

PCAT7 Enhances Doxorubicin Resistance of Osteosarcoma by Modulating TGF- β Signalling

(PCAT7 / doxorubicin resistance / osteosarcoma)

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Abstract. Long noncoding RNAs (lncRNAs) are known to play critical roles in the progression of osteosarcoma. Despite their recognized importance, the specific biological functions of lncRNAs in osteosarcoma remain unclear. In this context, prostate cancer-associated transcript 7 (PCAT7) has been identified as a bone metastasis-related lncRNA through the analysis of The Cancer Genome Atlas dataset. In this study, we investigated the expression of PCAT7 in osteosarcoma cells, particularly those exhibiting resistance to doxorubicin, a widely used chemotherapeutic agent in clinic. Functional assays including cell growth, invasion and apoptosis were conducted to elucidate the impact of PCAT7 inhibition on osteosarcoma cells, focusing on sensitivity to doxorubicin treatment. To understand the underlying molecular mechanisms, the interaction between PCAT7, miR-324-5p, and the TGF- β /SMAD signalling pathway was further explored. The study revealed that PCAT7 is up-regu-

lated in osteosarcoma cells with doxorubicin resistance. Inhibition of PCAT7 could enhance the sensitivity to doxorubicin treatment by reducing cell growth, suppressing cell invasion and increasing cell apoptosis. Mechanistically, PCAT7 was shown to activate the TGF- β /SMAD signalling pathway by up-regulating the expression of TGFBR1 through sponging miR-324-5p. These findings unveil a novel mechanism contributing to the constitutive activation of TGF- β signalling in osteosarcoma. Targeting PCAT7 may offer a promising avenue for therapeutic interventions in osteosarcoma by disrupting the aberrant TGF- β signalling, thus presenting a potential strategy to improve treatment outcomes in this challenging cancer.

Introduction

Osteosarcoma (OS) is a rare and aggressive form of bone cancer that primarily affects the long bones, such as the arms and legs (Bian et al., 2023). It most commonly occurs in adolescents and young adults, but it can also affect individuals of any age. It is the second most fatal cancer in children and young adults, which is often associated with poor prognosis due to cancer progression and metastasis (Du et al., 2023). Treatment of osteosarcoma usually involves a combination of surgery, chemotherapy, and sometimes radiation therapy (Anninga et al., 2011).

Chemotherapy is often administered before and after surgery to shrink the tumour and eliminate any cancer cells that may have spread. Doxorubicin is a chemotherapy drug that is commonly used in the treatment of osteosarcoma (Yang and Zhang, 2013; Hurkmans et al., 2022). Doxorubicin is a cytotoxic drug that works by interfering with the DNA inside cancer cells. It inhibits the replication of cancer cells and induces cell death. However, several studies have reported doxorubicin resistance in osteosarcoma (Gallego et al., 2022; Garcia-

Received June 3, 2025. Accepted September 12, 2025.

This study is supported by Wuhan Municipal Health and Medical Research Fund (No. WX21Q26).

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Abbreviations: ceRNAs – competitive endogenous RNAs, EMT – epithelial-mesenchymal transition, lncRNAs – long non-coding RNAs, PCAT7 – prostate cancer-associated transcript 7, SMAD – Mothers against decapentaplegic homolog transcription factor, TGF- β – transforming growth factor beta, TGFBR1 – transforming growth factor beta receptor 1.

Ortega et al., 2022). As metastasis and drug resistance are both significant challenges in the treatment of osteosarcoma, our study investigated the factors that induce metastasis and doxorubicin resistance in osteosarcoma.

