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LncRNA PCAT-1 promotes the progression of osteosarcoma *via* miR-508-3p/ZEB1 axis

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is an adolescent idiopathic malignancy with a poor prognosis. Accumulating evidence has verified that long non-coding RNAs (lncRNAs) were implicated in the initiation and development of various tumors. We aimed to clarify the functions and underlying mechanism of lncRNA PCAT-1 in OS progression.

PATIENTS AND METHODS: RT-qPCR was performed to examine the relative expressions of PCAT-1, miR-508-3p and ZEB1 in OS tissues or cells. The proliferation capacities of OS cells with different transfection were detected by CCK-8 assays. Transwell assays were carried out to determine the functions of PCAT-1 and miR-508-3p in OS cell migration and invasion. Moreover, bioinformatical analysis and Luciferase reporter assay were applied to verify the association between PCAT-1 and miR-508-3p, miR-508-3p and ZEB1.

RESULTS: Data of current study revealed that PCAT-1 was markedly upregulated in OS, which indicated poor prognosis of OS patients. CCK-8 and transwell assays indicated that PCAT-1 upregulation could promote OS cell proliferation, invasion and migration. Additionally, we found that miR-508-3p was a direct target of PCAT-1, and PCAT-1 regulated the development of OS via decreasing miR-508-3p and activating its target gene ZEB1.

CONCLUSIONS: All data demonstrated that PCAT-1 promoted OS progression, and miR-508-3p/ZEB1 axis was implicated in the functional roles of PCAT-1 in OS, suggesting that PCAT-1/miR-508-3p/ZEB1 might serve as candidate therapeutic targets for OS patients.

Key Words:
Osteosarcoma, LncRNA PCAT-1, MiR-508-3p, ZEB1.

Introduction

Osteosarcoma (OS) is one common invasive malignant bone tumor, accounting for approximately 1% of all newly diagnosed malignancies each year[1]. OS usually occurs in the extremity of the long bones, with high incidences especially in children and adolescents[2]. Thanks to the usage of adjuvant and neoadjuvant chemotherapy after operation, survival rate for OS patients without metastasis has increased to 70%[3]; unfortunately, survival rate is fairly low and the survival for metastatic patients is far from satisfactory after recurrence and metastasis[4]. Moreover, the specific molecular mechanisms of tumorigenesis are still unclear.

A series of external and internal factors are involved in OS progression. In OS patients, the genome is extremely unstable, and the mutation of tumor suppressors or activation of oncogenes has been considered to be part of the causes of OS[5]. Above 90% of the human genomes are widely transcribed, whereas only about 2% of them are protein-coding genes[6]. Non-coding RNA (ncRNA)s without protein coding abilities accounts for most of the remaining transcripts. According to the transcript sizes, ncRNAs included long non-coding RNA (LncRNA), microRNA (MiRNA) and small ncRNA.

LncRNAs are a class of RNAs with a length of more than 200 nucleotides[7]. LncRNA has no ability to encode proteins, but it can regulate a group of physiological and pathological processes via regulation of transcription and/or post-transcription[8,9]. LncRNA plays an inhibitory or carcinogenic role in various tumors through a variety of molecular mechanisms, such as interaction with miRNA, messenger RNA (mRNA) or protein, mRNA splicing and epigenetic silencing[10,11]. Many up-regulated or down-regulated lncRNAs have been identified in OS, which exerted important effects on tumorigenicity[12,13].

Unlike lncRNA, miRNA is a kind of evolutionarily conserved endogenous non-coding RNA,

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typically 21-24 nucleotides in length, and essentially regulates post-transcription of mRNA[14]. miRNA exerts crucial functions in varieties of molecular and cellular biological processes, including carcinogenesis[15-17]. As for OS, multiple dysregulated miRNAs serve as key contributing factors for the malignant characteristics of tumors[18-20]. Accordingly, targeted therapies based on lncRNA and miRNA may be a promising strategy against OS.

In the current study, we investigated the functional roles of lncRNA PCAT-1 in OS and explored the interaction between PCAT-1 and its target miR-508-3p *in vitro*. The results indicated that PCAT-1 acted as an oncogene in OS by sponging miR-508-3p and regulating its downstream target gene ZEB1.

Patients and Methods

Clinical Samples

49 OS tissues and matched normal tissue samples were from OS patients undergoing surgical excision in Jiyang People's Hospital. All tissues were obtained from the enrolled patients with the written informed consents. No patient had received chemotherapy or radiotherapy. The collected tissues were frozen in liquid nitrogen for further use immediately after the surgical operation. The current investigation was approved by the Ethics Committee of Jiyang People's Hospital. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

Two human OS cells (U2OS and MG-63) and osteoblastic cell line hFOB 1.19 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All the cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Invitrogen, Carlsbad, CA, USA) in a humidified chamber containing 5% CO_2 at 37°C.

Cell Transfection

pcDNA-PCAT-1, pcDNA-ZEB1, empty pcDNA vector (vector), siRNA targeting PCAT-1, miR-508-3p mimic/inhibitor and their corresponding negative controls (NC) were obtained from GenePharma Co., Ltd., (Shanghai, China). Then, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect the plasmids into OS cells. The efficiencies were validated using qPCR 48h post the transfection.

