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Hsa_circ_0078767 Enhances Osteosarcoma Chemoresistance to Doxorubicin Through the Regulation of the miR-188-3p/GPX4 Axis

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Background: Osteosarcoma (OS) is a primary malignancy of bone. The emergence of chemoresistance represents a persistent barrier to effective cancer patient care. This analysis sought to examine hsa_circ_0078767 as a mediator of doxorubicin (DOX) resistance in OS.

Methods: Levels of hsa_circ_0078767, miR-188-3p, and glutathione peroxidase 4 (GPX4) in OS clinical tissue samples and cell lines were evaluated by quantitative polymerase chain reaction (qPCR) and Western blotting. Associations between hsa_circ_0078767 levels in clinical samples and patient overall survival were assessed with Kaplan-Meier curves. CCK-8 assays were utilized as a means of examining DOX half-inhibitory concentration (IC_{50}) values. RNA immunoprecipitation and pull-down, as well as reporter assays, investigated interactions between hsa_circ_0078767, miR-188-3p, and GPX4 within OS cells exhibiting DOX resistance.

Results: OS patient tissues and cell lines resistant to DOX exhibited elevated hsa_circ_0078767 and GPX4 expression together with a reduction in miR-188-3p levels. Inhibiting hsa_circ_0078767 expression contributed to a profound decrease in the ability of OS tumors to resist DOX. Mechanistically, it was determined that hsa_circ_0078767 can enhance DOX chemoresistance through its ability to bind and sequester miR-188-3p, which otherwise negatively modulates GPX4 to enhance chemosensitivity. Accordingly, the sequestration of miR-188-3p by hsa_circ_0078767 led to the derepression and upregulation of GPX4.

Conclusion: Hsa_circ_0078767 was found to modulate miR-188-3p/GPX4 signaling to enhance OS cell resistance to DOX treatment and facilitate disease progression. As such, hsa_circ_0078767 may represent a valuable biomarker or target for use in the context of OS patient management.

Keywords: GPX4, hsa_circ_0078767, doxorubicin, miR-188-3p, osteosarcoma

Introduction

Osteosarcoma (OS) is a bone tumor that usually occurs during adolescence or childhood.[1] Prognostic OS patient outcomes have improved recently due to improvements in surgical approaches and chemotherapeutic technologies such that the 5-year overall survival rate is now roughly 70%.[2] Chemotherapeutic resistance, however, is a persistent barrier to the durable treatment of this devastating form of cancer, underscoring a need to develop new strategies for mitigating such resistance so as to improve patient responses to treatment.

Circular RNAs (circRNAs) harbor binding sites for certain microRNAs (miRNAs), enabling them to function as competing endogenous RNAs (ceRNAs) that modulate a range of biological processes.[3,4] Dysregulated expression of circRNAs can alter these processes and contribute to tumor progression in some cases.[5,6] Certain circRNAs have been tied to tumor chemoresistance.[7,8] Through its ability to interact with staphylococcal nuclease and tudor domain

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containing (SND1), for instance, circ_0004087 can increase prostate tumor cell resistance to docetaxel by enhancing the ability of these cells to correct for mitotic error.[9] CircFARP1 can further target the LIF/STAT3 axis to facilitate resistance to gemcitabine in pancreatic cancer.[10] Owing to its ability to target the miR-348/ATG7 pathway, hsa_circ_0092276 can increase resistance to doxorubicin (DOX) in breast cancer cells.[11] Given these effects, circRNAs represent promising targets for efforts aimed at restoring tumor chemosensitivity.

Silencing of hsa_circ_0078767 and its subsequent effects on miR-1205/PTBP1 expression have been shown to suppress OS development.[12] The specific mechanistic roles played by hsa_circ_0078767 in the context of chemoresistance in individuals with OS, however, remain to be clarified. As such, this study was developed with the goal of clarifying the mechanisms through which hsa_circ_0078767 shapes OS tumor cell resistance to DOX.

Materials and Methods

Patient Samples

OS tissues were obtained from 126 patients diagnosed with primary OS from the Second Affiliated Hospital of Zhejiang University School of Medicine. To be eligible for inclusion, patients had to (1) be free of any history of chemotherapy or surgery, (2) be histopathologically diagnosed with OS, and (3) provide written informed consent from the participants and/or their guardians. Patients were excluded if they (1) exhibited other tumors, (2) exhibited nervous system diseases, or (3) suffered from serious diseases impacting any other organ systems. The collected tumor tissue samples included 65 and 61 non-respond and respond OS samples. This study received approval from the Second Affiliated Hospital of Zhejiang University School of Medicine. Our study complies with the Declaration of Helsinki.

Cellular Transfection

The KHOS and U2OS human OS cell lines from Biovector (Beijing, China) were grown in DMEM (Gibco, MA, USA) supplemented with 10% FBS (Gibco) and penicillin/streptomycin (Invitrogen, MA, USA) in a 5% CO_2 37°C incubator. To generate DOX-resistant versions of these cell lines (U2OS/DOX and KHOS/DOX), the parental cells were cultured in media containing progressively increasing DOX (Zhejiang Hisun Pharmaceutical CO., LTD, Taizhou, China) concentrations (2.5 ng/mL1–μg/mL) over a 6-month interval as in past studies.