Long noncoding RNAs (lncRNAs) are a class of RNA transcripts with no protein-coding capacity that are greater than 200 nt in length. They regulate many biological processes through various mechanisms, including scaffolds or guides to regulate interactions between proteins and genes, decoys to bind proteins, and enhancers to modulate transcription of their target genes (Mathy and Chen, 2017; Peng et al., 2017). Notably, recent studies have identified that lncRNAs can function as competitive endogenous RNAs (ceRNA) and are able to serve as a 'miRNA sponge' to derepress miRNA-targeted mRNA expression (Qiu et al., 2020; Shen et al., 2020; Lin et al., 2021). Increasing evidence has indicated that lncRNAs play an important role in the metastasis of human cancers, including osteosarcoma (Chen et al., 2017; Hu et al., 2017; Li et al., 2020; Sun et al., 2020). The prostate cancer-associated transcript 7 (PCAT7) is a lncRNA that has been studied in the context of several cancers such as non-small cell lung cancer (NSCLC), breast cancer and prostate cancer. It has been found to play a role in the regulation of gene expression and is associated with the development and progression of tumours (Horie et al., 2017; Liu et al., 2017a, 2017b; Lang et al., 2020; Zhou et al., 2021; Geng et al., 2022). In NSCLC, PCAT7 is over-expressed and correlates with advanced TNM stage, histological grade, lymph node metastasis and poor prognosis; it enhances cell proliferation, migration and invasion while inhibiting apoptosis and inducing epithelial-mesenchymal transition (EMT) by sponging miR-134-5p (Liu et al., 2017b). In breast cancer, PCAT7 drives malignant progression by activating the ErbB/PI3K/Akt pathway, thereby potentiating cell proliferation, migration, invasion, and apoptosis suppression (Zhou et al., 2021). In prostate cancer with bone metastasis, PCAT7 forms a constitutive active loop with TGF- β /SMAD3/SP1 signalling, up-regulating transforming growth factor beta (TGF- β) pathway activity to enhance metastasis and drug resistance (Lang et al., 2020). However, there is no extensive research specifically linking PCAT7 to the drug resistance of osteosarcoma.

In this study, we demonstrated that PCAT7 was elevated in osteosarcoma with doxorubicin resistance. Further, we found that PCAT7 up-regulated transforming growth factor beta receptor 1 (TGFBR1) expression by sponging miR324-5p as a ceRNA, leading to unrestrained activation of the TGF- β pathway, which reciprocally promoted osteosarcoma progression, suggesting that PCAT7 can act as a potential therapeutic target for doxorubicin resistance of osteosarcoma.

Material and Methods

Cell culture and transfection

Human osteosarcoma cell line SaOS was purchased from iCell Bioscience Inc. (Shanghai, China). The SaOS

cells were maintained in RPMI1640 medium (GIBCO, Thermo Fisher Scientific, Waltham, MA) supplemented with 10 % foetal bovine serum (FBS, Biological Industries, Beit Haemek, Israel) plus 100 units/ml penicillin and 100 mg/ml streptomycin (Cell Research, Shanghai, China). Doxorubicin (Santa Cruz Biotechnology, Santa Cruz, CA) was dissolved in RPMI1640 at an initial concentration of 2.85 mg/ml and 0.1 mg/ml and stored at -20°C . Both concentrations were diluted in PBS as working concentrations. The doxorubicin-resistant cell line SaOS-DoxRes was established by gradual exposure to doxorubicin in a stepwise manner (from 5 nM to 100 nM) over a period of six months. The cells were incubated in drug-free medium for one week before use.

Plasmids with PCAT7 cDNA and miR-314-5p were constructed by amplification and introduction of PCAT7 and miR-314-5P cDNA sequences into the pGLV5 vector (ABM, Vancouver, Canada). The siRNA sequences targeting PCAT7 and shScr were purchased from Genepharma Co., Ltd (Shanghai, China). The Lipofectamine 2000 kit (Invitrogen, Waltham, MA) was used to transfect cells according to the manufacturer's protocol.

RNA extraction and quantitative real-time PCR

Total RNA extracted from tissues and cells was isolated with Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The isolated RNA was first reversely transcribed to cDNA using the PrimeScript RT reagent kit (Takara Bio, Japan) in compliance with the manufacturer's protocol. qRT-PCR was performed with SYBR Prime Script RT-PCR Kits (Takara Bio) based on the manufacturer's protocol. The primers were: PCAT7: forward 5'-AAACAAGCCAACCGCACAAT-3', reverse 5'-CCTGCTTGCTGTGTTACTGC-3'; miR-324-5p: forward, 5'-ACACTGCATCCTGGCAATTC-3'; reverse, 5'-CGTGGTGAATCGAGACTCAC-3'; all assays were performed in triplicate. The relative expression levels were first calculated using the $2^{-\Delta\Delta C_t}$ method and then normalized to the expression of GAPDH mRNA.

Immunoblot analysis

Cell protein lysates were separated by 10 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Roche) and then incubated with indicated antibodies including E-cadherin (1 : 1000, Cell Signaling Technology, Danvers, MA, #3195), vimentin (1 : 1000, Cell Signaling Technology #5741), pSMAD3 (1 : 500, Cell Signaling Technology #8769) and actin (1 : 3000, Proteintech, Rosemont, IL, #20536-1-AP). The incubation was performed with shaking overnight at 4°C , followed by HRP secondary antibodies. ECL chromogenic substrate was used for quantification by densitometry using the ImageJ software.

