Long non-coding RNA PCAT6 promotes the development of osteosarcoma by increasing MDM2 expression

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Received May 12, 2020; Accepted August 19, 2020

DOI: 10.3892/or.2020.7813

Abstract. Osteosarcoma is a severe malignant tumor. Several studies indicated that lncRNA prostate cancer-associated transcript 6 (PCAT6) promoted the development of multiple types of cancers. Studies have also revealed that MDM2 could aggravate tumor symptoms inhibiting P53 expression. However, whether lncRNA PCAT6 could affect the proliferation and metastasis of osteosarcoma cells by regulating P53 expression is unclear. The present study established lncRNA PCAT6-overexpressing osteosarcoma cells. Cell Counting Kit-8, wound healing and Transwell assays were performed to detect the change in proliferation, migration and invasion of these cells, respectively. Subsequently, E3 ubiquitin-protein ligase Mdm2 (MDM2), P53 and P21 expression were determined using western blotting. Finally, MDM2 expression was inhibited and the proliferation, migration and invasion of these cells was determined again. The present study found that the proliferation, migration and invasion of osteosarcoma cells increased following overexpression of lncRNA PCAT6. MDM2 expression was upregulated while the levels of P53 and P21 decreased following overexpression of lncRNA PCAT6. However, the proliferation, migration and invasion of osteosarcoma cells were inhibited following MDM2 knockdown. Additionally, P53 and P21 was rescued following MDM2 knockdown. To conclude, lncRNA PCAT6 promoted the proliferation, migration and invasion of osteosarcoma cells by promoting the expression of MDM2 and suppressing the expression of P53 and P21.

Introduction

Osteosarcoma is a common malignant bone tumor, which is highly lethal to adolescents and the elderly (1,2). Osteosarcoma cells have strong metastatic and invasion abilities, the disease could be aggravated by lung metastases at early stages (3). Although numerous studies in recent years have provided diverse treatment options, including gene therapy targeting long non-coding RNA (lncRNA) or microRNA (miR/miRNA) for osteosarcoma, the disease is still not fully understood (4,5).

Long non-coding RNA (lncRNA) is a type of the non-coding RNA molecule composed of >200 nucleotides (6,7). A study showed that lncRNAs play a critical regulatory role in the proliferation, differentiation and apoptosis of osteosarcoma cells (8). Several studies also suggested that lncRNA expression could regulate the proliferation, migration and invasion of multiple types of malignant tumor cells (9,10). LncRNA prostate cancer-associated transcript 6 (lncRNA PCAT6) promoted the proliferation of prostate cancer cells in an androgen-independent manner (11). Furthermore, high levels of lncRNA PCAT6 also promoted the proliferation, migration and invasion of non-small cell lung cancer cells and ovarian cancer (12,13). LncRNA PCAT6 was reported to act as a miRNA sponge to regulate the proliferation, migration and invasion of osteosarcoma (14). However, the effect of lncRNA PCAT6 on the occurrence of osteosarcoma remains to be elucidated.

P53 is a classic tumor suppressor gene (15). Higher levels of P53 expression could inhibit the proliferation and metastasis of breast, liver and lung cancer cells (15). P21, also known as CDKN1A, plays a crucial role in cell cycle progression (16). P21 could also suppress the proliferation of multiple cancer cells by inducing cell cycle arrest (17). A study revealed that P53 could activate the expression of P21 and therefore enhance the antitumor effects of P53/P21 (18). Another study indicated that MDM2 could promote the occurrence and development of malignant tumors by inducing P53 ubiquitination (19). Several studies also indicated that E3 ubiquitin-protein ligase Mdm2 (MDM2), which negatively regulates p53 protein, could promote the development of ovarian (20) and lung cancer (21). However, whether lncRNA PCAT6 could promote the development of osteosarcoma by promoting the expression of MDM2 is unclear.

Keywords: osteosarcoma, long non-coding RNA prostate cancer-associated transcript 6, E3 ubiquitin-protein ligase Mdm2, P53, proliferation
The present study established IncRNA PCAT6-overexpressing osteosarcoma cell lines. Subsequently, the proliferation, migration and invasion of these cells were determined with Cell Counting Kit (CCK)-8, wound healing and Transwell assays, respectively. MDM2 expression was then suppressed in the IncRNA PCAT6-overexpressing osteosarcoma cell line by transfection. Following which, the proliferation and metastasis of these cells were detected again. The results of these assays could reveal the effect of IncRNA PCAT6 on osteosarcoma development.