An siRNA specific for hsa_circ_0078767 (si-hsa_circ_0078767), a miR-188-3p mimic, a miR-188-3p inhibitor (anti-miR-188-3p), a GPX4 overexpression vector, and negative controls (miR-NC, si-NC, pcDNA, and anti-miR-NC) were from GenePharma (Shanghai, China). Transfection was performed with Lipofectamine 3000 (Invitrogen).

qPCR

RNA was extracted with TRIzol (Invitrogen), after which a HiScript II One-Step RT-PCR Kit (Vazyme, Nanjing, China) or a miRNA 1st Strand cDNA Synthesis Kit (Vazyme) were employed for reverse transcription. AceQ qPCR SYBR Green Master Mix (Vazyme) was then used for qPCR analyses, with GAPDH and U6 serving as normalization controls. The following primers were used: hsa_circ_0078767, Forward: 5′-ACCTGCCTAGCTGTCAAGGA-3′, Reverse: 5′-GGATCTAGAGATGCGCCAAC-3′; miR-188-3p, loop primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAAACC-3′, Forward: 5′-TGCGCCTCCCACATGCAGGGT-3′; GPX4, Forward: 5′-ACAAGAACGGCTGCGTGGTGAA-3′, Reverse: 5′-GCCACACACTTGTGGAGCTAGA-3′; GAPDH, Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′, Reverse: 5′-ACCACCCTGTTGCTGTAGCCAA-3′; U6, Forward: 5′- CTCGCTTCGGCAGCACA-3′, Reverse: 5′-AACGCTTCACGAATTTGCGT-3′.

CCK-8

Cells were added to a 96-well plate, with 3000 cells in each well, followed by incubation with a range of DOX doses for appropriate intervals. Then, cells were incubated in the presence of 10 μL of CCK-8 reagent (Solarbio) for 2 h after which absorbances at 450 nm were read in a MultiMode Reader (BioTek, VT, USA). IC_{50} values for DOX were then calculated based upon these results.

Western Immunoblotting

RIPA buffer (Solarbio) was employed for protein extraction, after which proteins were electrophoresed on SDS-PAGE prior to transfer onto a PVDF membrane (Millipore, MA, USA). Anti-GPX4 (ab116703; Abcam, Cambridge, United Kingdom) and anti-GAPDH (ab9485; Abcam) were then used to probe the blots that were then probed with a secondary antibody (ab7090; Abcam). An enhanced chemiluminescence system (Millipore) was then employed to detect protein bands.

Luciferase Reporter Assays

The wild-type or mutated hsa_circ_0078767 and GPX4 3'UTR sequences harboring putative sites for miR-188-3p binding were initially amplified after which they were cloned into the pmirGLO vector (Promega, WI, USA), yielding the hsa_circ_0078767-wt, hsa_circ_0078767-mut, GPX4-wt, and GPX4-mut reporters. These reporter vectors were then transfected into HOS/DOX and U2OS/DOX cells along with miR-188-3p or miR-NC, and a Dual-Luciferase Reporter Assay Kit (Promega) was then used to assess levels of promoter activity.

RNA Immunoprecipitation (RIP)

The lysis buffer from the EZ-Magna RIP kit (Millipore) was employed to extract the intracellular contents from DOX-resistant cells, followed by the incubation of these lysates with anti-Ago2 or control IgG-coated magnetic beads. A qPCR approach was subsequently used to evaluate hsa_circ_0078767, miR-188-3p, or GPX4 enrichment in different samples.

RNA Pull-Down Assay

Biotinylated hsa_circ_0078767 (Bio-hsa_circ_0078767) and control (Bio-NC) probes (GenePharma) were added to M-280 streptavidin Dynabeads (Invitrogen) and allowed to incubate at 37°C for 2 h. Cell lysates were incubated for a further 3 h with the beads at 4°C. A qPCR approach was then employed to assess miR-188-3p expression.

Statistical Analysis

Data are shown as means with standard deviations and were analyzed with GraphPad Prism 7.0 (GraphPad, CA, USA). Results were compared with one-way ANOVA or *t*-tests. $P < 0.05$ was selected as a threshold to identify significantly different results.

Results

hsa_circ_0078767 Upregulation is Evident in DOX-Resistant OS Patient Tissue Samples and Cell Lines

Initial analyses revealed a significant rise in hsa_circ_0078767 levels within OS patient tumors that were non-respond relative to respond tumors (Figure 1A). Kaplan–Meier analyses revealed hsa_circ_0078767 upregulation in non-respond group to be associated with poorer overall survival among patients diagnosed with OS (Figure 1B). A notable increase in hsa_circ_0078767 levels was also detected in the DOX-resistant HOS and U2OS cell lines as compared to the parental varieties thereof (Figure 1C). Greater RNase R resistance was also noted for hsa_circ_0078767 relative to DYNC1H1, which is the linear gene from which this circRNA is derived (Figure 1D and E). Moreover, hsa_circ_0078767 was mainly located in the cytoplasm (Figure 1F). When actinomycin D was utilized to treat DOX-resistant cells, subsequent qPCR analyses indicated that the hsa_circ_0078767 half-life was over 24 h relative to < 6 h for the DYNC1H1 mRNA (Figure 1G). Together, these results provided preliminary support for an oncogenic function for hsa_circ_0078767 in OS.