CCK-8 assay

The CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) was used to assess the viability of the cells, which were then seeded in a 96-well plate at a density of $1 \times$

10^4 cells per well. After being cultured for 24 h, the corresponding shPCAT7 and shScr were transfected and cultured in normal media. After adding the CCK-8 solution at 0 h, 24 h, 48 h, 72 h and 96 h after transfection, the relative number of cells was assessed by measuring at OD 570 nm. All assays were performed in triplicate.

Cell migration and invasion assays

Transwell chambers (8- μ m pore membrane filters; Costar, Corning, NY) and Matrigel invasion (Becton Dickinson, Franklin Lakes, NJ) were used to measure the SaOS cell migration and invasion. Forty-eight h after transfection, the cells were cultured in serum-free medium for 24 h before being transferred into the upper chamber without or with 10 μ g/ml Matrigel. Medium with 10 % FBS was added into the lower chamber. The remaining cells on the upper surface of the filter were wiped off with a cotton swab, while those that had migrated were fixed in methanol, stained with 0.1 % crystal violet and counted using a microscope in 10 random fields per filter.

Statistical analysis

All statistical analyses were conducted using the SPSS 22.0 software (SPSS Inc., Chicago, IL). Two-group comparisons were made using the *t*-test and one-way ANOVA, and the χ^2 test was used to analyse multiple group comparisons, where $P < 0.05$ was considered statistically significant.

Results

PCAT7 is up-regulated and enhances doxorubicin resistance in osteosarcoma cells

To confirm the doxorubicin resistance of SaOS-DoxRes cells compared to the parental SaOS cells, cell proliferation was examined by the CCK-8 assay. As shown in

Fig. 1A, the proliferation of SaOS-DoxRes cells was significantly higher than that of parental SaOS cells after four days of treatment with doxorubicin (100 nM). To assess the expression of PCAT7 in the progression of OS, we isolated total RNA from parental SaOS and SaOS-DoxRes cells; qPCR results showed that the expression of PCAT7 at the mRNA level significantly increased in the SaOS-DoxRes cell line (Fig. 1B). Immunoblotting results indicated that E-cadherin expression was down-regulated while vimentin levels were up-regulated in SaOS-DoxRes cells compared to parental SaOS cells (Fig. 1C), suggesting that doxorubicin resistance is related to TGF- β signalling in osteosarcoma.

Knockdown of PCAT7 suppressed the proliferation, migration and invasion of osteosarcoma cells

To investigate the effects of PCAT7 on SaOS-DoxRes cells, PCAT7 shRNA was designed and transfected into the SaOS-DoxRes cells. PCAT7 expression was significantly decreased in the shPCAT7 cells compared to shScr (scrambled) control by qPCR (Fig. 2A). Interestingly, the resistance to doxorubicin of the shPCAT7 cells was dramatically decreased and cell proliferation was remarkably slower than in the shScr control group by the CCK-8 assay, indicating that PCAT7 plays a critical role in doxorubicin resistance of SaOS-DoxRes cells (Fig. 2B). Additionally, the migration and invasion capabilities of SaOS-DoxRes cells were decreased by PCAT7 depletion (Fig. 2C and D). PCAT7 depletion resulted in increased E-cadherin expression and reduced vimentin expression in SaOS-DoxRes cells by immunoblot (Fig. 2E). Taken together, PCAT7 knockdown suppressed proliferation, inhibited migration and invasion, and reversed the TGF- β signalling in SaOS-DoxRes cells.