Materials and methods

Cell culture and transfection. Normal osteoblasts (hFOB1.19) and human osteosarcoma cell lines (Saos2, MG63, U2OS and HOS) used in the present study were purchased from American Type Culture Collection. Cells were cultured with RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% FBS and 1% penicillin/streptomycin ( Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in 37°C humid atmosphere with 5% CO2. IncRNA PCAT6 overexpression lentiviruses (4 µg/ml; 1 ml) were designed and purchased from Shanghai GeneChem Co., Ltd. Polybrene (Shanghai GeneChem Co., Ltd.) was used to increase transfection efficacy. After 24 h of transfection, MG63 cells were used for further experiments.

Animal assays. A total of 30 BALB/c nude mice (male, 4-week-old; 18-20 g) were obtained from the Shanghai Lingchang Biotechnology Company (Shanghai, China). Mice were raised in a sterile laminar flow cabinet with a 12-h light/dark cycle at 25±1°C and 40-60% humidity and free access to food and water.

Subsequently, MG63 cells (1x106) were injected into the thigh joint of nude mice by subcutaneous injection. After two weeks, these mice were anesthetized with ketamine (80 mg/kg) and xylazine (7 mg/kg). The mice were then placed in a carbon dioxide (CO2) euthanasia chamber (Shanghai Yuyan Instruments Co., Ltd.) and sacrificed by excess CO2. The controlled flow rate of CO2 was 20% of the volume of the euthanasia chamber per minute. Once the animal lost consciousness, the flow rate was increased to 100% of the euthanasia chamber volume per minute. Mice tumors were collected for subsequent experimentation. The percentage of weight loss was also calculated using the following formula: \( \text{Weight loss} = \text{Weight at 0 h} - \text{Weight at 24 h} \)

Wound healing assay. Prior to experimentation, cells of different groups were plated into six-well plates (4x10^5 cells/well). Once adhered, cells were cultured in serum-free medium for 12 h. A scratch was then created with a pipette tip. The width of the scratch was photographed and recorded at 0 h (magnification, x100). Subsequently, the width of the scratch was photographed after 24 h (magnification, x100) with a light microscope (Olympus Corporation). The migration rates were calculated with the following formula:

\[
\text{Migration rate} = \frac{[(\text{width at 0 h}) - \text{width at 24 h}] \times 100}{\text{width at 0 h}}
\]

Transwell assay. Cells were cultured in serum-free medium for 12 h before experimentation. Cells (1x10^6/ml) were then plated into Transwell chambers (Corning Inc.). Complete medium with 10% FBS was also added to the lower Transwell chamber. Following 24 h, the cells in the opposite basement membrane of the Transwell chamber was fixed with 4% paraformaldehyde for 10 min and then stained with crystal violet for 5 min at room temperature (Thermo Fisher Scientific, Inc.). Stained cells were calculated under a light microscope (magnification, x100; Olympus Corporation).

Hematoxylin and eosin (HE) staining. Tumor tissues were collected and used for experiments after mice euthanasia. After tumor tissues were fixed in 4% paraformaldehyde for 48 h at 4°C, tumor tissues were embedded with paraffin and then cut into 5-µm sections. Slices were subsequently stained with hematoxylin and eosin (Thermo Fisher Scientific, Inc.) for 5 min at room temperature. Three slices were observed and photographed under a light microscope (magnification, x200; Olympus Corporation).
**Immunohistochemistry.** Tumor tissues were fixed in 4% paraformaldehyde for 48 h at 4°C. Subsequently, tumor tissues were dehydrated and cut into 5-µm sections. An appropriate amount of normal goat serum (Beyotime Institute of Biotechnology) was added to the slide tissue for non-specific antigen blocking for 30 min at 37°C. Subsequently, Ki-67 expression in tumor tissues was detected with Ki-67 antibody (1:5,000; cat. no. ab51580; Abcam) according to the manufacturer's instructions. Following which, goat anti-rabbit biotinylated secondary antibody (1:40,000; cat. no. ab7089; Abcam) was added to detect the primary antibody. Neutral balsam was used to mount the sections. The staining results were observed under a light microscope (magnification, x200; Olympus Corporation).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using a reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. SYBR Green (Thermo Fisher (cat. no. 4897030001; Roche Diagnostics) according to the manufacturer's instructions. The following primer pairs were used for the qPCR: MDM2 forward, 5'-AGT AGC AGT GAA TCT ACA GGG C-3' and reverse, 5'-CTG ATC CAA CCA ATC ACC TGA AT-3'; and GAPDH forward, 5'-AGA AGG CTG GGG CTC ATT G-3' and reverse, 5'-AGGGGCCCATCCACAGTCTTC-3'.