Silencing hsa_circ_0078767 Reduces in vitro Resistance to DOX

To better understand the regulatory functions of hsa_circ_0078767, the DOX-resistant HOS and U2OS cell lines were next transfected with the si-hsa_circ_0078767 construct to knock down this circRNA. A qPCR approach confirmed the successful silencing of hsa_circ_0078767 without any corresponding change in DYNC1H1 expression levels (Figure 2A

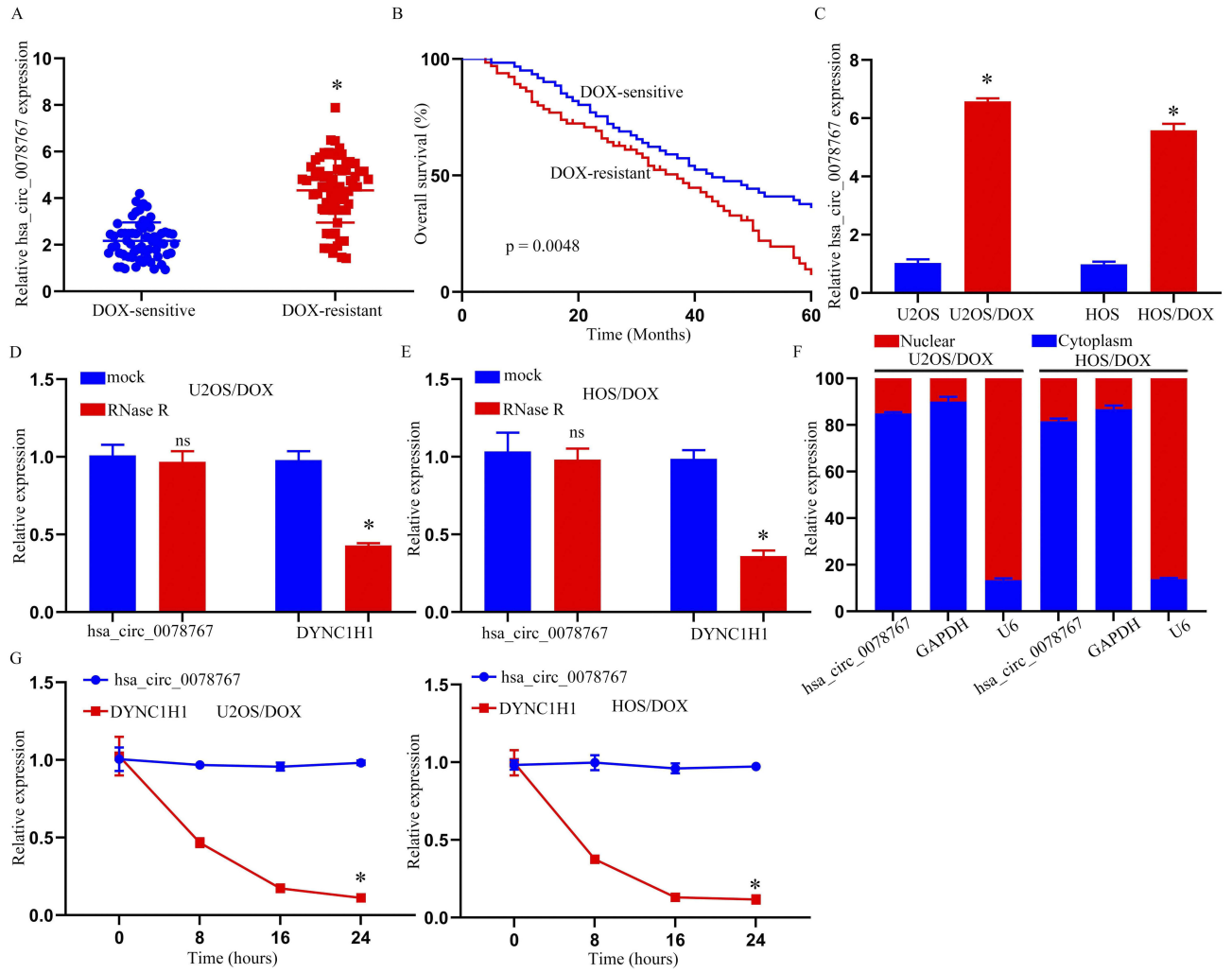

Figure 1 DOX-resistant OS tumors and cell lines express higher levels of hsa_circ_0078767. (**A**) qPCR was used to detect hsa_circ_0078767 levels in 65 and 61 non-respond and respond OS tissue samples, respectively. (**B**) Kaplan-Meier curves for assessment of the association between OS patient survival andhsa_circ_0078767 levels. (**C**) hsa_circ_0078767 expression in parental (HOS, U2OS) and DOX-resistant (HOS/DOX, U2OS/DOX) OS cell lines, shown by qPCR. (**D** and **E**) RNase R was used to treat U2OS/DOX and HOS/DOX cells, after which qPCR was utilized to assess hsa_circ_0078767 and DYNC1H1 expression. (**F**) Hsa_circ_0078767, GAPDH, and U6 were detected from the nuclear and cytoplasmic fractions of U2OS/DOX and HOS/DOX cells. (**G**) hsa_circ_0078767 and linear DYNC1H1 transcript stability levels were analyzed in U2OS/DOX and HOS/DOX cells. *P < 0.05.