RT-qPCR

Total RNA was isolated from OS cell lines and tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the PrimerScript RT Reagent Kit (TaKaRa, Dalian, Japan). Later, PCR was performed using the SYBR Premix Ex Taq II (TaKaRa, Dalian, Japan) on the ABI7500 system (Applied Biosystems, Foster City, CA, USA). The expression levels were normalized to U6 (for miR-508-3p) or GAPDH (for PCAT-1 or ZEB1). The expression level was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation ability was assessed by CCK-8 assay. The transfected OS cells were seeded into 96-well plates and cultured for 24, 48, 72, 96 h. At the time of harvest, 10 μl CCK-8 was added to each well and cultured for another 4h. Cell viability was determined by examining the OD_{450} using a microplate reader (Olympus Corp., Tokyo, Japan).

Transwell Assay

The cell invasion and migration potentials were evaluated by transwell assays. OS cell suspension in FBS-free medium was inserted into the top chamber of transwell device (pre-coated with Matrigel for invasion assay; Corning, Corning, NY, USA). Meanwhile, complete medium was added to the bottom chamber. After incubation for 48 h, the cells remained in the top chamber were removed and those adhered to the bottom chamber were fixed and stained. Finally, the number of invasion or migration cells was quantified under a light microscope (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Assay

The wild-type (WT) or mutant (MUT) sequences of PCAT-1 or ZEB1 were inserted into pGL3 vectors (Promega, Beijing, China) to obtain WT/MUT-PCAT-1 or -ZEB1. Then, miR-508-3p mimic was co-transfected into OS cells with the above reporter plasmids. After 48 h of transfection, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was applied to evaluate the luciferase activity.

Statistical Analysis

All data were from at least 3 independent experiments. SPSS software version 17.0 (SPSS

Inc., Chicago, IL, USA) was utilized to complete data analysis. Student's *t*-test or one way ANOVA followed by Tukey's post-hoc test was used to analyze difference between 2 or multiple groups. Significant difference was defined as $p< 0.05$.

Results

Increased PCAT-1 In OS Indicated Poor Prognosis

First, PCAT-1 levels in OS tissues were measured by RT-qPCR. We found that PCAT-1 expressions in OS tissues were significantly increased compared to those in corresponding normal tissues (Figure 1A). Similarly, upregulated expressions of PCAT-1 in OS cells were also indicated by RT-qPCR (Figure 1B). As the increased PCAT-1 levels in OS have been observed, we further investigated its prognostic value in OS. The enrolled patients were assigned into two groups based on the median PCAT-1 level and Kaplan-Meier analysis demonstrated that patients in high PCAT-1 level group presented poorer survival (Figure 1C).

PCAT-1 Promoted OS Cell Proliferation, Invasion and Migration

To investigate the functional roles of PCAT-1 in OS development, pcDNA-PCAT-1or si-PCAT-1 was transfected into U2OS or MG63 cells to enhance or knockdown PCAT-1 expression. The successful transfection was indicated by a further RT-qPCR analysis (Figure 2A and 2B). Results of CCK-8 assay disclosed that elevated PCAT-1 expressions in U2OS cells significantly promoted cell proliferation whereas PCAT-1 knockdown markedly suppressed MG63 cell proliferation (Figure 2C and 2D). Transwell invasion and migration assays were further carried out to explore the biological functions of PCAT-1 in OS cell invasion and migration. As expected, PCAT-1 overexpression significantly promoted U2OS cell invasion and migration whereas PCAT-1 downregulation strikingly inhibited MG63 cell invasion and migration (Figure 2E and 2F). Therefore, these findings showed that PCAT-1 may play oncogenic roles in OS progression.

PCAT-1 Functioned as a Sponge for MiR-508-3p

Bioinformatics analysis was performed using starBase version 2.0 (http://starbase.sysu.edu.cn/) to predict potential target miR of PCAT-1, which may be involved in the mechanisms underlying the oncogenic roles of PCAT-1 in OS progression. As shown in Figure 3A, PCAT-1 contained complementary binding sites of miR-508-3p. Then, Dual-Luciferase reporter assay was performed to verify the association between PCAT-1 and miR-508-3p. Results revealed that the Luciferase activity of WT-PCAT-1 vector was considerably decreased by miR-508-3p mimic while the Luciferase activity of MUT-PCAT-1 vector was not significantly influenced (Figure 3B). Moreover, RT-qPCR analysis demonstrated that miR-508-3p in pcDNA-PCAT-1 transfected U2OS cells was prominently downregulated and remarkably increased by si-PCAT-1 in MG63 cells (Figure 3C). In addition, miR-508-3p mimic could reduce PCAT-1 expressions and miR-508-3p inhibitor enhanced PCAT-1 expressions (Figure 3D). Taken together, these results indicated the direct interaction between PCAT-1 and miR-508-3p. miR-508-3p expressions in OS tissues were mark-