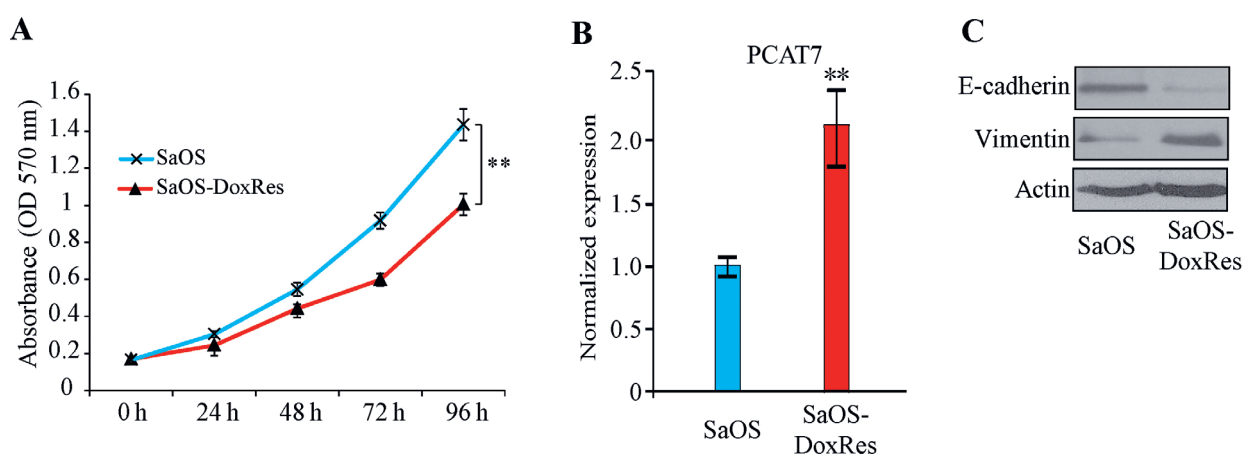


Fig. 1. PCAT7 expression was up-regulated in the doxorubicin resistant SaOS-DoxRes cells compared to the parental cells. (A) Cells were treated with doxorubicin (100 nM) for 0 h, 24 h, 48 h, 72 h and 96 h. Then the cell viability was evaluated using the CCK-8 assay. (B) Quantitative real-time PCR detection of PCAT7 expression in MG-63 and MG-63/Dox cells. Data are expressed as mean \pm SD (N = 3). ** $P < 0.01$ compared with SaOS cells. (C) Western blot for E-cadherin, vimentin and N-cadherin protein expression in SaOS and SaOS-DoxRes cells. For all panels, data are shown as mean \pm SD (N = 3). ** $P < 0.01$ compared with SaOS cells.