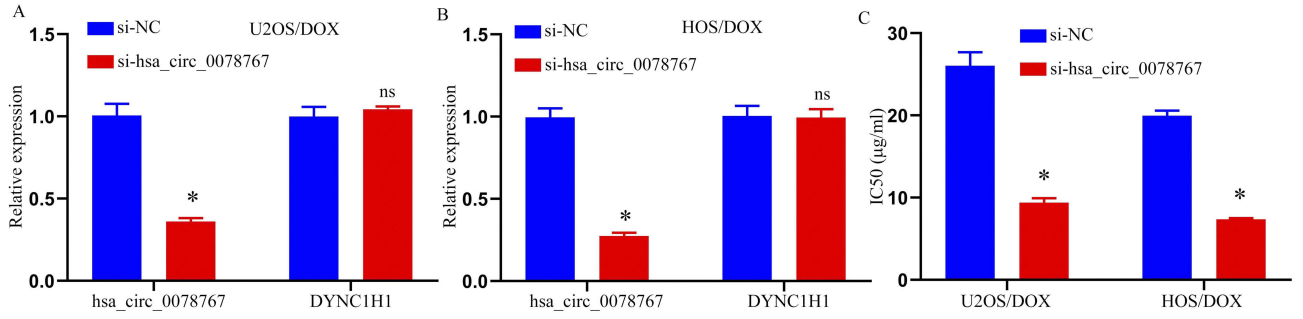

Figure 2 Knocking down hsa_circ_0078767 reduces in vitro OS cell resistance to DOX. (**A** and **B**) qPCR was employed to gauge knockdown efficiency in U2OS/DOX and HOS/DOX cells following si-NC or si-hsa_circ_0078767 transfection. (**C**) CCK-8 assays were utilized to calculate IC_{50} values for DOX-treated OS cells. *P < 0.05.

and B). CCK-8 assays further demonstrated that silencing hsa_circ_0078767 lowered the DOX IC_{50} values in both of DOX-resistant cell lines (Figure 2C).

miR-188-3p is Directly Targeted by hsa_circ_0078767

Analysis by the Circular RNA Interactome database showed sequence complementarity between hsa_circ_0078767 and miR-188-3p (Figure 3A). A marked rise in miR-188-3p levels was detected when analyzing OS tissue samples from non-respond patients relative to those exhibiting respond (Figure 3B). The levels of hsa_circ_0078767 and miR-188-3p