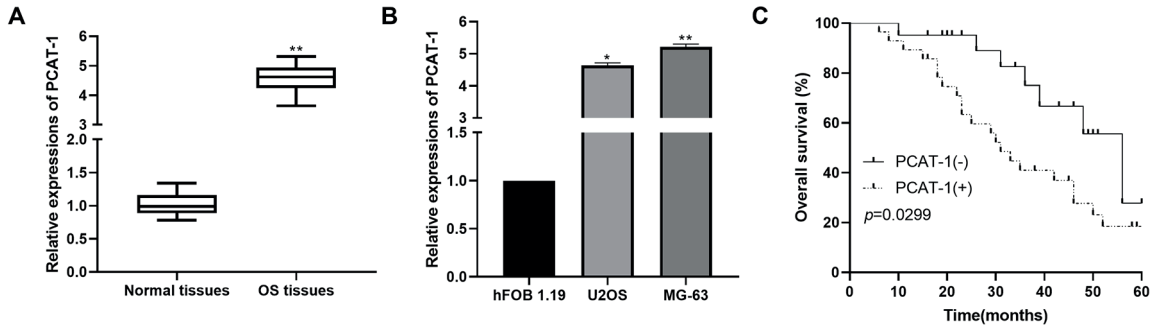

Figure 1. Increased PCAT-1 in OS indicated poor prognosis. **A**, PCAT-1 was upregulated in OS tissues. **B**, Elevated PCAT-1 expressions in OS cells were detected by RT-qPCR. **C**, Kaplan-Meier analysis of patients with high and low PCAT-1 expressions. *p<0.05, **p<0.01.

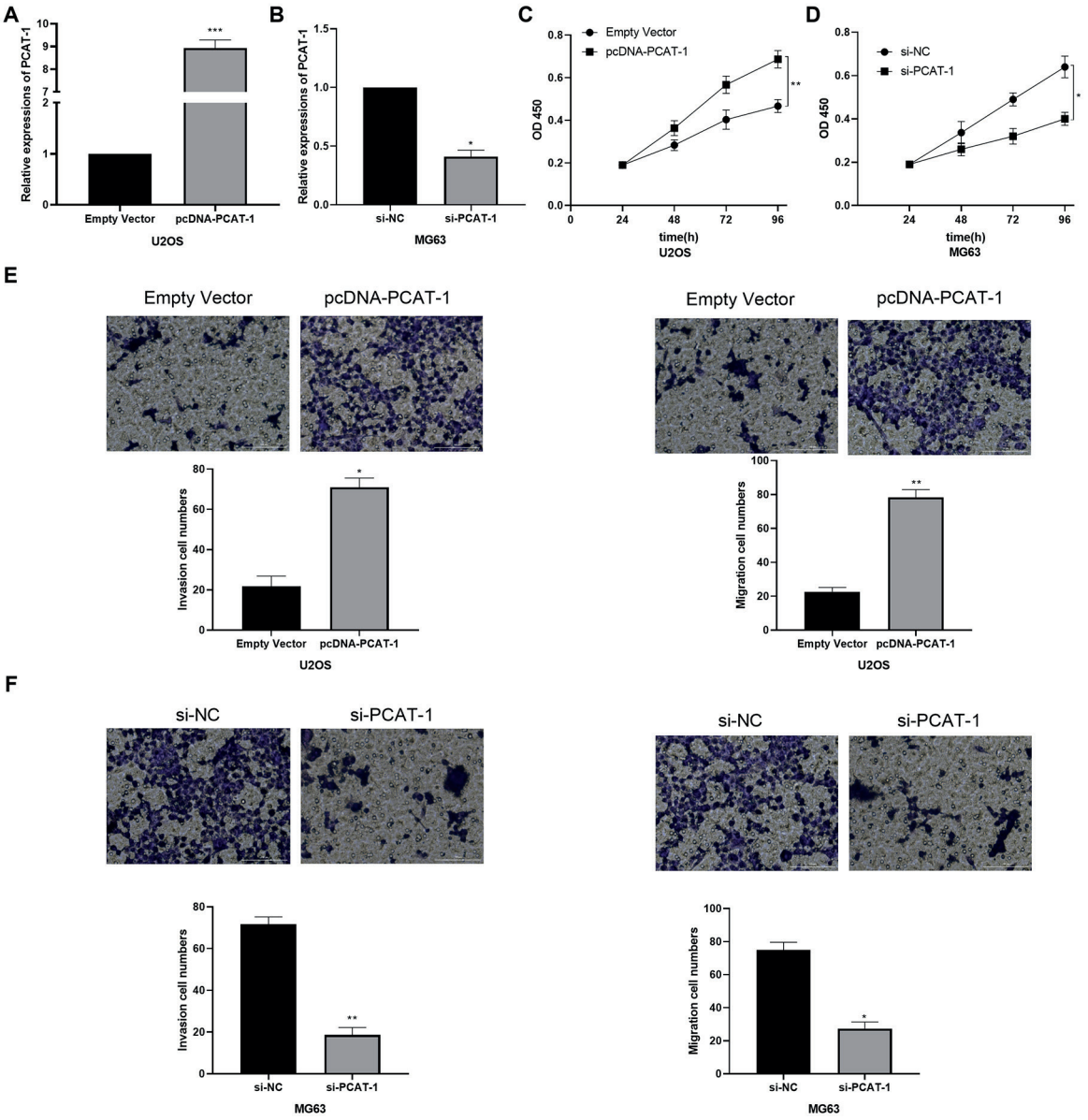

Figure 2. PCAT-1 promoted OS cell proliferation, invasion and migration. **A, B,** RT-qPCR analysis was performed to confirm the overexpression or knockdown of PCAT-1. **C, D,** PCAT-1 overexpression promoted while PCAT-1 knockdown inhibited OS cell proliferation. **E, F,** The influence of PCAT-1 on OS cell invasion and migration were detected by transwell assays, (magnification 100X). *p<0.05, **p<0.01, ***p<0.001.

