Circ_0002060 enhances doxorubicin resistance in osteosarcoma by regulating miR-198/ABCB1 axis

CURRENT STATUS: UNDER REVIEW

World Journal of Surgical Oncology BMC

Jun Liu
Weifang People's Hospital

Wenshuai Zhu
Weifang People's Hospital

Jianqin Ji
Weifang People's Hospital

ylua9ap@163.com*Corresponding Author*

DOI:
10.21203/rs.3.rs-16003/v1

SUBJECT AREAS
Oncology

KEYWORDS
Osteosarcoma, circ_0002060, miR-198, ABCB1, doxorubicin

Abstract

Background Osteosarcoma (OS) is a common aggressive primary sarcoma of bone. Drug resistance is a huge obstacle to chemotherapy for cancer. This study aimed to investigate the role and mechanism of circ_0002060 in OS resistance to doxorubicin (DOX). Methods The levels of circ_0002060, miR-198 and ATP binding cassette subfamily B member 1 (ABCB1) were measured by quantitative real-time polymerase chain reaction or western blot assay. Kaplan-Meier analysis was performed to determine the relationship between circ_0002060 expression and overall survival. The half inhibition concentration (IC50) of doxorubicin was calculated by Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was assessed by colony formation assay. Cell apoptosis was monitored by flow cytometry. The levels of apoptosis-related proteins were measured by western blot assay. Xenograft assay was utilized to analyze the effect of circ_0002060 on DOX resistance in vivo . The interaction among circ_0002060, miR-198 and ABCB1 were confirmed by dual-luciferase reporter assay, RNA immunoprecipitation assay or RNA pull-down assay. Results Circ_0002060 and ABCB1 were up-regulated, while miR-198 was down-regulated in OS tissues and DOX-resistant OS cells. Circ_0002060 silence reduced DOX resistance in vitro and in vivo . Moreover, circ_0002060 enhanced DOX resistance via sponging miR-198. Besides, miR-198 decreased DOX resistance by binding to ABCB1. In addition, circ_0002060 sponged miR-198 to up-regulate ABCB1 expression. Conclusion Circ_0002060 enhanced doxorubicin resistance of OS by regulating miR-198/ABCB1 axis, which provides potential therapeutic targets for OS therapy.

Background

Osteosarcoma (OS) is a common malignant bone cancer that occurs in children and adolescents [1]. With the development of treatment technology, OS therapy is not limited

to surgical resection. The introduction of chemotherapy improves the prognosis of OS patients, resulting in a 5-year survival rate of 70% [2]. Therefore, the use of chemical drugs to improve the prognosis of OS patients is promising.

Circular RNAs (circRNAs) are a new type of non-coding RNAs that can modulate tumorigenesis through a competing endogenous RNA mechanism (ceRNA) [3]. Increasing studies have unveiled that several circRNAs that are abnormally expressed in OS are closely related to OS pathogenesis [4]. For instance, circ-0000285 was highly expressed in OS and facilitated OS development by targeting microRNA-599 and increasing TGFB2 expression [5]. CircMMP9 sponged microRNA-1265 to up-regulate CHI3L1, thus accelerating OS progression [6]. In osteoporosis, circ_0002060, a transcript of dynein cytoplasmic 1 heavy chain 1 (DYNC1H1), was significantly elevated and might be a potential therapeutic target [7]. Therefore, circ_0002060 expression was analyzed in OS, and we first found that circ_0002060 was markedly up-regulated, hinting its potential role in OS development.

Moreover, many microRNAs (miRNAs) have been identified as playing a vital role in OS progression [8]. For example, miR-150 exerted an anti-cancer effect on OS progression and enhanced the sensitivity of OS cells to doxorubicin via regulating RUNX2 [9]. Dong et al. presented that miR-193b could sensitize OS cells to epirubicin via modulating FEN1-induced autophagy [10]. Furthermore, previous research discovered that miR-198 was an underlying molecular target for OS treatment [11]. Bioinformatics analysis showed that miR-198 and circ_0002060 have complementary binding sites, so it is speculated that circ_0002060 may interact with miR-198 in OS.

ATP binding cassette subfamily B member 1 (ABCB1) is strongly related to multidrug resistance [12]. In breast cancer, miR-381 targeted ABCB1 to reduce cisplatin resistance [13]. In osteosarcoma, TIPE2 strengthened cisplatin sensitivity by decreasing ABCB1

expression [14]. However, the mechanism of miR-198 and ABCB1 in doxorubicin resistance of OS is still barely reported.

Hence, we further investigated the function and potential mechanism of circ_0002060, miR-198 and ABCB1 in doxorubicin resistance and tumor progression in OS.

Materials And Methods

Clinical samples

Forty OS tissues and adjacent normal tissues were obtained from OS patients who received surgery at Weifang People's Hospital. This research was ratified by the Ethics Committee of Weifang People's Hospital. All participants signed written informed consent. All tissues were immediately frozen in liquid nitrogen and then stored at -80°C.

Cell culture

Two OS cell lines (U2OS and HOS) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). DOX-resistant cell lines (U2OS/DOX and HOS/DOX) were produced by U2OS and HOS cells exposed to gradient doses of doxorubicin (DOX) (Solarbio, Beijing, China). All cells were incubated in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco).

Cell transfection

Small interfering RNA (siRNA) against circ_0002060 (si-circ_0002060#1, si-circ_0002060#2 and si-circ_0002060#3), the siRNA control (si-NC), miR-198 mimics (miR-198), the mimics control (miR-NC), circ_0002060 overexpression vector (circ_0002060), the empty vector (pCD-ciR), miR-198 inhibitor (anti-miR-198), the inhibitor control (anti-miR-NC), ABCB1 overexpression vector (ABCB1) and the empty vector (pcDNA) were commercially obtained from GenePharma (Shanghai, China). The vectors and oligonucleotides were transfected into U2OS/DOX and HOS/DOX cells using Lipofectamine

3000 (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

After extracting RNA with Trizol (Invitrogen), RNA was reversely transcribed using HiScript II One Step RT-PCR Kit (Vazyme, Nanjing, China) or miRNA 1st Strand cDNA Synthesis Kit (Vazyme). Then, qRT-PCR was carried out using AceQ qPCR SYBR Green Master Mix (Vazyme). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were taken as internal controls. Primers were shown below: miR-198-F: 5'-GGTCCAGAGGGGAGAT-3', miR-198-R: 5'-GAATACCTCGGACCCTGC-3'; ABCB1-F: 5'-CCCATCATTGCAATAGCAGG-3', ABCB1-R: 5'-GTTCAAACTTCTGCTCCTGA-3'; GAPDH-F: 5'-TGCACCACCAACTGCTTAGC-3', GAPDH-R: 5'-GGCATGGACTGTGGTCATGAG-3'; U6-F: 5'-CTCGCTTCGGCAGCACA-3', U6-R: 5'-AACGCTTCACGAATTTGCGT-3'; 18sRNA-F: 5'-AAACGGCTACCACATCCA-3', 18sRNA-R: 5'-CACCAGACTTGCCCCTCCA-3'. The primers of circ_0002060 were purchased from GenePharma.

Cell Counting Kit-8 (CCK-8) assay

Cells (3×10^3) were plated into 96-well plates and incubated with escalating doses of DOX for indicated time. Subsequently, cells were reacted with 10 μL CCK-8 solution (Solarbio) for 2 h. Next, a Multi-Mode Reader (BioTek, Burlington, VT, USA) was used to measure the optical density at 450 nm to assess cell viability. The half-maximal inhibitory concentration (IC50) of DOX is the concentration at which cell viability is reduced to 50%.