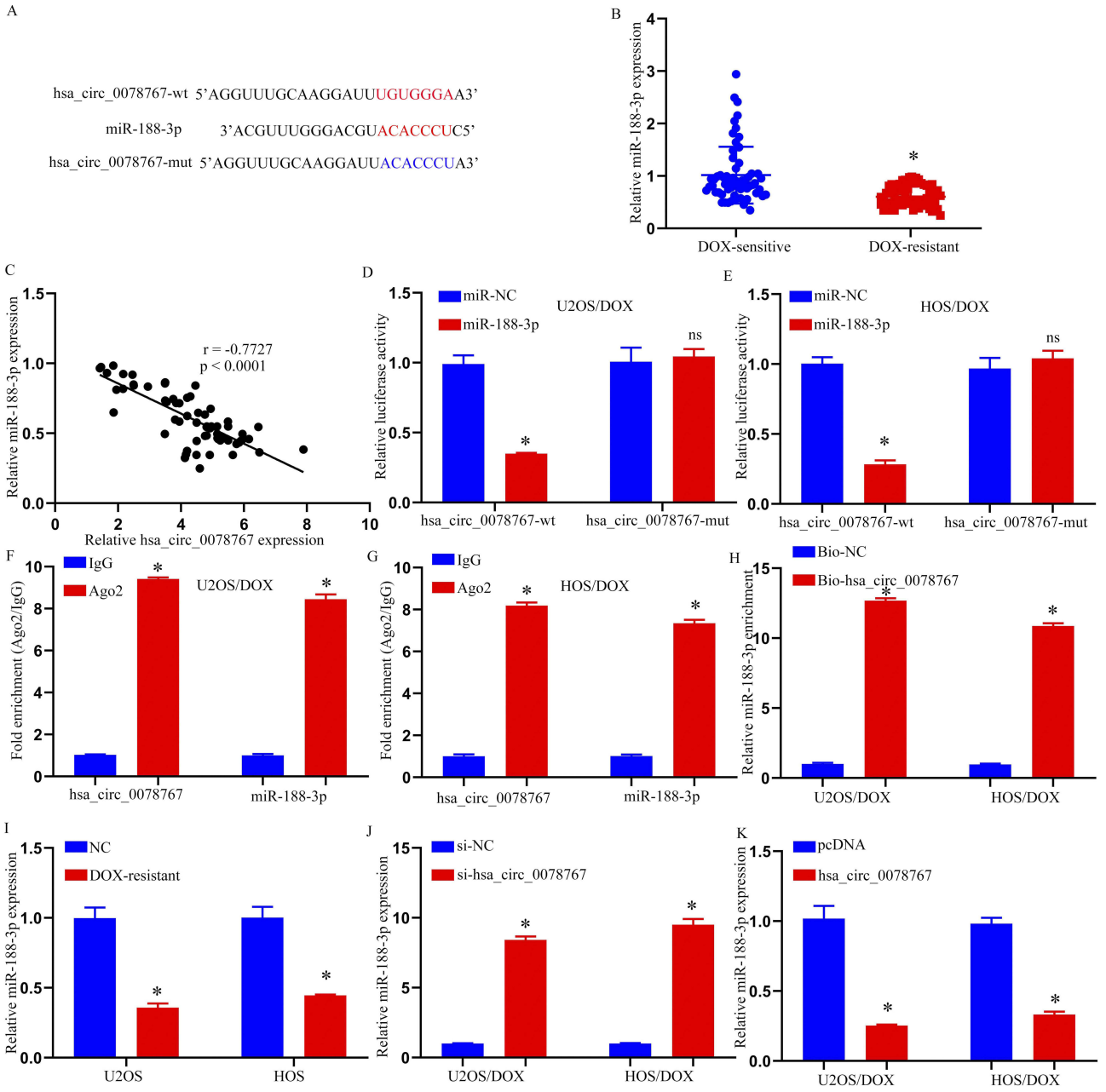

Figure 3 miR-188-3p is targeted directly by hsa_circ_0078767. (**A**) Putative hsa_circ_0078767 and miR-188-3p binding sites. (**B**) miR-188-3p levels in 65 and 61 non-respond and respond OS tissue samples, respectively, shown by qPCR. (**C**) Spearman correlation coefficients between hsa_circ_0078767 and miR-188-3p levels in DOX-resistant OS patient tissue samples. (**D** and **E**) Dual-luciferase reporter assays for assessment of HOS/DOX and U2OS/DOX cells following hsa_circ_0078767-wt or hsa_circ_0078767-mut co-transfection with the miR-188-3p or miR-NC constructs. (**F** and **G**) Interaction betweenhsa_circ_0078767 and miR-188-3p shown by RIP. (**H**) Interaction between hsa_circ_0078767 and miR-188-3p shown by RNA pull-down. (**I**) miR-188-3p levels in parental and DOX-resistant OS cells. (**J** and **K**) qPCR determination of miR-188-3p expression in U2OS/DOX and HOS/DOX cells following si-NC, si-hsa_circ_0078767, pCD-ciR, or hsa_circ_0078767 transfection. *P < 0.05.

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expression were negatively correlated in DOX-resistant OS patient tumor tissue samples (Figure 3C). Luciferase reporter assays further confirmed that the miR-188-3p mimic reduced binding to hsa_circ_0078767 (Figure 3D and E) and both RNA pull-down and RIP verified a direct interaction between hsa_circ_0078767 and miR-188-3p (Figure 3F–H), as evidenced by clear hsa_circ_0078767 and miR-188-3p enrichment in the Ago2 group versus the IgG control group and the ability of biotinylated hsa_circ_0078767 but not the negative control construct to pull down miR-188-3p (Figure 3H). A marked reduction in miR-188-3p expression was also noted when comparing U2OS/DOX and HOS/DOX cells to the corresponding parental lines (Figure 3I). The silencing of hsa_circ_0078767 resulted in pronounced miR-188-3p upregulation, whereas the opposite was observed when hsa_circ_0078767 was overexpressed (Figure 3J and K). Together, these findings suggest that hsa_circ_0078767 is sponged by miR-188-3p in OS cells.

hsa_circ_0078767 Acts as a Molecular Sponge for miR-188-3p, Thereby Mediating Resistance to DOX

To determine how hsa_circ_0078767 and miR-188-3p shape the ability of OS cells to resist DOX treatment, the si-NC, si-hsa_circ_0078767, si-hsa_circ_0078767+antimiR-NC, or si-hsa_circ_0078767+anti-miR-188-3p constructs were next transfected into DOX-resistant U2OS and HOS cells. This approach revealed that anti-miR-188-3p co-transfection was sufficient to reverse the observed upregulation of this miRNA in response to the knockdown of hsa_circ_0078767 (Figure 4A). Importantly, inhibiting miR-188-3p reverse the observed decreases in decline in DOX resistance detected in these OS cells upon the silencing of hsa_circ_0078767 (Figure 4B).

GPX4 is a miR-188-3p Target Gene

TargetScan predicted miR-188-3p interaction sites in the GPX4 3′-UTR (Figure 5A). To test for a potential regulatory relationship between these factors, qPCR were next employed to assess GPX4 expression levels, revealing its pronounced mRNA level upregulation in non-respond OS tissue samples relative to those respond (Figure 5B). MiR-188-3p levels were negatively associated with those of GPX4 in DOX-resistant tumor tissue samples from patients with OS (Figure 5C). A reporter assay verified the ability of miR_188-3p to regulate the activity of a reporter construct harboring the wild-type version of the 3′-UTR of GPX4 (Figure 5D and E). RIP assays additionally confirmed the significant enrichment of miR-188-3p and GPX4 in the Ago2 sample relative to the IgG control sample, supporting the ability of the two to directly interact with one another (Figure 5F and G). Significant increases in GPX4 mRNA expression were also noted in DOX-resistant HOS and U2OS cells relative to their corresponding parental lines (Figure 5H). In addition, transfection with miR-188-3p mimic constructs lowered GPX4 mRNA levels, whereas the opposite was observed when miR-188-3p was instead knocked down (Figure 5I and J). These results thus confirm the status of GPX4 as a bona fide miR-188-3p target.