edly downregulated and inversely correlated with PCAT-1 expressions (Figure 3E and 3F).

MiR-508-3p Inhibited OS Cell Proliferation, Invasion and Migration

We further investigated the potential roles of miR-508-3p in OS progression. As demonstrated by RT-qPCR results, miR-508-3p levels in OS cells were significantly decreased (Figure 4A). Then, miR-508-3p mimic or inhibitor was transfected into OS cells and the successful overexpression or knockdown of miR-508-3p in MG63 or U2OS cells was also confirmed by RT-qPCR (Figure 4B). Functional assays, including CCK-8 and transwell assays, demonstrated that elevated miR-508-3p markedly repressed MG63 cell proliferation, invasion and migration; on the other hand, miR-508-3p

inhibitor posed the opposite impact on U2OS cells (Figure 4C and 4D).

MiR-508-3p Targeted ZEB1 in OS

The probable targets of miR-508-3p were searched by TargetScan. ZEB1 was identified as a candidate target of miR-508-3p (Figure 5A). Dual-Luciferase reporter assay indicated that miR-508-3p overexpression led to a significantly decrease in Luciferase activity of WT-ZEB1 vector, but had no evident influence on Luciferase activity of MUT-ZEB1 (Figure 5B). Moreover, ZEB1

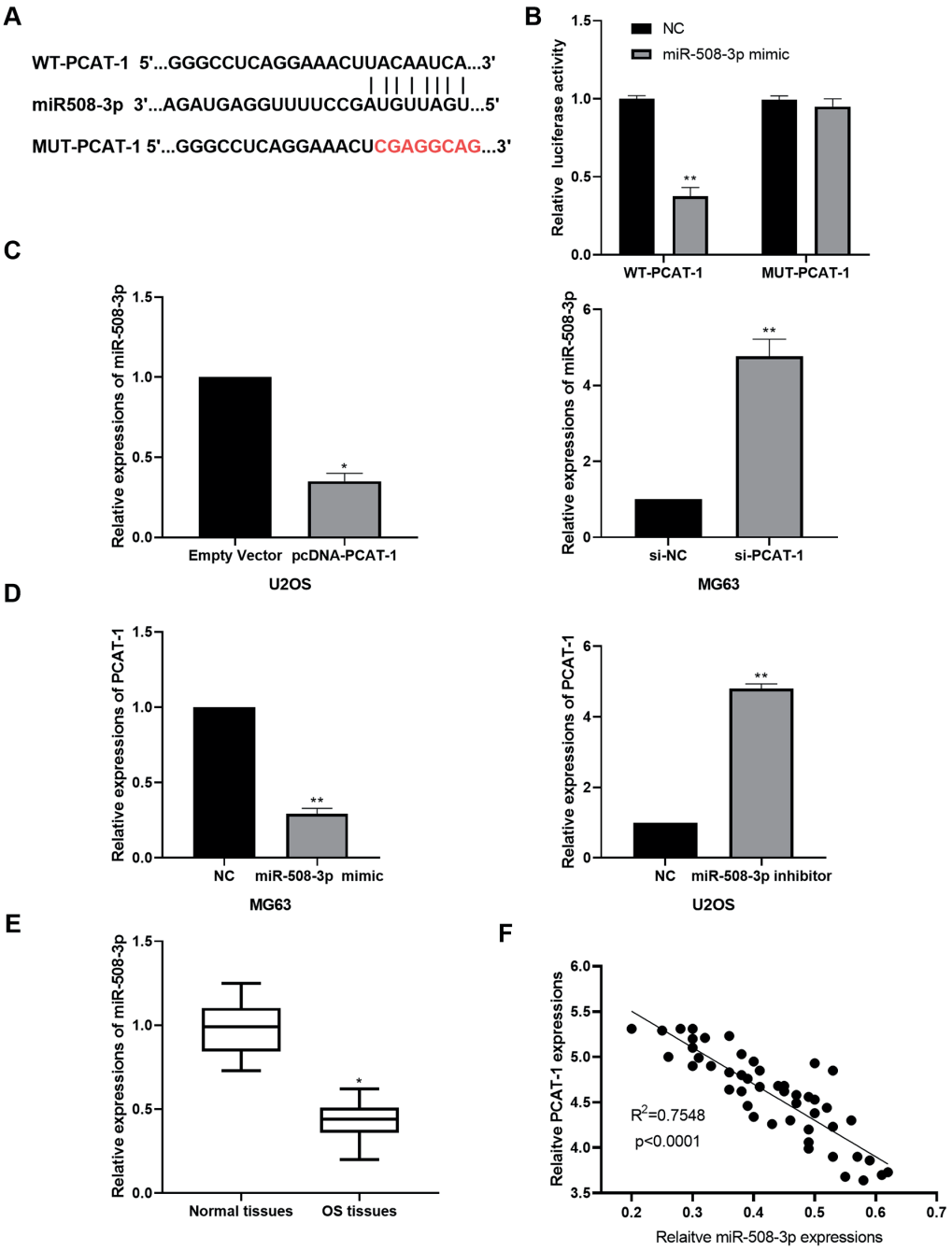

Figure 3. PCAT-1 functioned as a sponge for miR-508-3p. **A**, The potential binding sites of PCAT-1 on miR-508-3p were shown. **B**, Luciferase activity of cells which were co-transfected with miR-508-3p mimic and WT/MUT-PCAT-1 were detected. **C, D,** MiR-508-3p expressions were regulated by PCAT-1. **E**, MiR-508-3p expressions in OS tissues were significantly downregulated. **F**, MiR-508-3p was inversely correlated with PCAT-1 expressions in OS tissues. **p*<0.05, ***p*<0.01.

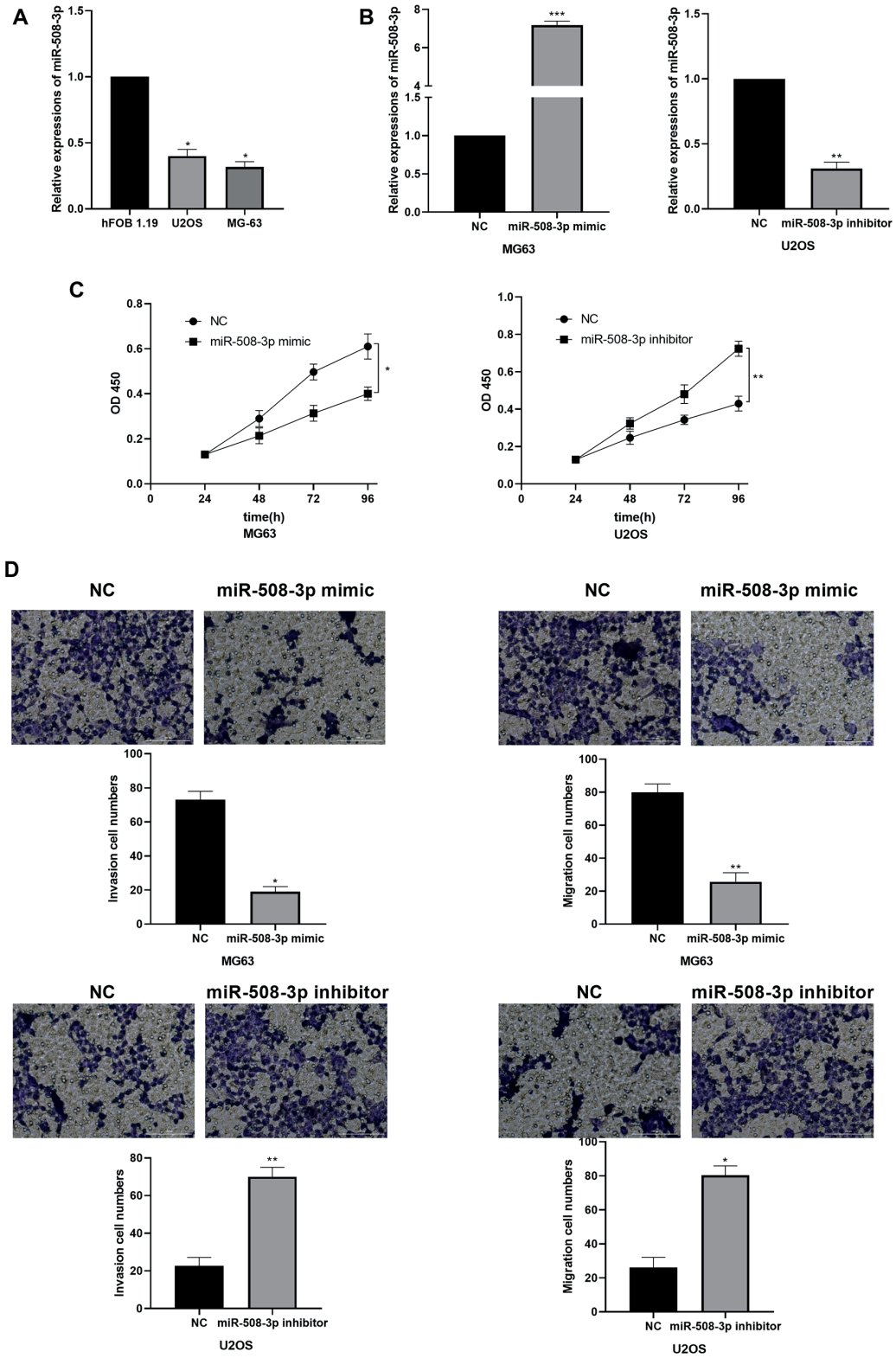

Figure 4. MiR-508-3p inhibited OS cell proliferation, invasion and migration. **A**, MiR-508-3p levels in OS cells were significantly decreased. **B**, MiR-508-3p mimic or inhibitor was transfected into OS cells to overexpress or inhibit miR-508-3p expressions. **C, D,** The influence of miR-508-3p on OS cell, proliferation, invasion and migration was investigated, (magnification 100X). *p<0.05, **p<0.01, ***p<0.001.