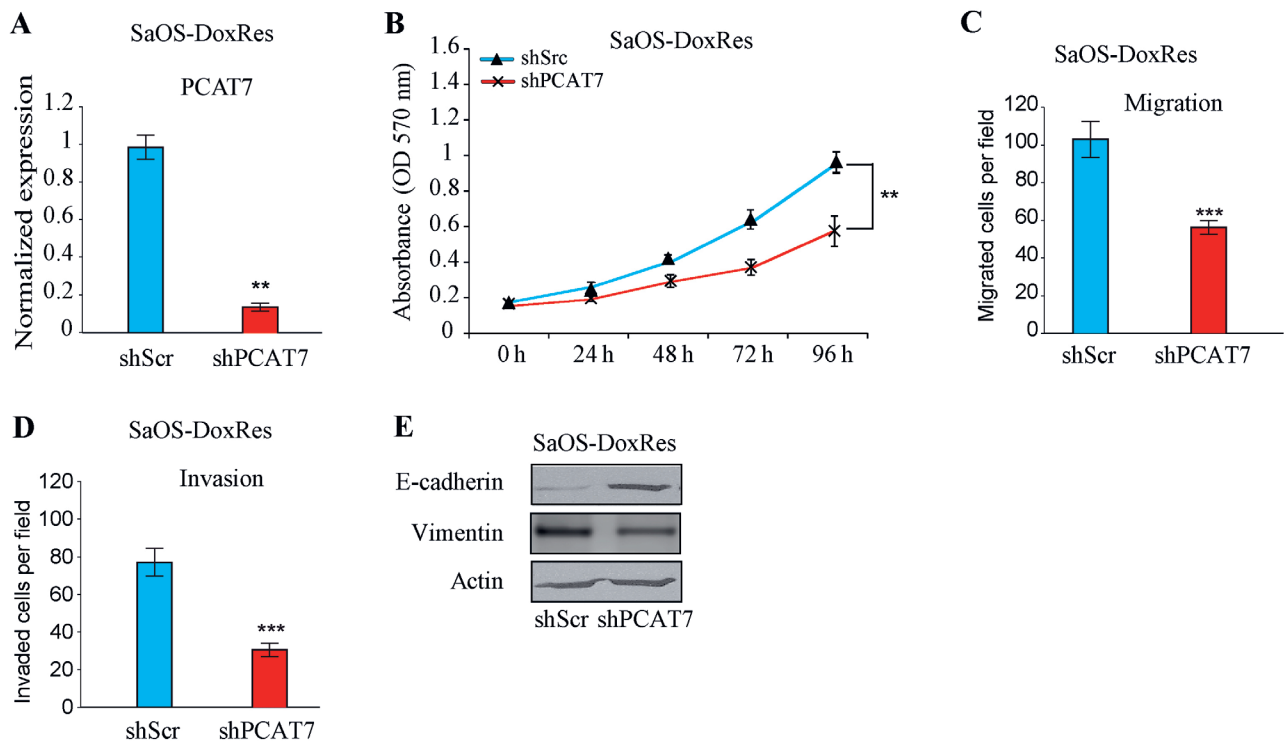


Fig. 2. Depletion of PCAT7 reduced proliferation, migration and invasion of SaOS-DoxRes cells. **(A)** qPCR of PCAT7 expression in SaOS-DoxRes cells transfected with shPCAT7 or shScr for 48 h. **(B)** shPCAT7-transfected cells were treated with doxorubicin (100 nM) for 0 h, 24 h, 48 h, 72 h and 96 h. Then the cell viability was evaluated using the CCK-8 assay. **(D–E)** Analysis for migration and invasion of the transfected SaOS-DoxRes cells by Transwell assay. **(F)** Western blot for E-cadherin and vimentin in SaOS-DoxRes cells transfected with shPCAT7 or shScr for 48 h. Data are expressed as mean \pm SD (N = 3). **P < 0.01 compared with the shScr group. For all panels, data are shown as mean + SD (N = 3). ***P < 0.01 compared with the shScr group.

PCAT7 directly targets miR-324-5p and promotes doxorubicin resistance of osteosarcoma by activating TGF- β signalling

To explore the targets of PCAT7, miRanda and TargetScan algorithms were employed to predict the downstream target of PCAT7; miR-324-5p was then followed for further analysis (Fig. 3A). Dual-luciferase reporter assay showed the binding sites of PCAT7 and miR-324-5p. After over-expression of miR-324-5p, the luciferase activity of PCAT7 was significantly inhibited. However, miR-324-5p over-expression exhibited limited effects on the luciferase activity of mutant PCAT7 (Fig. 3B). Additionally, PCAT7 activated TGF- β signalling by sponging miR-324-5p, as evidenced by increased pSMAD3 levels and luciferase activity of the *SMAD3* promoter (Fig. 3C–3D). To further validate PCAT7's activation of the TGF- β pathway, we knocked down SMAD3 in SaOS-DoxRes cells. Western blot confirmed SMAD3 knockdown and reduced pSMAD3 (Fig. 3E). Co-transfection of shSMAD3 with PCAT7 over-expression in SaOS cells abrogated the PCAT7's effects on proliferation, migration and invasion (Fig. 3F–3H). Thus, SMAD3 is crucial for PCAT7-mediated TGF- β activation and doxorubicin resistance in osteosarcoma.

PCAT7 exerts effects on SaOS-DoxRes cells by suppressing miR-324-5p expression

To confirm the effects of PCAT7 and miR-324-5p on SaOS-DoxRes cells, we performed the rescue assay. MiR-324-5p expression was significantly up-regulated after transfection with shPCAT7 in SaOS-DoxRes cells. In addition, a specific inhibitor targeting miR-324-5p reversed the shPCAT7-induced miR-324-5p expression (Fig. 4A). Furthermore, miR-324-5p inhibition reversed the effects of shPCAT7 on cell proliferation, migration, invasion, and expression of TGF- β signalling markers in SaOS-DoxRes cells (Fig. 4B–E). Thus, miR-324-5p could rescue the effect of shPCAT7 on cell proliferation in SaOS-DoxRes cells.

PCAT7 regulates through the PCAT7/miR-324-5p/TGFBR1 axis

Next, we found that TGFBR1 expression at both mRNA and protein levels was highly up-regulated in SaOS-DoxRes cells compared to SaOS cells (Fig. 5A and B). Subsequently, luciferase reporter assays revealed that miR-324-5p mimics reduced whereas miR-324-5p inhibitor enhanced the luciferase activity of wild-type 3'-UTR