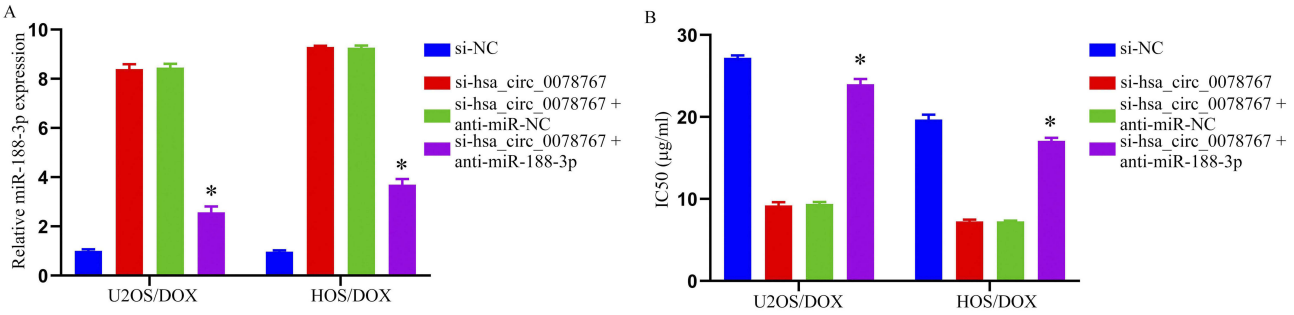

Figure 4 miR-188-3p sponges hsa_circ_0078767 sponges to regulate DOX chemosensitivity. (**A**) qPCR determination of miR-188-3p expression. (**B**) CCK-8 assays were used to compute DOX IC_{50} values. *P < 0.05.

