol contributes to attenuate tumors chemoresistance [33,34]. Given that AMPKα1 phosphorylates and inactivates HMGCR [26], it was likely that miR-301a promoted HMGCR expression by suppressing AMPKα1, which might explain doxorubicin-induced the parallel up-regulation of miR-301a and HMGCR. Then, it was necessary to clarify whether miR-301a invigorated HMGCR expression to protect OS cells from doxorubicin-induced death by inhibiting AMPKα1. Thus, we next examined the effects of miR-301a or anti-miR-301a on OS cells death in the presence or absence of doxorubicin. The data showed that miR-301a significantly prevented cells from doxorubicin-induced cell death, whereas anti-miR-301a overexpression facilitated doxorubicin-induced cell death, suggesting that up-regulation of miR-301a awarded OS cells for resistance to doxorubicin. Following this finding, we further observed that doxorubicin treatment reduced AMPKα1 level, enhanced HMGCR expression, and activated ERK1/2, which were further strengthened by miR-301a overexpression while were reversed by anti-miR-301a overexpression. Notably, miR-301a attenuated doxorubicin-induced caspase 3 cleavage whereas anti-miR-301a accentuated doxorubicin-induced caspase 3 cleavage. DNA fragmentation assay and flow cytometer analysis demonstrated that anti-miR-301a accelerated doxorubicin-induced cell death. These results strongly suggested that up-regulation of miR-301a promoted HMGCR expression by suppressing AMPKα1, leading to inhibit doxorubicin-induced cell death. Several studies have demonstrated that statins

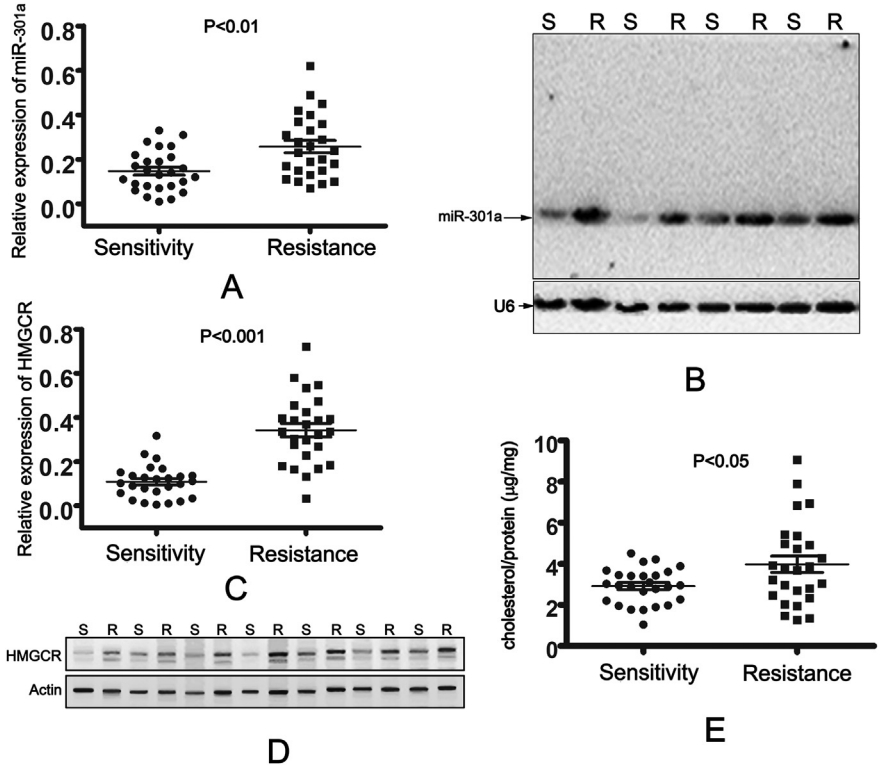


**Fig. 4.** Differential analysis of miR-301a, HMGCR and free cholesterol in chemotherapy-resistant and chemotherapy-sensitive OS tissues. A and B. Analysis of miR-301a in chemotherapy-resistant and chemotherapy-sensitive OS tissues by RT-PCR and Northern blot. C and D. Analysis of HMGCR in chemotherapy-resistant and chemotherapy-sensitive OS tissues by RT-PCR and Western blot. E. The relative levels of free cholesterol in chemotherapy-resistant and chemotherapy-sensitive OS tissues.

sensitize tumor cells to chemotherapy-induced cell death by inhibiting HMGCR expression [35,36], suggesting the key role of HMGCR in chemoresistance of tumors. Therefore, it was reasonable that elevated HMGCR level contributed to resistance of OS cells to doxorubicin.