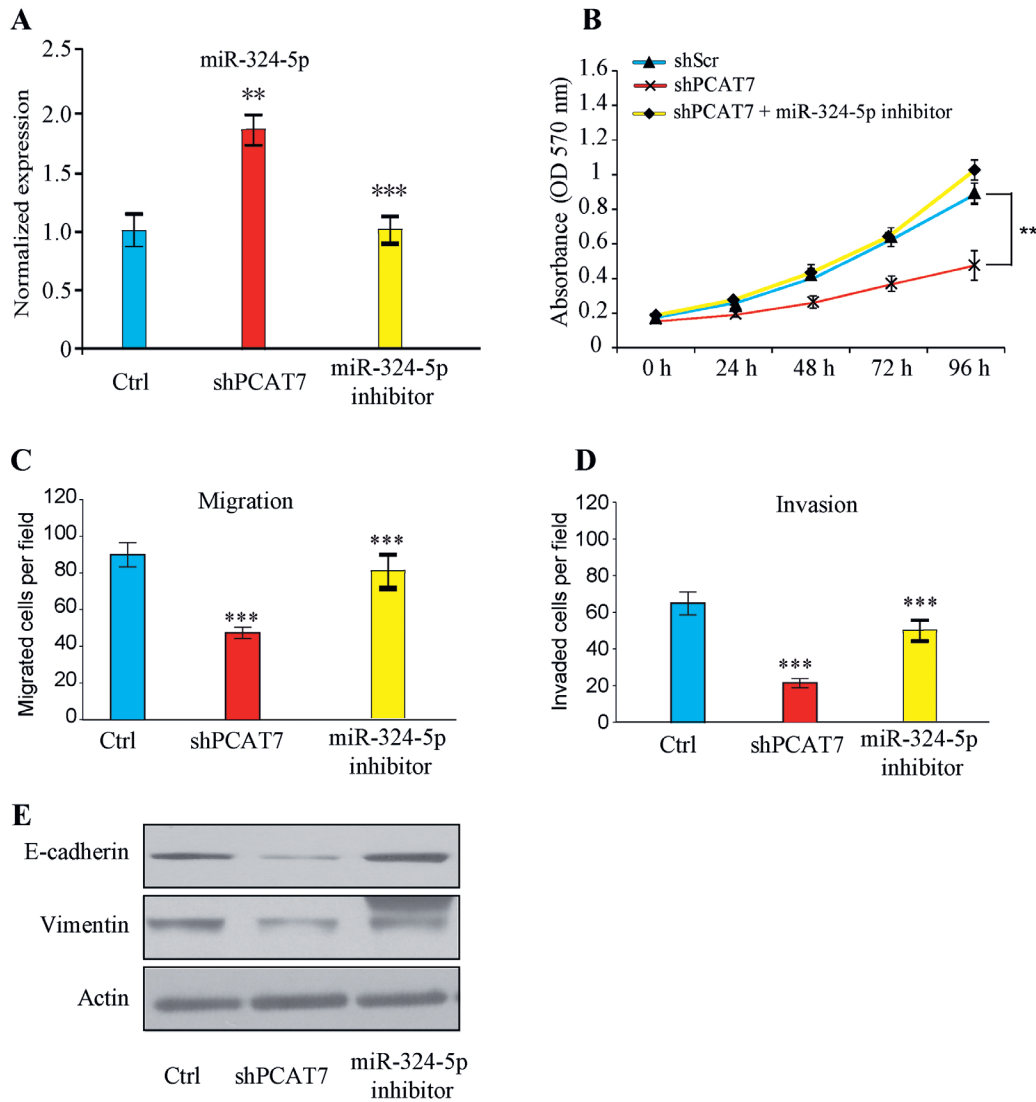


Fig. 4. PCAT7 regulated proliferation, migration and invasion in SaOS-DoxRes cells via binding with miR-324-5p. **(A)** Quantitative real-time PCR detection of miR-324-5p expression in SaOS-DoxRes cells after transfection of shPCAT7, shScr or miR-324-5p inhibitor. **(B)** CCK-8 assay for assessing cell proliferation of SaOS-DoxRes cells after transfection of shPCAT7, shScr or miR-324-5p inhibitor. **(C–D)** Transwell assay for assessing cell migration and invasion of SaOS-DoxRes cells after transfection of shPCAT7, shScr or miR-324-5p inhibitor. **(E)** Western blot for E-cadherin and vimentin expression in SaOS-DoxRes cells after transfection of shPCAT7, shScr or miR-324-5p inhibitor. Data are expressed as means \pm SD (N = 3). *P < 0.05, **P < 0.01 compared with the shScr group. For all panels, data are shown as mean + SD (N = 3). *P < 0.05, **P < 0.01 compared with the shScr group.

2016; Chen et al., 2017). Several lncRNAs have been reported to be differentially expressed in doxorubicin-resistant osteosarcoma cells and tissues (Kun-Peng et al., 2017; Wang et al., 2017; Zhang et al., 2017; Zhu et al., 2017; Zhou et al., 2018). In this study, we established the doxorubicin-resistant SaOS-DoxRes cell line to examine the PCAT7-mediated doxorubicin resistance in osteosarcoma. PCAT7 has been reported to be an oncogene in multiple cancers including osteosarcoma (Horie et al., 2017; Liu et al., 2017a, 2017b; Lang et al., 2020; Zhou et al., 2021; Geng et al., 2022). Consistent with previous studies, we found that PCAT7 contributed to doxorubicin resistance in osteosarcoma. PCAT7 expression was high-

er in SaOS-DoxRes cells than in parental SaOS cells. Knockdown of PCAT7 inhibited cell proliferation, migration and invasion in SaOS-DoxRes cells.