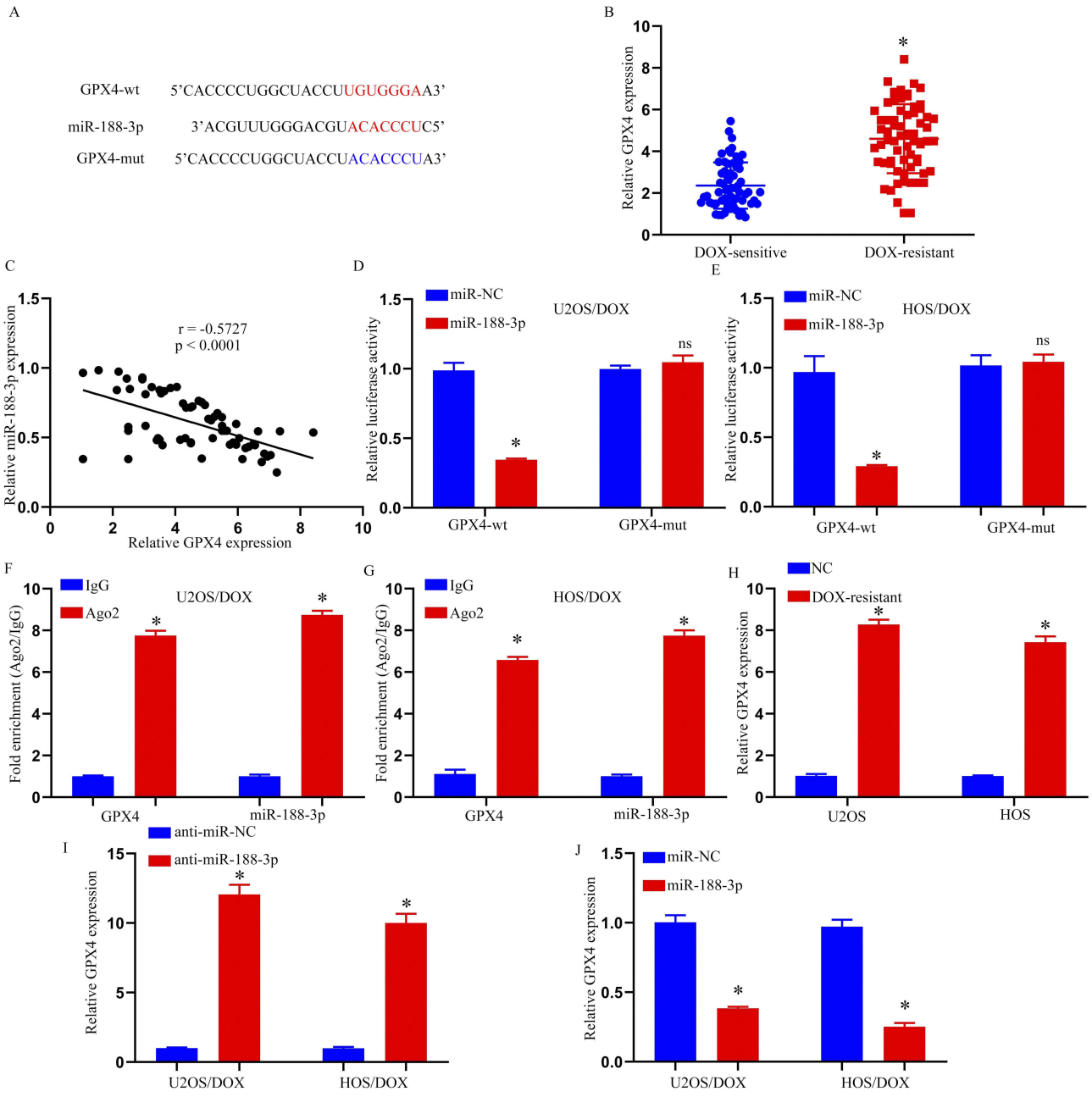

Figure 5 GPX4 is a miR-188-3p target (**A**) Overlap between predicted miR-188-3p and GPX4 interaction sites. (**B**) GPX4 levels in 65 and 61 non-respond and respond OS tissue samples, respectively, shown by qPCR. (**C**) Spearman correlations of miR-188-3p and GPX4 levels in DOX-resistant OS tissue samples. (**D** and **E**) Interaction between miR-188-3p and GPX4 shown by dual-luciferase reporter assays. (**F** and **G**) Interaction betweenmiR-188-3p and GPX4 shown by RIP. (**H**) GPX4 mRNA levels were analyzed in U2OS, U2OS/DOX, HOS, and HOS/DOX cells. (**I** and **J**) GPX4 levels were detected via qPCR in U2OS/DOX and HOS/DOX cells following miR-NC, miR-188-3p, anti-miR-NC, or anti-miR-188-3p transfection. *P < 0.05.

miR-188-3p Targets GPX4 to Enhance OS Cell Sensitivity to DOX

To further investigate associations between miR-188-3p and GPX4 in the modulation of DOX resistance, DOX-resistant OS cells were transfected with miR-NC, miR-188-3p, miR-188-3p+pcDNA, or miR-188-3p+GPX4 constructs. As expected, GPX4 transfection was sufficient to reverse GPX4 inhibition observed in response to miR-188-3p over-expression (Figure 6A and B). Moreover, increased in the miR-188-3p levels reduced the DOX IC_{50} value, whereas this change was eliminated when these cells were simultaneously transfected with GPX4 (Figure 6C).

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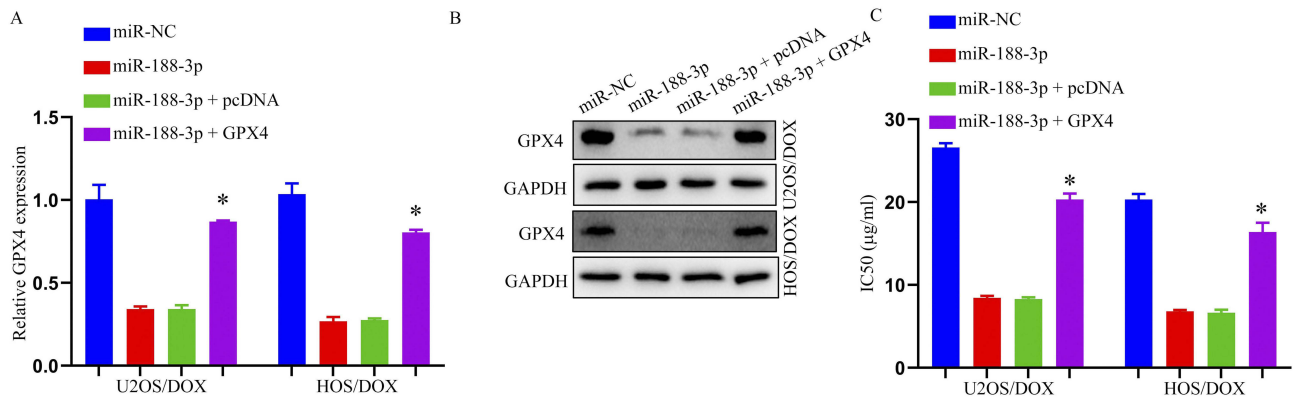

Figure 6 miR-188-3p targets GPX4 to suppress resistance to DOX. (**A** and **B**) GPX4 mRNA and protein levels shown by qPCR and Western immunoblotting. (**C**) DOX sensitivity evaluated by CCK-8 assays. *P < 0.05.

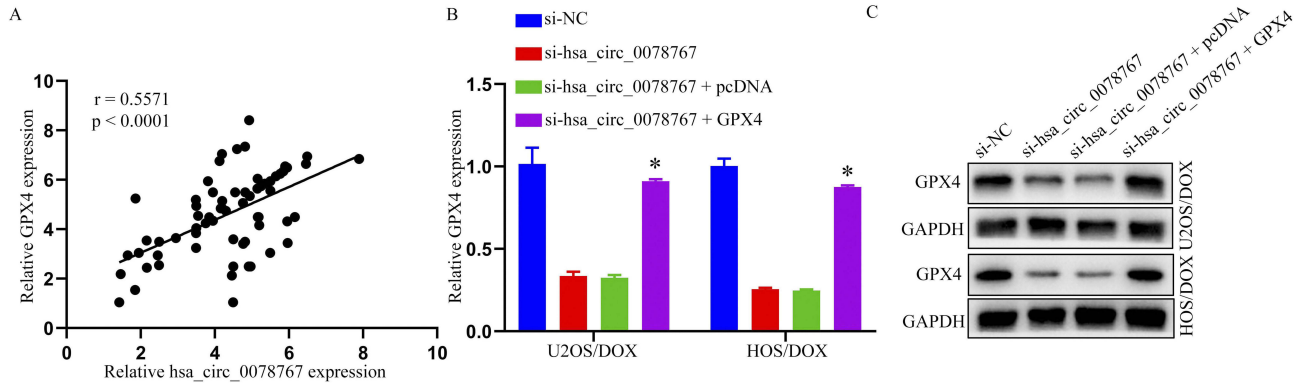

Figure 7 Hsa_circ_0078767 interacts with miR-188-3p to derepress the expression of GPX4. (**A**) Associations between hsa_circ_0078767 and GPX4 within DOX-resistant OS tissue samples were examined through Spearman correlation analyses. (**B** and **C**) GPX4 expression in U2OS/DOX and HOS/DOX cells following si-NC, si-hsa_circ_0078767, si-hsa_circ_0078767+anti-miR-NC, or si-hsa_circ_0078767+anti-miR-188-3p transfection, shown by qPCR and Western immunoblotting. *P<0.05.