Colony formation assay

The treated cells were seeded into six-well plates and cultured in DMEM medium containing 10% FBS at 37°C for 14 days. After staining with 0.5% crystal violet, colonies were imaged and counted at least three times.

Flow cytometry

The treated cells were inoculated into six-well plates and washed with cold PBS. Then,

AnnexinV-fluorescein isothiocyanate (AnnexinV-FITC)/Propidium Iodide (PI) Apoptosis Detection kit (Invitrogen) was used to detect cell apoptosis. Final, Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to monitor the apoptosis rate.

Western blot assay

After lysing cells with RIPA buffer (Solarbio), the extracted proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were probed with primary antibodies against caspase 3 (ab13847, Abcam, Cambridge, UK), B-cell lymphoma 2 (Bcl-2) (ab196495, Abcam), Bcl-2 associated X protein (Bax) (ab53154, Abcam), ABCB1 (ab129450, Abcam) and GAPDH (ab9485, Abcam). Next, the membranes interacted with secondary antibody (ab7090, Abcam). The signal intensity was measured using the enhanced chemiluminescence system (Millipore).

Xenograft tumor experiment

U2OS/DOX cells were transfected with the lentivirus carrying circ_0002060 short hairpin RNA (sh-circ_0002060) or the negative control (sh-NC). Subsequently, stably transfected cells (5×10^6) were subcutaneously injected into the right-back of BALB/c nude mice (n=6 per group). Then, DOX (15 mg/kg) was intravenously administered to nude mice twice a week. Tumor volume was monitored every week. After five weeks, the mice were sacrificed, and xenografts were weighted. The xenograft assay was ratified by the Animal Research Committee of Weifang People's Hospital.

Dual-luciferase reporter assay

The sequences of circ_0002060 or ABCB1 3'UTR containing miR-198 binding sites or mutant were amplified and then inserted into pmirGLO vector (Promega, Madison, WI, USA), named circ_0002060-wt, circ_0002060-mut, ABCB1 3'UTR-wt or ABCB1 3'UTR-mut

reporter. Then, the corresponding luciferase reporter and miR-198 or miR-NC were co-transfected into U2OS/DOX and HOS/DOX. Finally, the luciferase intensity was monitored using a Dual-Luciferase Reporter Assay Kit (Promega).

RNA immunoprecipitation (RIP) assay

RIP analysis was carried out using EZ-Magna RIP kit (Millipore). Briefly, U2OS/DOX and HOS/DOX cells were lysed by RIP lysis buffer. Then, cell lysates were incubated with magnetic beads conjugated with Ago2 antibody or IgG antibody. The enrichment of circ_0002060, miR-198 or ABCB1 was measured by qRT-PCR.

RNA pull-down assay

Biotin-labeled circ_0002060 probe (Bio-circ_0002060) and the control probe (Bio-NC) were purchased from GenePharma. Briefly, biotinylated probes were reacted with M-280 Streptavidin Dynabeads (Invitrogen) at 37°C for 2 h to construct probe-coated beads. Next, the cells were lysed and incubated with probe-coated beads at 4°C for 3 h. Finally, the expression of miR-198 was measured by qRT-PCR.

Statistical analysis

All data were exhibited as mean ± standard deviation using Graphpad Prism 7.0 software (GraphPad, San Diego, CA, USA). Differences were tested by Student's *t*-test or one-way analysis of variance. When $P < 0.05$, the difference was considered statistically significant.

Results

Circ_0002060 was up-regulated in OS tissues and DOX-resistant OS cells

First, we detected the expression of circ_0002060 in OS tissues and adjacent normal tissues, and the results exhibited that circ_0002060 expression in OS tissues was strikingly increased compared with non-cancer tissues (Fig. 1A). Besides, Kaplan-Meier analysis was performed to explore the relationship between circ_0002060 expression and overall survival in OS patients. The results showed that high circ_0002060 was closely

related to poor prognosis in OS patients (Fig. 1B). Also, the expression of circ_0002060 in U2OS/DOX and HOS/DOX cells was overtly higher than that in U2OS and HOS cells (Fig. 1C). Moreover, circ_0002060 was resistant to RNase R compared to its linear isoform DYNC1H1 (Fig. 1D and 1E). In addition, DOX-resistant cells were treated with Actinomycin D, and qRT-PCR showed that circ_0002060 had a half-life of more than 24 h, while DYNC1H1 mRNA had a half-life of less than 6 h (Fig. 1F and 1G). These data revealed that circ_0002060 might be an oncogene in OS.

Circ_0002060 knockdown decreased DOX resistance *in vitro* and *in vivo*

In order to explore the function of circ_0002060 in OS cells, circ_0002060 was silenced by transfecting si-circ_0002060#1, si-circ_0002060#2 or si-circ_0002060#3 into U2OS/DOX and HOS/DOX cells. The knockdown efficiency of circ_0002060 was detected by qRT-PCR analysis, and the expression of DYNC1H1 was not affected (Fig. 2A and 2B). CCK-8 assay showed that down-regulation of circ_0002060 reduced the IC50 value of DOX in U2OS/DOX and HOS/DOX cells (Fig. 2C and 2D). Colony formation assay demonstrated that circ_0002060 silencing and DOX treatment significantly decreased the proliferation ability of U2OS/DOX and HOS/DOX cells (Fig. 2E and 2F). Additionally, flow cytometry revealed that circ_0002060 knockdown and DOX stimulation markedly promoted the apoptosis of DOX-resistant OS cells (Fig. 2G-2I). Consistently, circ_0002060 silencing and DOX stimulation resulted in a striking reduction in Bcl-2 expression and a significant increase in c-caspaes 3/caspaes 3 and Bax levels (Fig. 2J and 2K). Moreover, a xenograft mouse model was established to investigate the effect of circ_0002060 on DOX sensitivity *in vivo*. As shown in Fig. 2L and 2M, tumor volume and weight were observably reduced in sh-circ_0002060 group compared with sh-NC group, and DOX treatment enhanced this effect. These results unveiled that silencing of circ_0002060 reduced DOX resistance *in vitro* and *in vivo*.

Circ_0002060 directly targeted miR-198

Next, the Circular RNA Interactome online database determined that circ_0002060 and miR-198 had complementary sequences (Fig. 3A). Besides, the expression of miR-198 in OS tissues was significantly lower than that in normal tissues (Fig. 3B). In OS tissues, circ_0002060 expression was inversely correlated with miR-198 expression (Fig. 3C). First, dual-luciferase reporter assay suggested that miR-198 mimics inhibited the luciferase activity of circ_0002060-wt reporter (Fig. 3D and 3E). Then, the relationship between circ_0002060 and miR-198 was verified by RIP and RNA pull-down assays. As shown in Fig. 3F-3G, circ_0002060 and miR-198 were markedly enriched in Ago2 group compared to lgG group. Additionally, RNA pull-down assay revealed that miR-198 could be pulled down by Bio-circ_0002060, but not by Bio-NC (Fig. 3H). Compared with U2OS and HOS cells, miR-198 level in U2OS/DOX and HOS/DOX cells was significantly reduced (Fig. 3I). In addition, circ_0002060 silencing strikingly elevated miR-198 expression, and circ_0002060 overexpression markedly decreased miR-198 expression (Fig. 3J and 3K). These data indicated that circ_0002060 was a sponge of miR-198.