expression was suppressed by miR-508-3p mimic and increased by miR-508-3p inhibitor (Figure 5C and 5D). Additionally, overexpressed ZEB1 was observed in OS tissues and cells (Figure 5E). Finally, we explored whether ZEB1 expressions were regulated by PCAT-1. Results indicated that si-PCAT-1 reduced ZEB1 expressions, which could be restored by miR-508-3p inhibition (Figure 5F). Findings suggested that PCAT-1 promoted ZEB1 expressions via sponging miR-508-3p.

PCAT-1/MiR-508-3p/ZEB1 Axis Regulated OS Progression

Rescue assays were then performed to explore the roles of PCAT-1/miR-508-3p/ZEB1 in OS progression. Firstly, ZEB1 expressions in cells with

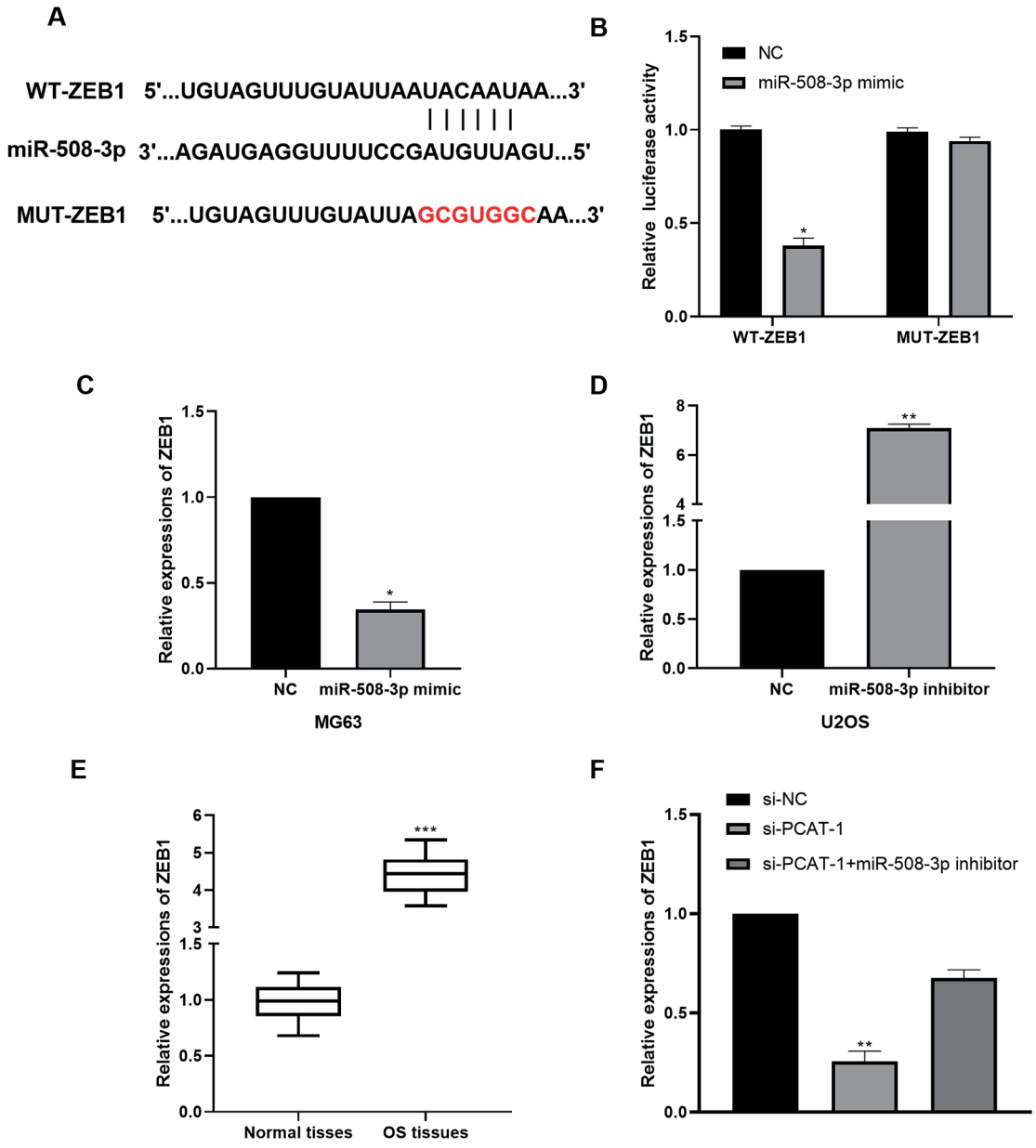

Figure 5. MiR-508-3p targeted ZEB1 in OS. **A**, The probable binding sequences of miR-508-3p on ZEB1 were predicted. **B**, MiR-508-3p mimic decreased luciferase activity in WT-ZEB1 vector. **C**, **D**, MiR-508-3p regulated ZEB1 expressions in OS cells. **E**, Overexpressed ZEB1 was observed in OS tissues and cells. **F**, MiR-508-3p inhibition restored the reduction of ZEB1 expression which was induced by si-PCAT-1. *p<0.05, **p<0.01, ***p<0.001.