In recent years, the hypothesis of ceRNAs related to lncRNAs has received widespread attention in the research of disease pathology. By binding to miRNAs, lncRNAs can modulate the expression and function of miRNA-targeted mRNAs (Yoon et al., 2014). Previous studies have demonstrated that PCAT7 regulates a number of miRNAs and mRNAs in various diseases (Liu et al., 2017a, 2017b; Wang et al., 2017; Lang et al., 2020; Zhou et al., 2021; Geng et al., 2022). For example, Zhou et al. (2021) reported that lncRNA PCAT7 promotes the

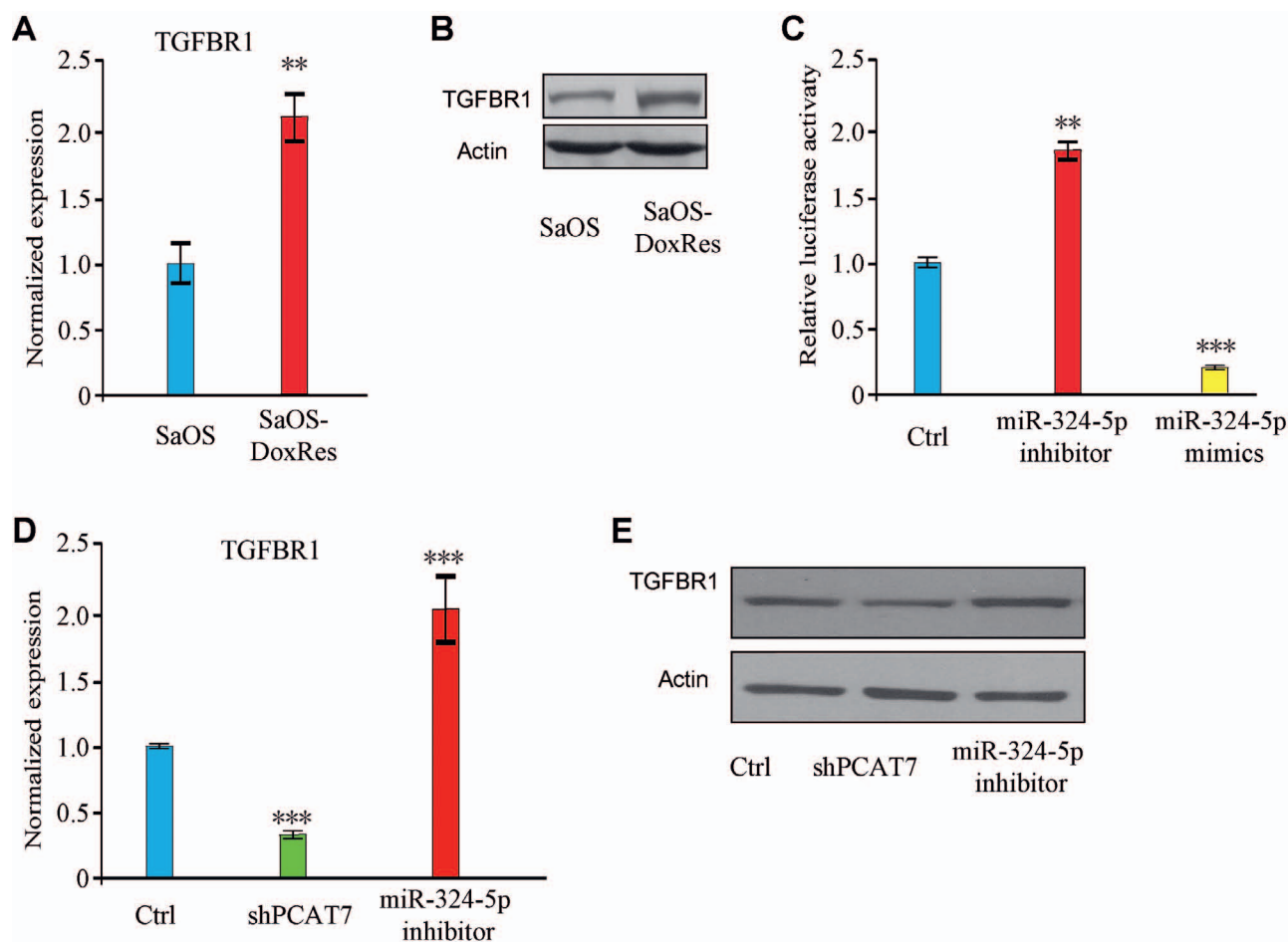


Fig. 5. PCAT7 promotes TGFBR1 expression to activate TGF- β signalling via sponging miR-324-5p. (A–B) qPCR (A) and immunoblot analysis (B) of TGFBR1 expression in the parental SaOS cells and SaOS-DoxRes cells. (C) Luciferase reporter assay for the luciferase activity of TGFBR1 expression in the indicated groups. Data are shown as mean + SD. * $P < 0.05$. (D–E) qPCR (D) and immunoblot analysis (E) of TGFBR1 expression in the cells transfected with shPCAT7, miR-324-5p mimics as indicated. For all panels, data are shown as mean + SD (N = 3). * $P < 0.05$.

malignant progression of breast cancer by regulating the ErbB/PI3K/Akt pathway. In nasopharyngeal carcinoma, lncRNA PCAT7 regulates ELF2 signalling through inhibition of miR-134-5p (Liu et al., 2017a).

The SMAD3/SP1 complex-mediated constitutive active loop between lncRNA PCAT7 and TGF- β signalling promotes prostate cancer bone metastasis (Lang et al., 2020). Similarly, lncRNA PCAT7 promotes non-small cell lung cancer progression by activating the miR-486-5p/CDK4 axis-mediated cell cycle (Geng et al., 2022). Therefore, PCAT7 might play a critical role in doxorubicin resistance in osteosarcoma similarly as the other types of cancers. However, the detailed mechanism remains unknown.

In this study, we confirmed that miR-324-5p is the target of PCAT7. MiR-324-5p has been found to be down-regulated in various cancers (Zhi et al., 2017; Tang et al., 2018; Zheng et al., 2018; Fan et al., 2020; Huang et al., 2020; Liu et al., 2020; Ghatak et al., 2021; Jiang et al., 2021; Zhang et al., 2023). However, there are few reports about the role of miR-324-5p in osteosarcoma. A couple