Circ_0002060 regulated DOX resistance by sponging miR-198

To investigate the roles of circ_0002060 and miR-198 in DOX resistance, U2OS/DOX and HOS/DOX cells were transfected with si-NC, si-circ_0002060#1, si-circ_0002060#1+anti-miR-NC or si-circ_0002060#1+anti-miR-198. The results showed that the addition of anti-miR-198 reversed the increase of miR-198 expression caused by circ_0002060 silencing (Fig. 4A). CCK-8 analysis revealed that inhibition of miR-198 eliminated the reduction in DOX resistance induced by circ_0002060 knockdown (Fig. 4B-4E). Colony formation assay exhibited that down-regulation of miR-198 partially attenuated the reduction in colony number caused by DOX treatment or circ_0002060 silencing (Fig. 4F and 4G). In addition, transfection with miR-198 partially reversed the effect of DOX stimulation or circ_0002060

depletion on cell apoptosis (Fig. 4H and 4I). Consistently, DOX treatment and circ_0002060 silencing increased the levels of c-caspase 3/caspase 3 and Bax, and decreased the level of Bcl-2, while miR-198 knockdown partially abolished the effects (Fig. 4J-4M). These data indicated that circ_0002060 modulated DOX resistance by sponging miR-198 in DOX-resistant OS cells.

ABCB1 was a target of miR-198

Further, TargetScan online database predicted that miR-198 and ABCB1 3'UTR had putative binding sites (Fig. 5A). First, qRT-PCR and western blot assays suggested that the mRNA and protein levels of ABCB1 were remarkably increased in OS tissues relative to normal tissues (Fig. 5B and 5C). Also, miR-198 and ABCB1 levels were negatively correlated in OS tissues (Fig. 5D). Then, dual-luciferase reporter assay demonstrated that mature miR-198 reduced the luciferase activity of ABCB1 3'UTR-wt reporter (Fig. 5E and 5F). RIP assay was performed to validate the interaction between miR-198 and ABCB1, and the results showed that miR-198 and ABCB1 were significantly enriched in Ago2 group compared with lgG group (Fig. 5G and 5H). Compared with U2OS and HOS cells, the mRNA and protein levels of ABCB1 in U2OS/DOX and HOS/DOX cells were significantly up-regulated (Fig. 5I and 5J). Besides, miR-198 mimics suppressed ABCB1 mRNA and protein expression, while miR-198 knockdown has the opposite effect (Fig. 5K-5N). These data unveiled that ABCB1 was a target of miR-198.

MiR-198 reduced DOX resistance via targeting ABCB1

To explore the relationship between miR-198 and ABCB1 in DOX resistance, U2OS/DOX and HOS/DOX cells were introduced with miR-NC, miR-198, miR-198+pcDNA or miR-198+ABCB1. First, transfection with ABCB1 reversed the decrease in ABCB1 expression caused by miR-198 overexpression (Fig. 6A-6C). In addition, up-regulation of miR-198 decreased the IC50 value of DOX, whereas the impact was abolished after transfection

with ABCB1 (Fig. 6D-6G). Colony formation assay revealed that overexpression of ABCB1 partially abrogated the decrease in colony number induced by DOX stimulation or miR-198 up-regulation (Fig. 6H and 6I). Additionally, introduction of ABCB1 partially rescued the promoting effect of DOX treatment or miR-198 overexpression on cell apoptosis (Fig. 6J and 6K). Similarly, DOX stimulation and miR-198 up-regulation resulted in increased c-caspase 3/caspase 3 and Bax expression, and decreased Bcl-2 expression, while these changes were partially reversed by overexpressing ABCB1 (Fig. 6L-6O). These data evidenced that miR-198 decreased DOX resistance via targeting ABCB1 in U2OS/DOX and HOS/DOX cells.

Circ_0002060 modulated ABCB1 expression by regulating miR-198

Firstly, Spearman's correlation analysis illustrated that circ_0002060 and ABCB1 levels were positively correlated in OS tissues (Fig. 7A). Then, in order to elucidate the regulatory mechanism of circ_0002060, miR-198 and ABCB1, U2OS/DOX and HOS/DOX cells were transduced with si-NC, si-circ_0002060#1, si-circ_0002060#1+anti-miR-NC or si-circ_0002060#1+anti-miR-198. As displayed in Fig. 7B-7D, inhibition of miR-198 reversed the decrease of ABCB1 expression caused by circ_0002060 knockdown. These results concluded that circ_0002060 silencing reduced ABCB1 expression via sponging miR-198.

Discussion

Recently, the occurrence of chemoresistance has become a major stumbling block in OS treatment [15]. Besides, adriamycin has significant medicinal properties in adjuvant chemotherapy of localized high-grade OS [16]. Additionally, large amounts of abnormally expressed non-coding RNAs (ncRNAs) play a crucial role in anti-tumor drug resistance [17]. Therefore, we strengthened the study of circRNA in doxorubicin resistance of OS. Accumulating evidence demonstrated that circRNAs could mediate the expression of

target mRNAs by serving as ceRNAs or miRNA sponges, thereby regulating chemotherapy resistance [18]. For example, circKDM4C potentiated doxorubicin sensitivity and hindered tumorigenesis in breast cancer via sponging miR-548p and up-regulating PBLD [19]. Additionally, Hu *et al*. presented that circ-LARP4 attenuated cisplatin and doxorubicin resistance in OS by binding to miR-424 [20]. This research demonstrated that circ_0002060 was remarkably up-regulated in OS tissues and DOX-resistant OS cells, and confirmed that circ_0002060 silencing reduced DOX resistance in OS. Further, we disclosed the negative regulatory and binding relationship between circ_0002060 and miR-198.

More and more researches corroborated that miR-198 was an inhibitor of various tumors, including gastric cancer [21], colorectal cancer [22] and breast cancer [23]. Furthermore, the mining of miRNAs provides a new pathway for early diagnosis of OS. Zhang *et al*. revealed that miR-198 functioned as a potential target for OS treatment by regulating ROCK1 [11]. Also, several studies demonstrated that miRNAs could regulate chemotherapy resistance of OS by mediating multiple potential molecular mechanisms such as DNA damage response, autophagy induction, and signal transduction [24]. Chen *et al*. found that miR-198 weakened temozolomide resistance via modulating MGMT in glioblastoma [25]. Huang *et al*. discovered that miR-198 reduced the increase of cisplatin resistance caused by circAKT3 by targeting PIK3R1 in gastric cancer [26]. Nevertheless, the effect of miR-198 on DOX resistance of OS remains unclear. In this study, we evidenced that miR-198 expression was remarkably decreased, and circ_0002060 sponged miR-198 to enhance DOX resistance in OS.

Meanwhile, the existence of multidrug resistance (MDR) has made tumor treatment more difficult [27]. ABCB1 (also known as MDR1) is considered to be the main factor inducing MDR [28]. In hepatocellular carcinoma, miR-122 improved oxaliplatin sensitivity by

repressing ABCB1 expression to inactivate Wnt/β-catenin pathway [29]. In colorectal cancer, overexpression of ABCB1 attenuated oxaliplatin sensitivity, which might be regulated by miR-302c-5p [30]. Also, many studies discovered that ABCB1 was remarkably elevated in OS [31], and ABCB1 inhibitor undermined OS resistance to doxorubicin and paclitaxel [32]. We first unveiled that ABCB1 was a target of miR-198 in DOX-resistant OS cells.