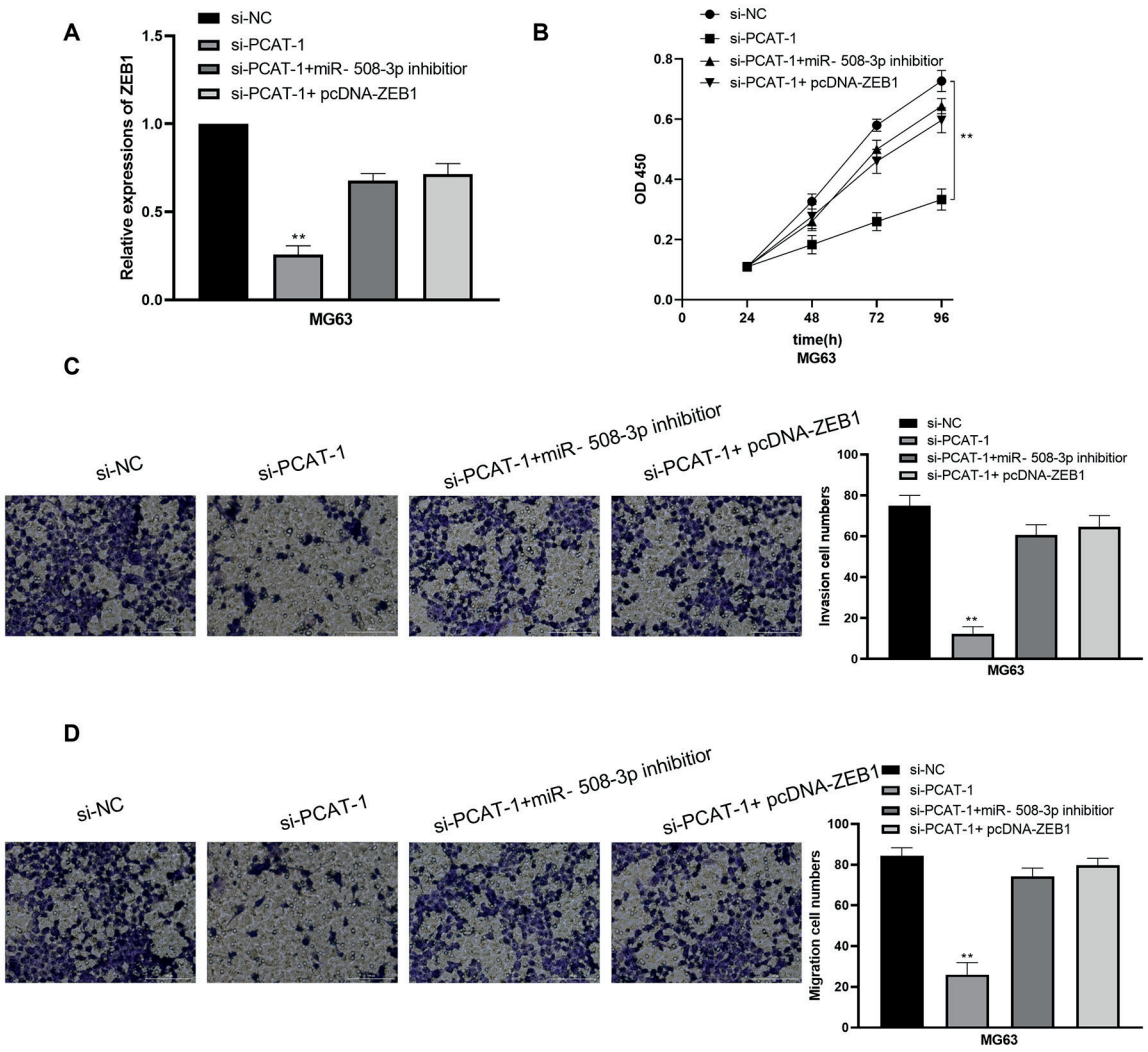

Figure 6. PCAT-1/miR-508-3p/ZEB1 axis regulated OS progression. **A**, RT-qPCR analysis was performed to detect the ZEB1 expressions in cells with different transfections. **B**, **C**, **D**, MiR-508-3p inhibition or ZEB1 overexpression could recovered the inhibited ability of OS cell proliferation, invasion and migration induced by PCAT-1 knockdown, (magnification 100X). $^{**}p<0.01$.

different transfection were detected by RT-qPCR. Results showed that knockdown of PCAT-1 significantly decreased ZEB1 expressions, and this reduction could be recovered by miR-508-3p inhibition or ZEB1 overexpression (Figure 6A). CCK-8 assay showed that miR-508-3p inhibition or ZEB1 overexpression could promote cell proliferation when compared to si-PCAT-1 transfected cells (Figure 6B). Similarly, the inhibitory effects on OS cell invasion and migration induced by si-PCAT-1 were recovered by miR-508-3p inhibition or ZEB1 overexpression (Figure 6C and 6D). Collectively, these findings suggested that PCAT-1 promoted OS progression by activating ZEB1 and decreasing miR-508-3p.