**Western blotting.** Total protein (20 µg) was extracted from cells or tumor tissues using RIPA buffer (Beyotime Institute of Biotechnology). Protein concentration was determined using the 2-ΔΔCq method (22). The following primer pairs were used for the qPCR: MDM2 forward, 5'-AGTACGACTGAATCTACAGGG A-3' and reverse, 5'-CTGATCCAACAAACATCAGTGAAT-3'; LncRNA PCAT6 forward, 5’-CCCTCCTTTACTCCTTGGAC CAAC-3’ and reverse, 5'-GACCCAATGAGGATGGGAGAC AC-3' and GAPDH forward, 5'-AGAAGGCTGGGCTTCATT TG-3' and reverse, 5'-AGGGGCCCATCCACAGTCTTC-3'.

**Statistical analysis.** Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, Inc.). One-way ANOVA was used for comparison among multiple groups, followed by Tukey's post hoc test. Data were expressed as the mean ± SD. All experiments in were repeated three times. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Overexpression of lncRNA PCAT6 promotes the proliferation of osteosarcoma cells.** LncRNA PCAT6 was reported to be upregulated in osteosarcoma tissue (14). The present study detected the expression of lncRNA PCAT6 and MDM2 in osteosarcoma cell lines (hFOB1.19, Sáo2, MG63, U2OS and HOS). As shown in Fig. 1A-C, LncRNA PCAT6 and MDM2 expression was highest in MG63 cells. Therefore, MG63 cells was selected for subsequent experiments. LncRNA PCAT6-overexpressing osteosarcoma cells were then established. Fig. 1D shows that LncRNA PCAT6 expression in the overexpression group was significantly higher compared with the NC group. CCK-8 assays were performed to detect the change in MG63 cell proliferation. Compared with the NC group, the proliferation of MG63 cells significantly increased following overexpression of LncRNA PCAT6 at 24, 48 and 72 h (Fig. 1E).

**Overexpression of LncRNA PCAT6 enhances the migration and invasion of osteosarcoma cells.** Wound healing and Transwell assays were performed to detect the migration and invasion abilities of MG63 cells following overexpression of LncRNA PCAT6. Based on the results (Fig. 2A), it was found that the migration and invasion of MG63 cells significantly increased following overexpression of LncRNA PCAT6 compared with the NC group. Increased levels of MMP-2 and MMP-9 were closely correlated with metastases of osteosarcoma (23,24). Subsequently, MMP-2 and MMP-9 expression was detected via western blotting. As shown in Fig. 2B, MMP-2 and MMP-9 expression in MG63 cells significantly increased following overexpression of LncRNA PCAT6 compared with the NC group.

**Overexpression of LncRNA PCAT6 promotes the proliferation of osteosarcoma cells in vivo.** MG63 cells (negative control and overexpression group) were injected into mice by subcutaneous injection. The weight of these mice was measured each day for 14 days after injection. There was no significant difference in the weight and weight loss of mice between the PCAT6 overexpression group and control group (Fig. 3A). The tumor volume was significantly increased to varying degrees following overexpression of LncRNA PCAT6 (Fig. 3B). The maximum tumor diameter measured was 0.94 cm. The tumor weight significantly increased following overexpression of LncRNA PCAT6 compared with the NC group (Fig. 3C-E). Additionally, the maximum percentage of tumor weight out of total animal body weight was 8.59%. Tumor nuclei were heavily stained and the atypia was obvious using HE staining in the OE PCAT6 group compared with the NC group (Fig. 3F). Immunohistochemistry results (Fig. 3F) also showed that ki-67 expression was enhanced in tumor tissues overexpressing LncRNA PCAT6.

**Overexpression of LncRNA PCAT6 promotes MDM2 expression.** Subsequently, the expression of MDM2, P53 and P21 in MG63 cells and tumors was detected. The results showed...
that MDM2 expression significantly increased following lncRNA PCAT6 overexpression compared with the NC group (Fig. 4A and B). However, the levels of P53 and P21 significantly decreased following overexpression of lncRNA PCAT6 compared with the NC group.