Conclusion

Circ_0002060 potentiated doxorubicin resistance and facilitated tumor progression in OS via modulating miR-198/ABCB1 axis. Our research found a novel molecular mechanism for improving OS chemotherapy.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Weifang People’s Hospital and written informed consents were collected from all patients and hospitals.

The animal experiment was permitted by the Animal Research Committee of Weifang People’s Hospital

and performed in accordance with the guidelines of the National Animal Care and Ethics Instituti

Consent for publication

Not applicable

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

None

Authors' contributions

Jun Liu performed experiments, analyzed data, and wrote the manuscript. Wenshuai Zhu designed research, performed experiments, and analyzed data. Jianqin Ji conceived and designed research.

Acknowledgment

None

References

1. Whelan JS, Davis LE. Osteosarcoma, Chondrosarcoma, and Chordoma. J Clin Oncol. 2018;36:188-193.
2. Isakoff MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. J Clin Oncol. 2015;33:3029-3035.
3. Zhong Y, Du Y, Yang X, Mo Y, Fan C, Xiong F, et al. Circular RNAs function as ceRNAs to regulate and control human cancer progression. Mol Cancer. 2018;17:79.
4. Wang C, Ren M, Zhao X, Wang A, Wang J. Emerging Roles of Circular RNAs in Osteosarcoma. Med Sci Monit. 2018;24:7043-7050.
5. Zhang Z, Pu F, Wang B, Wu Q, Liu J, Shao Z. Hsa_circ_0000285 functions as a competitive endogenous RNA to promote osteosarcoma progression by sponging hsa-miRNA-599. Gene Ther. 2019.
6. Pan G, Hu T, Chen X, Zhang C. Upregulation Of circMMP9 Promotes Osteosarcoma Progression Via Targeting miR-1265/CHI3L1 Axis. Cancer Manag Res. 2019;11:9225-9231.
7. Huang Y, Xie J, Li E. Comprehensive circular RNA profiling reveals circ_0002060 as a potential diagnostic biomarkers for osteoporosis. J Cell Biochem. 2019;120:15688-

15694.

8. Sampson VB, Yoo S, Kumar A, Vetter NS, Kolb EA. MicroRNAs and Potential Targets in Osteosarcoma: Review. Front Pediatr. 2015;3:69.
9. Ling Z, Fan G, Yao D, Zhao J, Zhou Y, Feng J, et al. MicroRNA-150 functions as a tumor suppressor and sensitizes osteosarcoma to doxorubicin-induced apoptosis by targeting RUNX2. Exp Ther Med. 2020;19:481-488.
10. Dong S, Xiao Y, Ma X, He W, Kang J, Peng Z, et al. miR-193b Increases the Chemosensitivity of Osteosarcoma Cells by Promoting FEN1-Mediated Autophagy. Onco Targets Ther. 2019;12:10089-10098.
11. Zhang S, Zhao Y, Wang L. MicroRNA-198 inhibited tumorous behaviors of human osteosarcoma through directly targeting ROCK1. Biochem Biophys Res Commun. 2016;472:557-565.
12. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the role of ABC transporters in multidrug-resistant cancer. Nat Rev Cancer. 2018;18:452-464.
13. Yi D, Xu L, Wang R, Lu X, Sang J. miR-381 overcomes cisplatin resistance in breast cancer by targeting MDR1. Cell Biol Int. 2019;43:12-21.
14. Zhao P, Wang S, Jiang J, Liu H, Zhu X, Zhao N, et al. TIPE2 sensitizes osteosarcoma cells to cis-platin by down-regulating MDR1 via the TAK1- NF-kappaB and - AP-1 pathways. Mol Immunol. 2018;101:471-478.
15. Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther. 2006;6:1075-1085.
16. Anninga JK, Gelderblom H, Fiocco M, Kroep JR, Taminiau AH, Hogendoorn PC, et al. Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? Eur J Cancer. 2011;47:2431-2445.

17. Jia L, Yang A. Noncoding RNAs in Therapeutic Resistance of Cancer. Adv Exp Med Biol. 2016;927:265-295.

18. Zhu KP, Zhang CL, Ma XL, Hu JP, Cai T, Zhang L. Analyzing the Interactions of mRNAs and ncRNAs to Predict Competing Endogenous RNA Networks in Osteosarcoma Chemo-Resistance. Mol Ther. 2019;27:518-530.

19. Liang Y, Song X, Li Y, Su P, Han D, Ma T, et al. circKDM4C suppresses tumor progression and attenuates doxorubicin resistance by regulating miR-548p/PBLD axis in breast cancer. Oncogene. 2019;38:6850-6866.

20. Hu Y, Gu J, Shen H, Shao T, Li S, Wang W, et al. Circular RNA LARP4 correlates with decreased Enneking stage, better histological response, and prolonged survival profiles, and it elevates chemosensitivity to cisplatin and doxorubicin via sponging microRNA-424 in osteosarcoma. J Clin Lab Anal. 2019:e23045.

21. Gu J, Li X, Li H, Jin Z, Jin J. MicroRNA-198 inhibits proliferation and induces apoptosis by directly suppressing FGFR1 in gastric cancer. Biosci Rep. 2019;39.

22. Li LX, Lam IH, Liang FF, Yi SP, Ye LF, Wang JT, et al. MiR-198 affects the proliferation and apoptosis of colorectal cancer through regulation of ADAM28/JAK-STAT signaling pathway. Eur Rev Med Pharmacol Sci. 2019;23:1487-1493.

23. Hu Y, Tang Z, Jiang B, Chen J, Fu Z. miR-198 functions as a tumor suppressor in breast cancer by targeting CUB domain-containing protein 1. Oncol Lett. 2017;13:1753-1760.

24. Chen R, Wang G, Zheng Y, Hua Y, Cai Z. Drug resistance-related microRNAs in osteosarcoma: Translating basic evidence into therapeutic strategies. J Cell Mol Med. 2019;23:2280-2292.

25. Nie E, Jin X, Wu W, Yu T, Zhou X, Shi Z, et al. MiR-198 enhances temozolomide sensitivity in glioblastoma by targeting MGMT. J Neurooncol. 2017;133:59-68.

26. Huang X, Li Z, Zhang Q, Wang W, Li B, Wang L, et al. Circular RNA AKT3 upregulates PIK3R1 to enhance cisplatin resistance in gastric cancer via miR-198 suppression. Mol Cancer. 2019;18:71.

27. Tan B, Piwnica-Worms D, Ratner L. Multidrug resistance transporters and modulation. Curr Opin Oncol. 2000;12:450-458.

28. Kumar A, Jaitak V. Natural products as multidrug resistance modulators in cancer. Eur J Med Chem. 2019;176:268-291.

29. Cao F, Yin LX. miR-122 enhances sensitivity of hepatocellular carcinoma to oxaliplatin via inhibiting MDR1 by targeting Wnt/beta-catenin pathway. Exp Mol Pathol. 2019;106:34-43.

30. Ghanbarian M, Afgar A, Yadegarazari R, Najafi R, Teimoori-Toolabi L. Through oxaliplatin resistance induction in colorectal cancer cells, increasing ABCB1 level accompanies decreasing level of miR-302c-5p, miR-3664-5p and miR-129-5p. Biomed Pharmacother. 2018;108:1070-1080.