Furthermore, we tested the differential expression of HMGCR in chemotherapy-resistant OS and chemotherapy-sensitive OS. The results showed up-regulation of HMGCR in chemotherapy-resistant OS. Considering that HMGCR mediates cholesterol synthesis, we further measured the differential level of free cholesterol in chemotherapy-resistant OS and chemotherapy-sensitive OS. We found an elevated free cholesterol level in chemotherapy-resistant OS. Cholesterol accumulation has been ascertained in several solid tumors, including prostate cancer and oral cancer [33,34]. Dysregulated cholesterol metabolism is due to loss of cholesterol feedback. Thus, HMGCR is up-regulated in many malignancies despite elevated cholesterol level [37]. The role of cholesterol in multidrug resistance has been a focus of recent attentions. Cholesterol is a key constituent of lipid raft that regulates multiple signal pathways, including caveolins and P-glycoprotein. These pathways are utilized to extrude the cytotoxic drugs, leading to chemoresistance of cells [38]. Therefore, up-regulation of cholesterol contributes to multidrug resistance of tumor cells. Consistent with these data, our results revealed that HMGCR expression was increased in chemotherapy-resistant OS accompanied with elevated cholesterol level, which should facilitate chemoresistance of OS.

In summary, we evidenced that miR-301a promoted HMGCR expression by targeting AMPK$\alpha$1, and enhanced resistance of OS cells to doxorubicin. Moreover, we demonstrated up-regulation of miR-301a and HMGCR in chemotherapy-resistant OS compared to chemotherapy-sensitive OS. Our data suggested that miR-301a might be a potential biomarker for chemotherapy-resistant OS and miR-301a attenuation contributed to overcome drug resistance of OS.

## Conflict of interest

None.

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.101.

## References

[1] L. Endo-Munoz, A. Cumming, S. Sommerville, I. Dickinson, N.A. Saunders, Osteosarcoma is characterised by reduced expression of markers of osteoclastogenesis and antigen presentation compared with normal bone, Br. J. Cancer 103 (2010) 73–81.
[2] A.J. Chou, R. Gorlick, Chemotherapy resistance in osteosarcoma: current challenges and future directions, Expert Rev. Anticancer Ther. 6 (2006) 1075–1085.
[3] J. Huang, J. Ni, K. Liu, et al., HMGB1 promotes drug resistance in osteosarcoma, Cancer Res. 72 (2012) 230–238.
[4] A. McTiernan, R.C. Jinks, M.R. Sydes, et al., Presence of chemotherapy-induced toxicity predicts improved survival in patients with localised extremity osteosarcoma treated with doxorubicin and cisplatin: a report from the European Osteosarcoma Intergroup, Eur. J. Cancer 48 (2012) 703–712.