MDM2 knockdown weakens the proliferation, migration and invasion of osteosarcoma cells. Lentiviruses were designed to establish MDM2-knockdown osteosarcoma cells. As shown in Fig. 5A, MDM2 expression was inhibited following transfection with si-MDM2. However, the inhibition efficiency of si-MDM2-1 was higher compared with si-MDM2-2. Therefore, si-MDM2-1 was selected for subsequent experiments. Next, CCK-8 assays were performed to MG63 cell proliferation. The results showed that MG63 cell proliferation was suppressed following MDM2 knockdown compared with the OE PCAT6 group at 48 and 72 h (Fig. 5B). Similarly, compared with the OE PCAT6 group, the proliferation of these cells was also promoted after treatment with RG7112, an MDM2 inhibitor. The migration and invasion of MG63 cells were also significantly inhibited after MDM2 knockdown and RG7112 treatment compared with PCAT6 overexpression (Fig. 5C).

MDM2 knockdown suppresses the proliferation of osteosarcoma cells in vivo. For these experiments, MG63 cells were injected into nude mice by subcutaneous injection. There was no significant difference in weight and weight loss between the mice of different groups (Fig. 6A). The tumor volume and tumor
Figure 2. LncRNA PCAT6 enhances the migration and invasion of osteosarcoma cells. (A) The migration and invasion of osteosarcoma cells increased following overexpression of LncRNA PCAT6. (B) MMP-2 and MMP-9 expression was detected using western blotting. *P<0.05, **P<0.01 and ***P<0.001. LncRNA PCAT6, long non-coding RNA prostate cancer-associated transcript 6; NC, negative control; OE, overexpression.

Figure 3. Overexpression of LncRNA PCAT6 promoted the proliferation of osteosarcoma cells in vivo. (A) The daily weight of mice was recorded, and the percentage of weight loss was calculated. (B) Tumor volume. (C) Tumor size. (D) Tumor diameter. (E) Tumor weight. (F) HE staining in osteosarcoma tissue was performed to analyze pathological characteristics and immunohistochemistry was performed to detect ki-67 expression. *P<0.05, **P<0.01 and ***P<0.001 vs. NC. LncRNA PCAT6, long non-coding RNA prostate cancer-associated transcript 6; NC, negative control; OE, overexpression; HE, hematoxylin-eosin.
weight declined following MDM2 knockdown and RG7112 treatment (Fig. 6B and F). The mice and tumors in different groups are shown in Fig. 6C-E. The maximum percentage of tumor weight, out of total animal body weight, was 11.65%
and the maximum tumor diameter measured was 0.98 cm. Compared with the OE PCAT6 group, the nucleolus partially disappeared while the nucleocytoplasmic ratio decreased in OE PCAT5+si-MDM2 and OE PCAT5+RG7112 groups through HE staining and immunohistochemistry results also showed that ki-67 expression was inhibited following MDM2 knockdown and RG7112 treatment (Fig. 6G). In previous studies, we found that lncRNA PCAT6 knockdown enhanced the expression of P53 (25). Therefore, the expression of P53 and P21 after MDM2 knockdown was detected. Knockdown of MDM2 rescued the expression of P21 and P53 (Fig. 7). MDM2 knockdown significantly increased p53 and p21 expression.
expression compared with the OE PCAT6 group, the effects of which were similar to RG7112 treatment.

Discussion

Osteosarcoma could induce bone damage and can lead to lung metastasis (26). The main features of osteosarcoma include the presence of mesenchymal spindle cells and production of the bone matrix (27). In terms of age distribution, patients with osteosarcoma are mainly adolescents and the elderly (28,29). The prognosis of patients with osteosarcoma has not been well improved or managed in the past 30 years (30). The genetic and biological complexity of osteosarcoma may be the main cause of this prognosis (31).

LncRNAs play critical roles during the development of multiple types of cancers (32,33). A study suggested that lower levels of lncRNA growth arrest-specific 5 promoted the proliferation and metastasis of ovarian cancer by targeting and suppressing the expression of miR-196-5p (34). In addition, lncRNA small nucleolar RNA host gene (SNHG) 3 could also modulate the proliferation, migration and invasion of ovarian cancer cells by regulating the energy metabolism of these cells (35). During osteosarcoma development, higher lncRNA SNHG4 expression could also promote the proliferation and metastasis of osteosarcoma cells by sponging miR-224-3p (31). LncRNA prostate cancer-associated transcript 6 (PCAT6) also enhanced the proliferation and colony formation ability of prostate cancer cells (11). The expression of lncRNA PCAT6 was also higher in the lung cancer tissues compared with adjacent normal tissues (25,36). The present study found that the expression of lncRNA PCAT6 in osteosarcoma cells was higher compared with hFOB1.19 cells. In addition, the proliferation, migration and invasion of osteosarcoma cells was promoted following lncRNA PCAT6 overexpression. Additionally, lncRNA PCAT6 overexpression also enhanced the proliferation of osteosarcoma cells in vivo.