31. Cheng FH, Zhao ZS, Liu WD. Long non-coding RNA ROR regulated ABCB1 to induce cisplatin resistance in osteosarcoma by sponging miR-153-3p. Eur Rev Med Pharmacol Sci. 2019;23:7256-7265.

32. Zhou Z, Wan L, Han Y, Meng X, Yang Q, Li Y, et al. ABCB1-overexpressing MG63/DOX cell xenograft model: maintain the MDR phenotype in vivo. Pharm Biol. 2013;51:968-973.

Figures

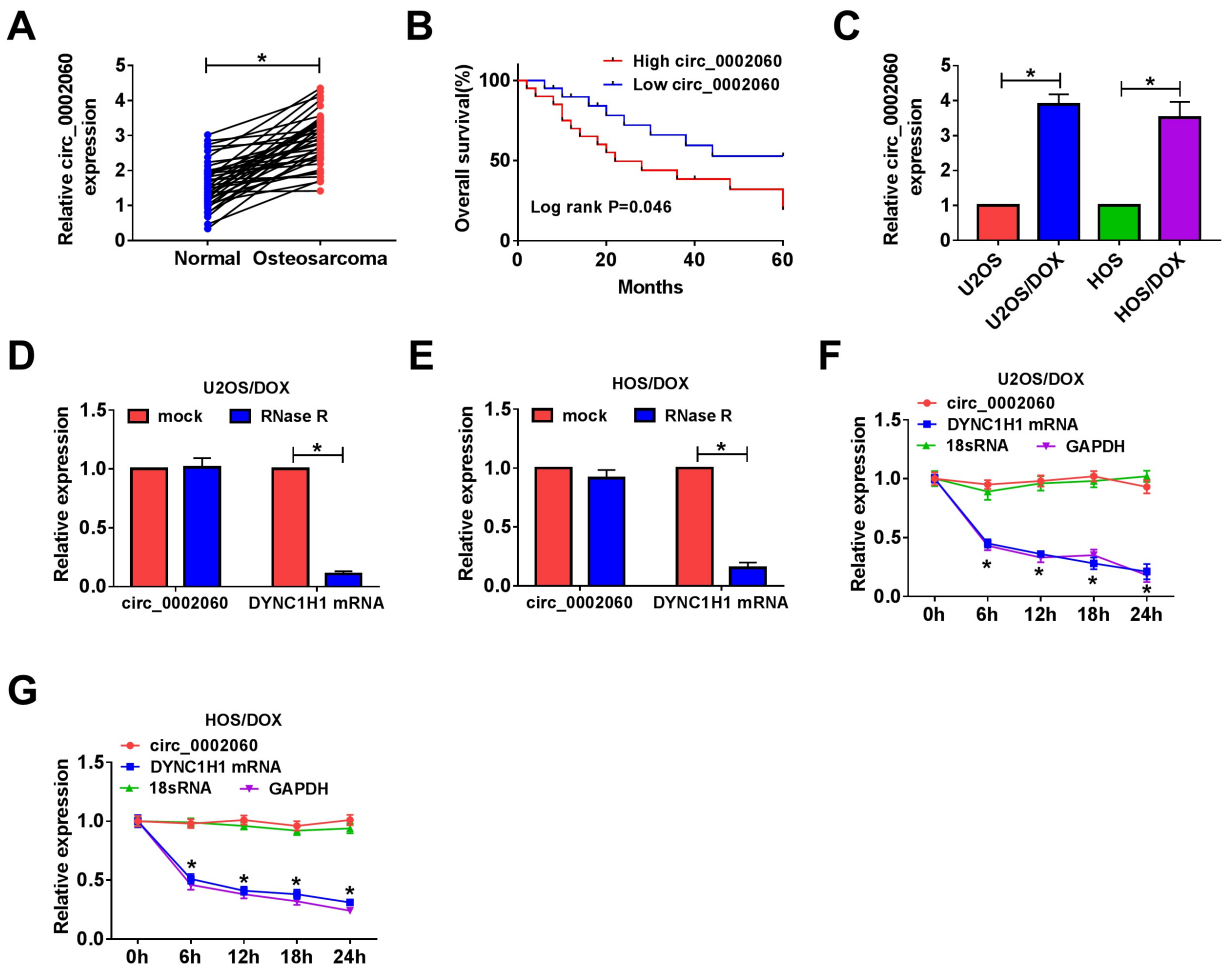

Figure 1

Circ_0002060 was up-regulated in OS tissues and DOX-resistant OS cells. (A) The expression of circ_0002060 in OS tissues (n=40) and adjacent normal tissues (n=40) was examined by qRT-PCR. (B) The relationship between circ_0002060 expression and overall survival of OS patients was detected using Kaplan-Meier survival analysis. (C) QRT-PCR was used to detect the expression of circ_0002060 in OS parental cells (U2OS and HOS) and DOX-resistant cells (U2OS/DOX and HOS/DOX). (D and E) U2OS/DOX and HOS/DOX cells were treated with or without RNase R, and the levels of circ_0002060 and DYNC1H1 mRNA were measured by qRT-PCR. (F and G) The stability of circ_0002060 and linear transcript DYNC1H1

was detected in U2OS/DOX and HOS/DOX cells. *P < 0.05.