of studies found that miR-324-5p was altered in osteosarcoma cells (Dai et al., 2013; Ma et al., 2019). In line with the previous study in various cancers, our study showed that miR-324-5p was suppressed in SaOS-DoxRes cells compared to parental SaOS cells. The miR-324-5p inhibitor abolished the inhibitory effects of PCAT7 on cell proliferation, migration and invasion in SaOS-DoxRes cells. Thus, these data indicate that PCAT7 promotes doxorubicin resistance in osteosarcoma cells by targeting miR-324-5p. Furthermore, TGFBR1 was identified downstream of the PCAT7/miR-324-5p axis in this study. As mentioned before, TGFBR1 has been reported to enhance cancer progression and drug resistance in several cancers, which eventually leads to poor prognosis (Petiti et al., 2018; Guillen Diaz-Maroto et al., 2019; Principe et al., 2019; Song et al., 2019). Similar to its role in other malignancies, we found that the expression of TGFBR1 was up-regulated in SaOS-DoxRes cells compared to parental SaOS cells, and its over-expression could reverse the effects of PCAT7 inhibition on doxorubicin resistance. Overall, the results revealed that

PCAT7 promoted doxorubicin resistance via the miR-324-5p/TGFBR1 axis in SaOS-DoxRes cells.

This is a preliminary study on the role of PCAT7 in resistance. Given the multitude of signalling pathways regulating tumour proliferation and metastasis (Ren et al., 2023), further research is needed to explore the involvement of PCAT7 in tumour resistance. Another limitation of this study is the lack of *in vivo* animal model data to validate the findings observed in the cell lines. Future studies using animal models are necessary to confirm the role of the PCAT7/miR-324-5p/TGFBR1 axis in doxorubicin resistance and its potential therapeutic implications in osteosarcoma.

To conclude, our study explains the mechanisms by which the PCAT7/miR-324-5p/TGFBR1 axis influences doxorubicin resistance in SaOS-DoxRes cells. Modulation of this axis could be utilized for novel adjuvant treatment to anti-osteosarcoma chemotherapy.

Consent for publication

All the authors agreed to publish this work.

Availability of data and material

All the data can be required reasonably.

Conflict of interests

All the authors declared that there is no competing interest.

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