[5] V. Saini, C.D. Hose, A. Monks, et al., Identification of CBX3 and ABCA5 as putative biomarkers for tumor stem cells in osteosarcoma, PLoS One 7 (2012) e41401.
[6] G. Moriceau, A.J. Roelofs, R. Brion, et al., Synergistic inhibitory effect of apomine and lovastatin on osteosarcoma cell growth, Cancer 118 (2012) 750–760.
[7] Y. Ma, Y. Ren, E.Q. Han, et al., Inhibition of the Wnt-β-catenin and Notch signaling pathways sensitizes osteosarcoma cells to chemotherapy, Biochem. Biophys. Res. Commun. 431 (2013) 274–279.
[8] E. Kobayashi, F.J. Hornicek, Z. Duan, MicroRNA involvement in osteosarcoma, Sarcoma 2012 (2012) 359739.
[9] C.M. Croce, Causes and consequences of microRNA dysregulation in cancer, Nat. Rev. Genet. 10 (2009) 704–714.
[10] S. Griffiths-Jones, H.K. Saini, S. van Dongen, A.J. Enright, miRBase: tools for microRNA genomics, Nucleic Acids Res. 36 (2008) 154–158.
[11] A. Markou, I. Sourvinou, P.A. Vorkas, G.M. Yousef, E. Lianidou, Clinical evaluation of microRNA expression profiling in non small cell lung cancer, Lung Cancer 81 (2013) 388–396.
[12] A.C. Godfrey, Z. Xu, C.R. Weinberg, et al., Serum microRNA expression as an early marker for breast cancer risk in prospectively collected samples from the sister study cohort, Breast Cancer Res. 15 (2013) R42.
[13] A. Schaefer, M. Jung, H.J. Mollenkopf, et al., Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma, Int. J. Cancer 126 (2010) 1166–1176.
[14] J. Guo, Y. Miao, B. Xiao, et al., Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues, J. Gastroenterol. Hepatol. 24 (2009) 652–657.
[15] N.A. Schultz, J. Werner, H. Willenbrock, et al., MicroRNA expression profiles associated with pancreatic adenocarcinoma and ampullary adenocarcinoma, Mod. Pathol. 25 (2012) 1609–1622.
[16] H.M. Namløs, L.A. Meza-Zepeda, T. Barøy, I.H. Østensen, et al., Modulation of the osteosarcoma expression phenotype by microRNAs, PLoS One 7 (2012) e48086.
[17] Y. Zhou, Z. Huang, S. Wu, X. Zang, M. Liu, J. Shi, miR-33a is up-regulated in chemoresistant osteosarcoma and promotes osteosarcoma cell resistance to cisplatin by down-regulating TWIST, J. Exp. Clin. Cancer Res. 33 (2014) 12.
[18] B. Song, Y. Wang, Y. Xi, et al., Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells, Oncogene 28 (2009) 4065–4074.
[19] B. Song, Y. Wang, M.A. Titmus, et al., Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells, Mol. Cancer 9 (2010) 96.
[20] H. Yu, H. Li, H. Qian, et al., Upregulation of miR-301a correlates with poor prognosis in triple-negative breast cancer, Med. Oncol. 31 (2014) 283.
[21] F. Ma, J. Zhang, L. Zhong, et al., Upregulated microRNA-301a in breast cancer promotes tumor metastasis by targeting PTEN and activating Wnt/β-catenin signaling, Gene 535 (2014) 191–197.
[22] M. Wang, C. Li, B. Yu, et al., Overexpressed miR-301a promotes cell proliferation and invasion by targeting RUNX3 in gastric cancer, J. Gastroenterol. 48 (2013) 1023–1033.
[23] J. Liu, G. Xian, M. Li, et al., Cholesterol oxidase from Bordetella species promotes irreversible cell apoptosis in lung adenocarcinoma by cholesterol oxidation, Cell. Death Dis. 5 (2014) e1372.
[24] R. Crespo, S. Montero Villegas, M.C. Abba, M.G. de Bravo, M.P. Polo, Transcriptional and posttranscriptional inhibition of HMGCR and PC biosynthesis by geraniol in Hep-G2 cell proliferation linked pathways, Biochem. Cell. Biol. 91 (2013) 131–139.
[25] Y. Liu, L. Zhao, Y. Ju, et al., A novel androstenedione derivative induces ROS-mediated autophagy and attenuates drugresistance in osteosarcoma by inhibiting macrophage migration inhibitory factor (MIF), Cell. Death Dis. 5 (2014) e1361.
[26] P.R. Clarke, D.G. Hardie, Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase in vitro and in intact rat liver, EMBO J. 9 (1990) 2439–2446.
[27] M. Bhandaru, M. Martinka, G. Li, A. Rotte, Loss of AMPKα1 expression is associated with poor survival in melanoma patients, J. Invest Dermatol 134 (2014) 1763–1766.
[28] J.J. Park, S.M. Seo, E.J. Kim, et al., Berberine inhibits human colon cancer cell migration via AMP-activated protein kinase-mediated downregulation of integrin β1 signaling, Biochem. Biophys. Res. Commun. 426 (2012) 461–467.
[29] Y.K. Lee, S.Y. Park, Y.M. Kim, et al., Suppression of mTOR via Akt-dependent and -independent mechanisms in selenium-treated colon cancer cells: involvement of AMPKalpha1, Carcinogenesis 31 (2010) 1092–1099.
[30] Y.K. Lee, W.S. Lee, G.S. Kim, O.J. Park, Anthocyanins are novel AMPKα1 stimulators that suppress tumor growth by inhibiting mTOR phosphorylation, Oncol. Rep. 24 (2010) 1471–1477.
[31] Y.C. Chiu, D.C. Shieh, K.M. Tong, et al., Involvement of AdipoR receptor in adiponectin-induced motility and alpha2beta1 integrin upregulation in humanchondrosarcoma cells, Carcinogenesis 30 (2009) 1651–1659.
[32] S. Wang, P. Song, M.H. Zou, Inhibition of AMP-activated protein kinase α (AMPKα) by doxorubicin accentuates genotoxic stress and cell death in mouse embryonic fibroblasts and cardiomyocytes: role of p53 and SIRT1, J. Biol. Chem. 287 (2012) 8001–8012.
[33] Y.C. Li, M.J. Park, S.K. Ye, C.W. Kim, Y.N. Kim, Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents, Am. J. Pathol. 168 (2006) 1107–1118.
[34] L. Zhuang, J. Kim, R.M. Adam, K.R. Solomon, M.R. Freeman, Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts, J. Clin. Invest 115 (2005) 959–968.
[35] Z. Cao, H. Fan-Minogue, D.I. Bellovin, et al., MYC phosphorylation, activation, and tumorigenic potential in hepatocellular carcinoma are regulated by HMG-CoA reductase, Cancer Res. 71 (2011) 2286–2297.
[36] K.K. Chan, A.M. Oza, L.L. Siu, The statins as anticancer agents, Clin. Cancer Res. 9 (2003) 10–19.
[37] M.D. Siperstein, Cholesterol, cholesterogenesis and cancer, Adv. Exp. Med. Biol. 369 (1995) 155–166.
[38] M. Liscovitch, Y. Lavie, Multidrug resistance: a role for cholesterol efflux pathways? Trends Biochem. Sci. 25 (2000) 530–534.