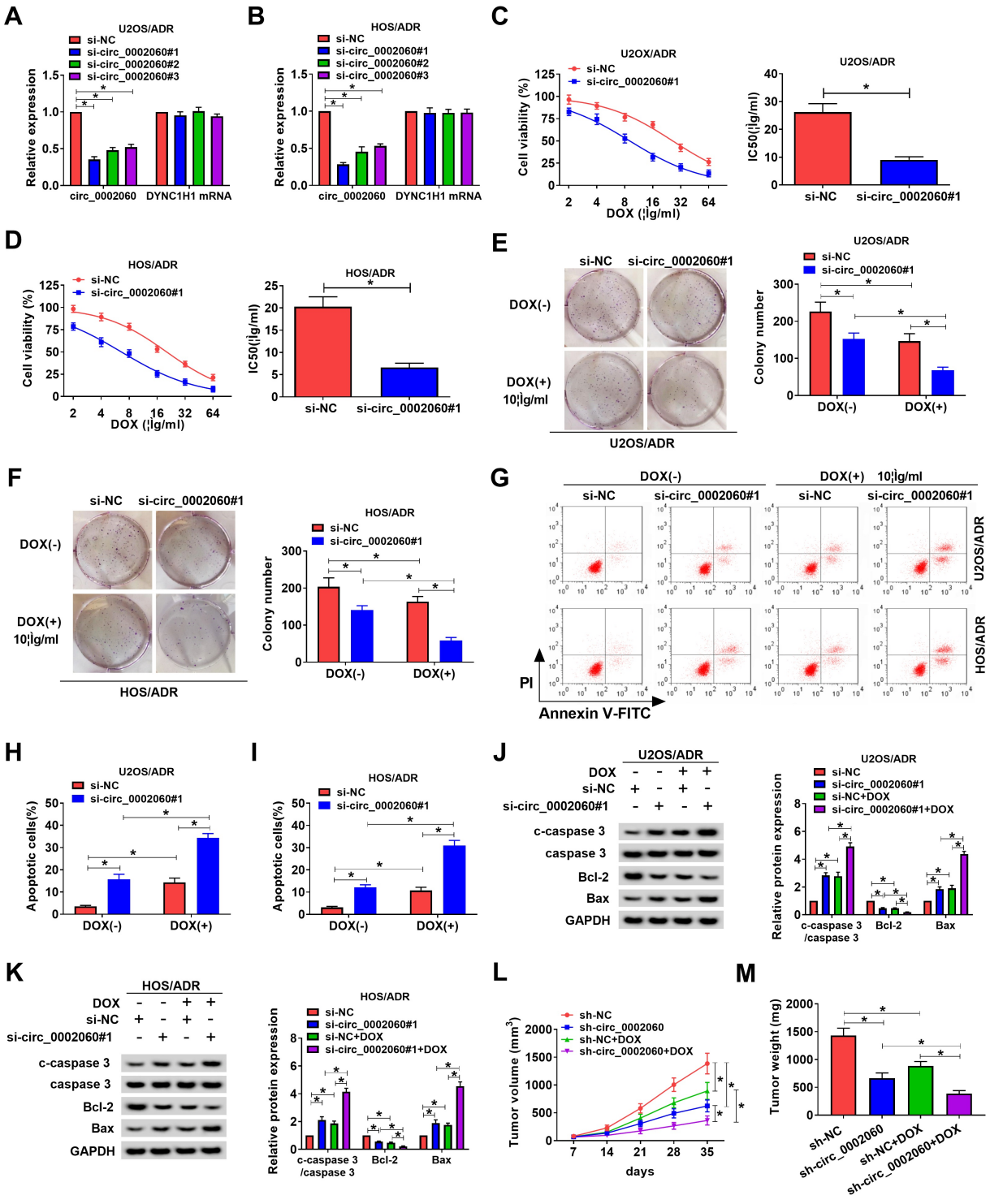

Figure 2

Circ_0002060 knockdown decreased DOX resistance in vitro and in vivo. (A and B) U2OS/DOX and HOS/DOX cells were transfected with si-NC, si-circ_0002060#1, si-

circ_0002060#2 or si-circ_0002060#3, and the knockdown efficiency was determined by qRT-PCR. (C and D) IC50 value was calculated by CCK-8 assay after treatment with different concentrations of DOX. (E and F) Cell proliferation was evaluated using colony formation assay. (G-I) The apoptosis rate of U2OS/DOX and HOS/DOX cells was examined by flow cytometry. (J and K) The levels of apoptosis-related proteins were detected by western blot. (L and M) The effect of circ_0002060 on DOX resistance was analyzed by xenograft assay. *P < 0.05.

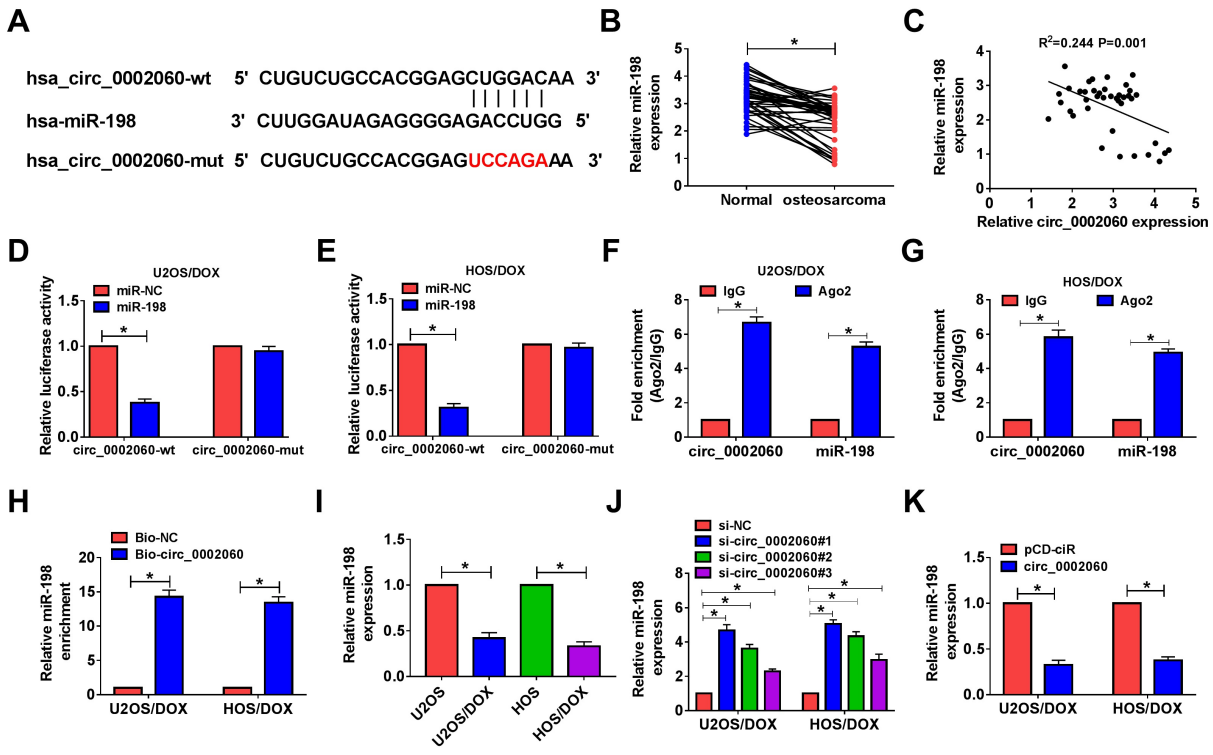

Figure 3

Circ_0002060 directly targeted miR-198. (A) The predicted binding sites of circ_0002060 and miR-198 were shown. (B) The expression of miR-198 in OS tissues and normal tissues was measured by qRT-PCR. (C) The correlation between circ_0002060 and miR-198 in OS tissues was assessed by Spearman's correlation analysis. (D and E) U2OS/DOX and HOS/DOX cells were co-transfected with circ_0002060-wt or circ_0002060-mut and miR-198 or miR-NC, and the luciferase activity was tested by dual-luciferase reporter assay. (F and G) RIP assay was used to verify whether circ_0002060 bound to miR-198. (H) The relationship between circ_0002060 and miR-198 was confirmed by RNA pull-down assay. (I) The level of miR-198 was measured in OS parental cells and DOX-resistant cells. (J and K) U2OS/DOX and HOS/DOX cells were introduced with si-NC, si-circ_0002060#1, si-circ_0002060#2, si-circ_0002060#3, pCD-ciR or circ_0002060, and miR-198 expression was examined using qRT-PCR. *P < 0.05.