Hsa_circ_0078767 Interacts with miR-188-3p to Derepress the Expression of GPX4

The levels of hsa_circ_0078767 and GPX4 were positively associated in OS patient tissue samples exhibiting DOX resistance (Figure 7A). To further determine the modulatory interactions between hsa_circ_0078767, miR-188-3p, and GPX4, DOX-resistant cells were next transfected with the si-NC, si-hsa_circ_0078767, si-hsa_circ_0078767+anti-miRNC, or si-hsa_circ_0078767+anti-miR-188-3p constructs. Inhibiting miR-188-3p was sufficient to reverse hsa_circ_0078767-mediated decreases in the expression of GPX4 (Figure 7B and C). Together, these data support a model wherein hsa_circ_0078767 sequesters mIR-188-2p, thereby indirectly derepressing the expression of its target mRNA GPX4.

Discussion

The emergence of chemoresistance remains a major barrier to effectively treating patients diagnosed with OS.[13] Adriamycin is often employed for the adjuvant treatment of localized high-grade OS cases.[14] DOX is a chemotherapeutic agent with robust antitumor activity that is widely used in clinical settings to treat diseases including OS and gastric, breast, and liver cancers.[15] Several circRNAs are reported to regulate chemoresistance in OS. One study, for example, identified hsa_circ_0001982 as a promoter of OS cell invasively, proliferative activity, and multidrug resistance.[16] Modulation of miR-342-3p/FBN1 by hsa_circ_0004674 was found to enhance DOX resistance in OS tumors,[17] whereas silencing circ_0081001 expression in OS cells can enhance their sensitivity to methotrexate treatment owing to its ability to regulate miR-494-3p/TGM2 signaling.[18] Building on these prior findings, high levels of hsa_circ_0078767 were observed in both OS tissues and cells resistant to DOX treatment. Strikingly, knocking down

hsa_circ_0078767 was sufficient to mitigate resistance to DOX in these cells while inhibiting their proliferative activity, enhancing DOX chemosensitivity, and driving higher rates of apoptotic death. These results support a model wherein higher levels of hsa_circ_0078767 expression is necessary to maintain OS cell resistance to DOX treatment, thus suggesting that knocking down hsa_circ_0078767 may represent a viable approach to overcoming chemoresistance in patients with OS.

It has been observed that miR-188-3p can reduce tumorigenesis is various cancers, including colon cancer,[8] hepatocellular carcinoma,[19] and cervical cancer.[20] Notably, reductions in the expression of miR-188-3p have been linked to chemoresistance in certain tumor types. In oral squamous cell carcinoma, for example, circ_0109291 can sponge miR-188-3p, thereby enhancing ABCB1 expression and contributing to the incidence of cisplatin resistance.[21] Through a similar mechanism, LINC00346 has been shown to sequester miR-188-3p, increasing BRD4 levels in pancreatic cancer cells and promoting proliferation and gemcitabine resistance.[22] These present analyses revealed that miR-188-3p levels were reduced in OS tissues and cells resistant to DOX, and the ability of anti-miR-188-3p to counteract the results of hsa_circ_0078767 silencing strongly suggests that this circRNA at least partially controls OS cell resistance to DOX by interacting with miR-188-3p.

Additional experimental analyses revealed GPX4 as a miR-188-3p target. As a phospholipid hydroperoxide glutathione peroxidase, GPX4 has recently been demonstrated to promote malignant behaviors in cancer through its ability to modulate ferroptotic induction. Specifically, GPX4 acts to suppress lipid peroxidation activity, thereby maintaining appropriate redox homeostasis within cells and protecting against the consequent induction of ferroptotic death.[23] High GPX4 expression levels have been reported in metastatic tumors and the expression of this gene is reportedly closely associated with disease progression.[24,25] Lu et al determined that KLF2 is capable of targeting GPX4 to regulate ferroptotic activity, thus reducing migratory activity and invasivity of renal cell carcinoma cells.[26] Through its ability to promote ferroptosis by targeting GPX4, miR-324-3p has also been shown to increase A549 lung adenocarcinoma cell sensitivity to cisplatin.[27] Moreover, one prior study found that OS chemosensitivity to cisplatin was enhanced following the impairment of the STAT3/Nrf2/GPX4 signaling axis and the consequent increase in the rate of ferroptotic death.[28] The present data build on these prior reports by demonstrating that miR-188-3p targets, increasing DOX sensitivity in OS cells.

Conclusion

In summary, these data offer novel evidence that hsa_circ_0078767 can act to enhance chemoresistant phenotypes in OS through the modulation of miR-188-3p/GPX4 signaling. As such, these results offer a foundation for future efforts aimed at the chemosensitization of OS tumors.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Acknowledgments

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Yin Tang and Yuzhe He contributed equally to this work and should be considered co-first authors.

Disclosure

The authors have no conflicts of interest to declare.

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