Discussion

OS is one of the rare but devastating tumors. Genetic factors have been proved to be related to OS occurrence and progression. However, its pathogenesis is still lack of characteristics. Although lncRNAs have no protein-coding capabilities, they have been shown to play important roles in almost all key pathological and biological processes[21]. Previous studies[22,23] showed that dysregulation of lncRNAs was implicated in OS progression through a variety of mechanisms: participation in transcriptional or post-transcriptional modifications, epigenetic alteration, splicing, and working as RNA baits. Basically, in OS,

underexpressed lncRNAs exert tumor-inhibitory functions, while overexpressed lncRNAs serve as oncogenes. Therefore, investigations on the functions and the mechanisms of OS-associated lncRNAs may reveal promising targets for prevention, diagnosis, treatment, and prognosis of OS. However, in addition to those lncRNAs which have clear functions in OS, there are still thousands of other lncRNAs that may be involved in OS development. These lncRNAs have not yet to be identified and to be studied thoroughly.

Prostate cancer-associated ncRNA transcripts 1 (PCAT-1) is a kind of lncRNA, the elevated expression of which was originally discovered in prostate carcinoma[24]. PCAT-1 upregulation was not only existed in prostate carcinoma, but also in a variety of other cancers, indicating a poor prognosis. In pancreatic carcinoma, PCAT-1 was found to promote cell migration by inhibition of RBM5, suggesting that PCAT-1served as oncogenes and novel therapeutic targets[25]. Moreover, highly expressed PCAT-1 was found in non-small cell lung cancer (NSCLC) cells and tissues, suggesting its oncogenic roles in NSCLC[26]. We found that PCAT-1 was upregulated in OS, which indicated a poor prognosis. Moreover, experimental evidence showing that PCAT-1 promoted the proliferation, invasion and migration of OS cells. As we all know, lncRNAs could regulate the expressions of their targets via sponging miRNAs, serving as ceRNAs in carcinogenesis. Accumulating studies have demonstrated that ceRNA could sponge miRNA, reducing the binding to the target genes and thereby modulating their expressions. In current study, we further identified the interactions between PCAT-1 and miR-508-3p and findings showed that PCAT-1 suppressed miR-508-3p expressions via serving as a ceRNA. miR-508-3p was identified as a predicted target of PCAT-1. Moreover, the low miR-508-3p levels in OS tissues and cells were identified and PCAT-1 functioned as a sponge of miR-508-3p. Investigation of the mechanisms further indicated that ZEB1 acted as a direct target of miR-508-3p in OS cells. In addition, PCAT-1 promoted OS tumorigenesis by sponging miR-508-3p to upregulate ZEB1.

In current study, zinc-finger E-box-binding homeobox 1(ZEB1) was identified as a direct target of miR-508-3p. ZEB1 is one of the most common zinc-finger transcription factors which could induce the epithelial–mesenchymal transition (EMT) in a range of pathological conditions, including tumorigenesis[27,28]. In normal physiological processes, ZEB1 could facilitate the development of cartilage and T cells, and while in pathological processes, ZEB1 was associated with the development of cancers. Upregulated ZEB1 in tumor tissues can downregulate E-cadherin and trigger EMT[29]. In thyroid cancer, ZEB1 was indicated to be implicated in invasion and migration via functioning as a direct target of FOXE1[30]. Additionally, high level of ZEB1 was related to the poor prognosis of colorectal cancer, and downregulation of ZEB1 caused by miR-551b overexpression led to the dysregulation of EMT signatures[31]. In our study, we found that ZEB1 was significantly upregulated in OS cells and results of dual-luciferase reporter assay showed that ZEB1 was a target of miR-508-3p. Thereafter, rescue assays were performed to further investigated whether ZEB1 was involved in the functions of PCAT-1/miR-508-3p in OS progression. Consistent with its known roles in tumorigenesis, we found that ZEB1 overexpression could recover the inhibitory functions in OS cell proliferation, invasion and migration caused by PCAT-1 knockdown. This study firstly reveals the potential involvement of PCAT-1 in the pathogenesis of OS and investigates its correlation between miR-508-3p/ZEB1 hitherto unreported in OS.

Conclusions

In short, the elevated PCAT-1 in OS indicated poor prognosis of OS patients, and PCAT-1 upregulation could promote OS cell proliferation, invasion and migration through sponging miR-508-3p. Additionally, ZEB1 was identified as a functional target of miR-508-3p, and the results of this finding demonstrated the decoying activity of PCAT-1 was to liberate ZEB1 from miR-508-3p, promoting OS metastasis. This study advanced our understanding of the role of PCAT-1 as regulators of OS progression and shed light on lncRNA-directed diagnostics and therapeutics.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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