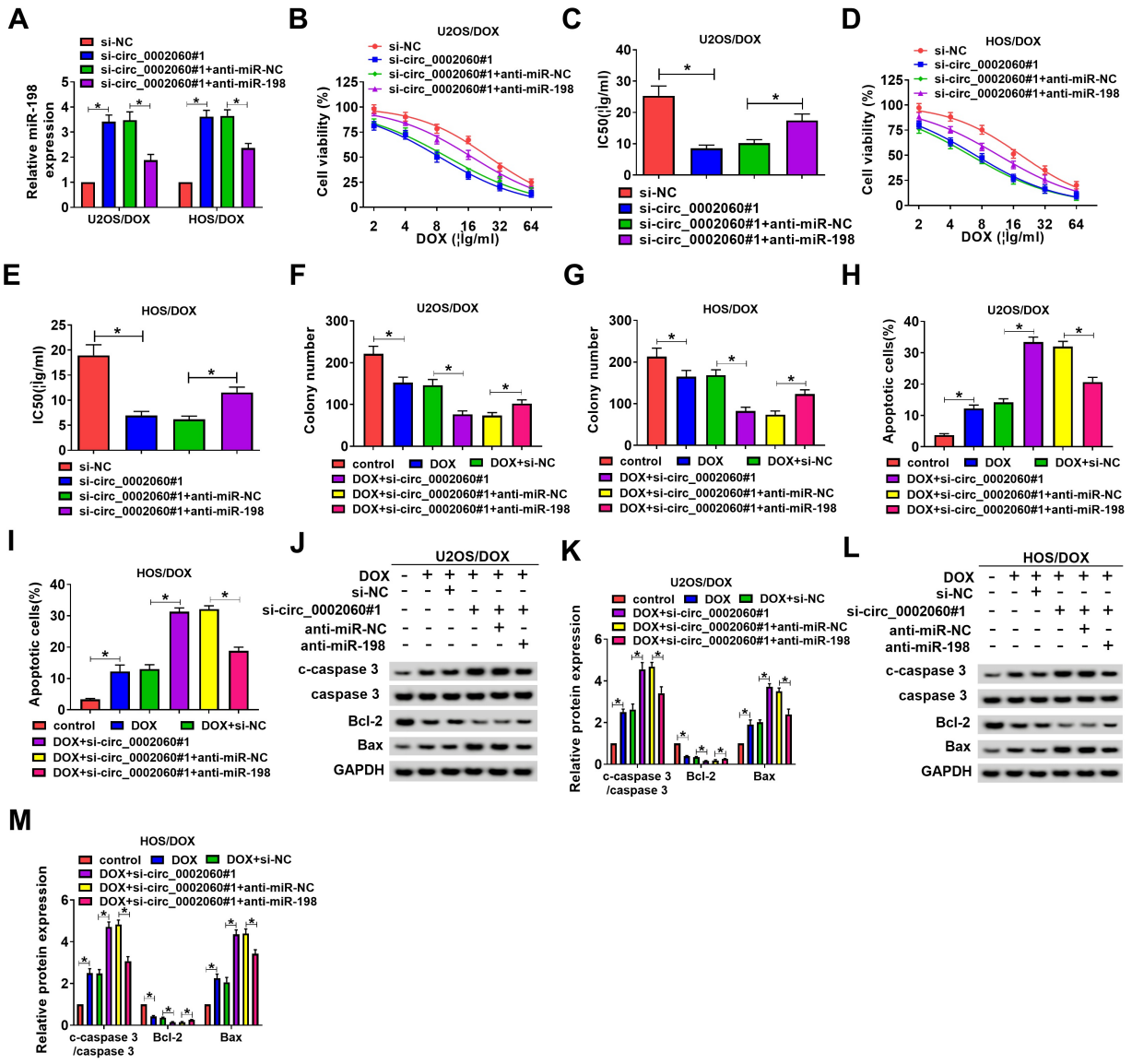

Figure 4

Circ_0002060 regulated DOX resistance by sponging miR-198. U2OS/DOX and HOS/DOX cells were introduced with si-NC, si-circ_0002060#1, si-circ_0002060#1+anti-miR-NC or si-circ_0002060#1+anti-miR-198, respectively. (A) The expression of miR-198 was detected by qRT-PCR. (B-E) IC50 of DOX was determined by CCK-8 assay. (F and G) Colony formation assay was used to evaluate cell proliferation. (H and I) The apoptosis rate of U2OS/DOX and HOS/DOX cells was measured by flow cytometry. (J-M) The protein levels of apoptosis-related markers were examined using western blot. *P < 0.05.

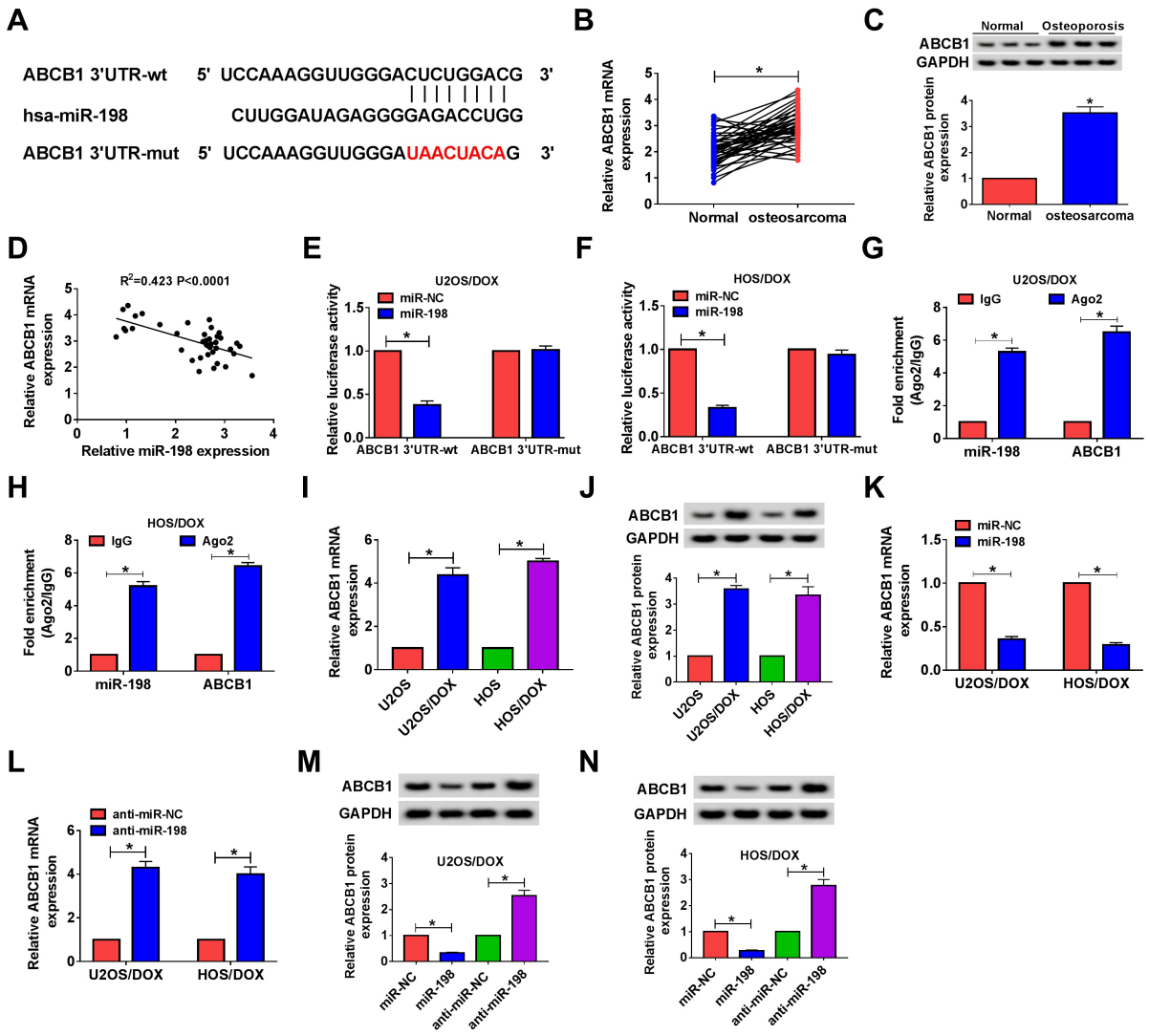

Figure 5

ABCB1 was a target of miR-198. (A) The putative binding sites of miR-198 and ABCB1 were exhibited. (B and C) The mRNA and protein levels of ABCB1 in OS tissues and adjacent normal tissues were examined by qRT-PCR and western blot. (D) The correlation between miR-198 and ABCB1 in OS tissues was detected by Spearman's correlation analysis. (E and F) Dual-luciferase reporter assay was performed to detect the relationship between miR-198 and ABCB1. (G and H) RIP assay was used to confirm whether miR-198 bound to ABCB1. (I and J) The mRNA and protein levels of ABCB1 were measured in U2OS, U2OS/DOX, HOS and HOS/DOX cells. (K-N) The expression of ABCB1 in U2OS/DOX and HOS/DOX cells