MDM2/p53 signaling was reported to be involved in the proliferation and apoptosis of osteosarcoma cells (37,38). However, how MDM2/p53 signaling is regulated in osteosarcoma remains unclear. Drugs targeting MDM-p53 interaction, such as nutlin-3a and RG-7112, have been designed as new drugs against well-differentiated liposarcomas and osteosarcomas (39). Blocking of MDM-p53 interaction exerted efficacy by suppressing osteosarcoma cell growth (38,40). LncRNAs have been considered as potential therapeutic targets for osteosarcoma and regulate osteosarcoma progression (41-43). MDM2 is implicated in regulating long noncoding RNA maternally expressed 3, which affected MMP2 and MMP9 expression (44). Therefore, it was hypothesized that MDM2 could regulate MMP2 and MMP9 levels to affect migration and invasion ability of osteosarcoma cells. As shown in several studies, lncRNA plasmacytoma variant translocation 1 (PVT1) regulated MMP2 and MMP9 expression and affected radiosensitivity in non-small-cell lung cancer and cell migration abilities in a murine abdominal aortic aneurysm model (45,46).

Furthermore, P53 and P21 expression could inhibit the proliferation and metastasis of cancer cells. P53 is a cancer suppressor gene that was identified in 1979 (47) and is a crucial tumor suppressor gene (48). P53 expression is declined in tumor tissues (49). Several studies revealed that P53 suppressed the proliferation of cancer cells by regulating P21 expression and inducing cell cycle arrest (50,51). MDM2 expression was also associated with the development of multiple types of cancer (52,53). A study revealed that higher levels of MDM2 promoted the proliferation and metastasis of colorectal tumor cells (54). miRNA-379-5p could also inhibit the proliferation and metastasis of bladder cancer cells by suppressing MDM2 expression (53). A study indicated that MDM2 could aggravate the symptoms of cancer by suppressing P53 expression (55). The present study found that MDM2 expression increased while the levels of P53 and P21 decreased following IncRNA PCAT6 overexpression. However, the proliferation, migration and invasion of osteosarcoma cells were inhibited after MDM2 knockdown. P53 and P21 expression was also rescued following MDM2 knockdown. These results indicated that IncRNA PCAT6 promoted the proliferation, migration and invasion of osteosarcoma cells by promoting MDM2 expression and inhibiting P53 and P21 expression.

However, higher MDM2 levels were expressed in MG63 cells, which may be due to IncRNA PCAT6 upregulating MDM2 expression. Therefore, IncRNA PCAT6, which was reported to regulate miRNA levels, affected the expression of MDM2 possibly through miRNAs, (56-58). Additionally, in MG63 cells, MDM2 was regulated by proteins including Ras-ERK1/2 signaling or GRIM-19 (59,60). Thus, MDM2 could be regulated by IncRNA PCAT6 possibly attributing to the regulation of IncRNA PCAT6 on the upstream proteins of MDM2.

Additionally, the present study detected the effects of IncRNA PCAT6 on the proliferation and metastasis of
osteosarcoma cells. The results indicated that IncRNA PCAT6 enhanced the proliferation, migration and invasion of osteosarcoma cells by promoting MDM2 expression and therefore inhibiting the expression of P53 and P21. This conclusion may provide a new target and therapy for the clinical treatment of osteosarcoma.

Acknowledgements

Not applicable.

Funding

This study was supported by the Science and Technology Project of Jiaxing (grant no. 2019AY32030), the Key Discipline of Jiaxing Oncology Medicine Construction Project (grant no. 2019-ze-11), Key Laboratory of Precision Discipline of Jiaxing Oncology Medicine Construction Committee of Zhejiang Province (grant no. JUMC2019-019).

Availability of data and materials

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

Authors' contributions

XG, YX and JZ wrote the manuscript and performed the experiments. XG and JZ made substantial contributions to the conception and design of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the animal experimental guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Experimental Animal Ethical Committee of Zhejiang Hospital (approval no. JUMC2019-019).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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