transfected with miR-NC, miR-198, anti-miR-NC or anti-miR-198 was tested by qRT-PCR and western blot. *P < 0.05.

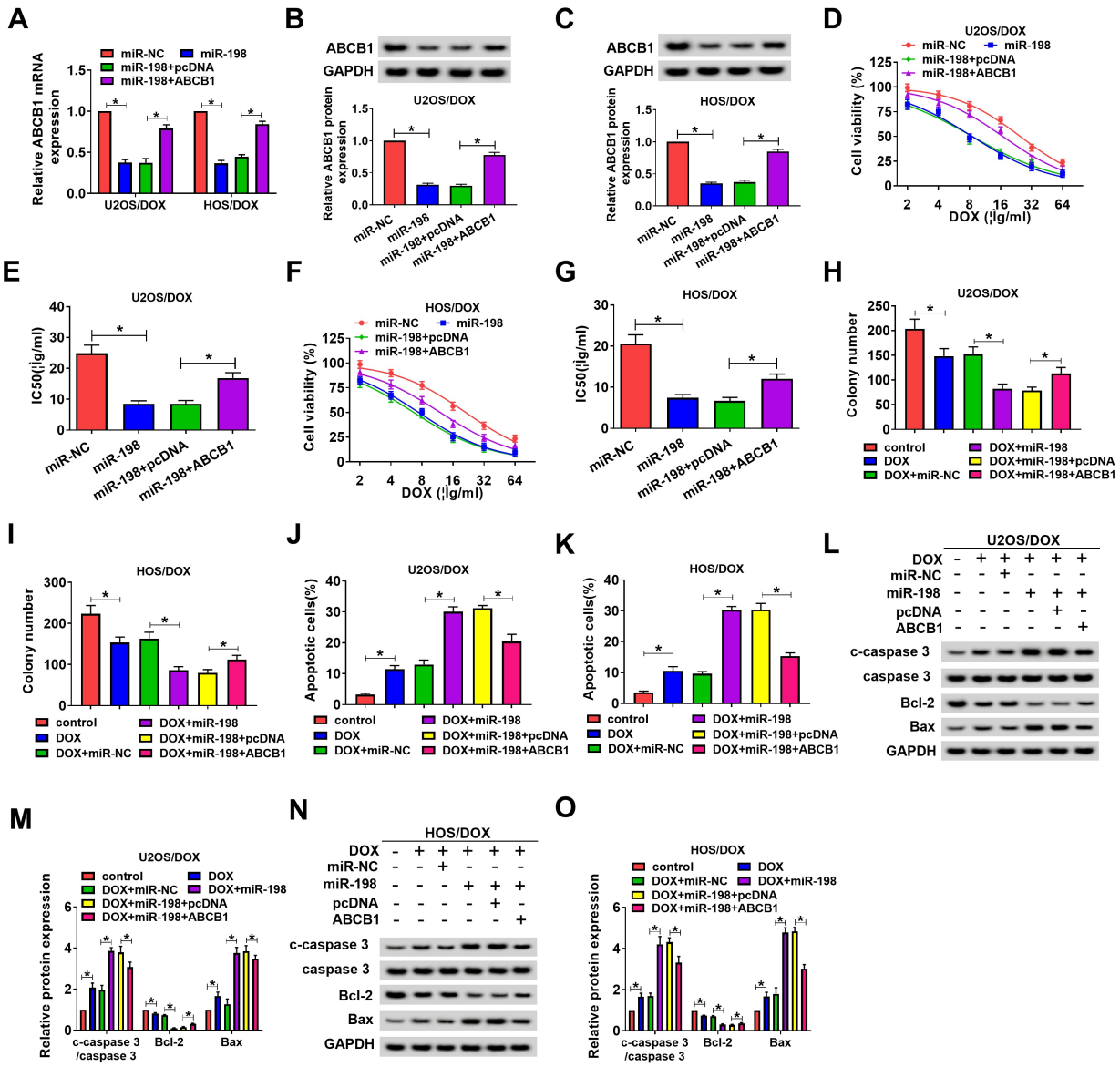

Figure 6

MiR-198 reduced DOX resistance via targeting ABCB1. U2OS/DOX and HOS/DOX cells were transduced with miR-NC, miR-198, miR-198+pcDNA or miR-198+ABCB1, respectively. (A-C) The mRNA and protein levels of ABCB1 were examined by qRT-PCR and western blot. (D-G) CCK-8 assay was used to assess cell sensitivity to DOX. (H and I) Cell proliferation was evaluated by colony formation assay. (J and K) Cell apoptosis was monitored by flow cytometry. (L-O) The protein levels of apoptosis-related proteins were measured by western blot. *P < 0.05.

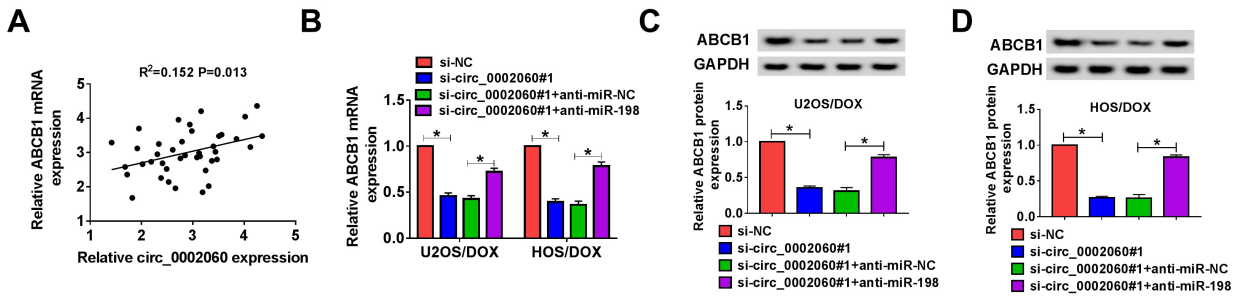

Figure 7

Circ_0002060 modulated ABCB1 expression by regulating miR-198. (A) Spearman's correlation analysis was used to detect the relationship between circ_0002060 and ABCB1 in OS tissues. (B-D) U2OS/DOX and HOS/DOX cells were transfected with si-NC, si-circ_0002060#1, si-circ_0002060#1+anti-miR-NC or si-circ_0002060#1+anti-miR-198, and ABCB1 expression was examined using qRT-PCR and western blot. *P < 0.05.