lncRNAPVT1 targets miR-152 to enhance chemoresistance of osteosarcoma to gemcitabine through activating c-MET/PI3K/AKT pathway

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ABSTRACT

Background: LncRNA PVT1 has been reported to be involved in a variety of biological processes, including cell proliferation, cell differentiation and cancer progression. However, the mechanism by which LncRNA PVT1 contributes to chemoresistance of osteosarcoma cell, has not been fully elucidated.

Methods: We first generatedLncRNA PVT1-overexpressed MG63 cells and LncRNA PVT1 knockdown MG63/DOX cells. Then, we examined the effect of LncRNA PVT1 on cell viability and colony formation ability by MTT assay and soft agar assay, respectively. In addition, we performed flow cytometry analysis to detect apoptosis induced by GEM. Dual luciferase reporter assay and RIP were used to confirmed the interaction between LncRNA PVT1 and miR-152. Finally, we determined protein level of c-MET, p-PI3K, and p-AKT by westernblot.

Results: LncRNA PVT1 overexpression promoted cell proliferation and exhibited the anti-apoptotic property in LncRNA PVT1-overexpressing MG63 cells treated with gemcitabine. While, LncRNA PVT1-depleted MG63/DOX cells treated with gemcitabine exhibited significant lower survival rate and high percentage of apoptosis. Next, we found that LncRNA PVT1 could target and downregulated the level of miR-152. Interestingly, miR-152 greatly rescued the biological outcomes of LncRNA PVT1 not only in MG63 but also in MG63/DOX cells. We observed that LncRNA PVT1 markedly induced PI3K/AKT pathway activation, which was abolished by miR-152 mimics overexpression. Finally, c-MET inhibitor was used to confirm the essential role of c-MET in LncRNA PVT1 and miR-152-regulated PI3K/AKT signaling.

Conclusion: We showed thatlncRNA PVT1 played a contributory role in chemoresistance of osteosarcoma cells through c-MET/PI3K/AKT pathway activation, which was largely dependent on miR-152. Our findings advance our understanding of how lncRNA PVT1 promotes chemoresistance of osteosarcoma cells and facilitate development of novel strategies for treating osteosarcoma.

1. Introduction

Osteosarcoma (OS) is the most common primary bone tumor in children and adolescents, which always metastasizes to the lung once diagnosis [1,2]. Surgical resection, chemotherapy and radiotherapy are the three main strategies used for treating patients with osteosarcoma. Cisplatin, doxorubicin, methotrexate and gemcitabineare are well-established chemotherapy regimen as standard protocol [3]. Although higher 5-year survival rate has been achieved with the use of these therapies, chemoresistance of osteosarcoma to these drugs is the major barrier for effectively curing this type of cancer [4]. To date, the detailed molecular mechanism underlying chemoresistance of osteosarcoma remains to be resolved.

For decades, increasing evidences indicate that many biological processes are regulated by regulatory regions of the noncoding portions of the genome [5]. Indeed, only about 1.2–1.5% of genome are protein-coding, whereas a number of noncoding regulatory elements are transcribed into noncoding RNA(ncRNA) [6]. Long ncRNAs (> 200 nt) are relatively long form of ncRNAs found to exert their gene transcription regulatory function through epigenetic regulatory mechanism [7]. Long-noncoding RNA plasmacytoma variant translocation 1 (LncRNA PVT1) has been reported to be associated with cell proliferation, invasion, metastasis, apoptosis and tumor chemoresistance [8,9]. Many studies revealed that LncRNA PVT1 promoted development of multi-drug resistance in gastric, lung, cervical, pancreatic, ovarian cancer [10–13]. Recently, LncRNA PVT1 has been demonstrated to promote osteosarcoma development by regulating miR-195 [14]. However, the question of how LncRNA PVT1 contributes to osteosarcoma

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chemoresistance to GEM has not been solved.

MicroRNA is a class of around 22-nucleotide-long non-coding RNAs. They regulates gene expression mainly through binding 3'-UTR of target gene mRNAs, leading to mRNA degradation or translational suppression [15]. A number of studies have revealed that miRNA plays a critical role in health and diseases, including cell growth, migration, apoptosis and cancer cell resistance [16–18]. In OS, many miRNAs were aberrantly expressed and involved in the development and progression of OS [19,20]. Among them, *MiR-152* was significantly decreased, which is associated with survival rate of patients with osteosarcoma [21]. In addition, zhang et al. [22] reported that *miR-152* reduce tamoxifen resistance in ER + breast cancer via downregulating ALCAM. As *lncRNA PVT1* was reported to have a role in inhibiting *miR-152* level, raising the question of whether *miR-152* is implicated in *lncRNA PVT1*-promoted chemoresistance of osteosarcoma cells.

Here, we demonstrate that *lncRNA PVT1* acts as a contributor for development of chemoresistance of osteosarcoma cells through activation of c-MET/PI3K/AKT pathway, which is remarkably attenuated by *miR-152*. Thus, we propose a novel mechanism by which *lncRNA PVT1* enhances chemoresistance of osteosarcoma cells, which will help make novel strategies for treating osteosarcoma.

2. Materials and methods

2.1. Cell lines and culture

Human osteosarcoma cell MG63 and 293 T cell were purchased from ATCC. The human MDR osteosarcoma cell line MG63/DOX, which was not only resistant to DOX but also highly resistant to doxorubicin and gemcitabine [23]. And MG63/DOX cellswere selected in a stepwise manner by exposing drug-sensitive MG63 cells to increasing doses of doxorubicin (0.05, 0.1, 0.2, 0.5, 1.0ug/ml) for a total period of 8 months. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, thermofisher, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 U/ml)/streptomycin (100 U/ml) at 37℃, 5% CO2.

2.2. Cell transfection

The short hairpin RNAs (shRNAs), *Hsa-miR-152* mimic/negative control mimic and *Has-miR-152* inhibitor/negative control inhibitor were synthesized from GenePharma. *lncRNA PVT1* was subcloned into pLenti-GIII-CMV-Puro vector, which was named as *Lv-PVT1*. Transfections were performed using the Lipofectamine 2000 kit (Invitrogen,USA) according to the manufacturer's instructions.

2.3. MTT assay

Cell proliferation of MG63 and MG63/DOX cells from various groups were examined via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The cells were counted and plated into 96-well plates treated with gemcitabine. After 24 h, 0.1 mg/ml MTT was added to cells at 37℃ for 3 h and then resolved in DMSO (dimethyl sulfoxide) at room temperature for 30 min. Finally, the absorbance was measured at OD490 nm by a microplate reader (Bio-Tek, USA).

2.4. qRT-PCR

The total RNA was extracted using Trizol method. In brief, the cells were lysed with Trizol buffer, add chloroform to mixture. Then, the resulting solution was centrifuged at 12,000 rpm for 10 min. The supernatant was harvested and mixed with equivalent volume of isopropanol, next, the resultant was centrifuged at 12,000 rpm for 10 min. Removing the supernatant and add 75% ethanol to wash the pellet and centrifuge. Finally, discard the ethanol and dry the pellet, use 20–30 u l

Rnase-free H₂O to elute the RNA pellet. For reverse transcription, we used 1ug of total RNA to undergo reverse transcription according to manufacturer instruction (TAKARA PrimeScript Kit, Japan). For real time PCR, we used SYBR as probe dye and detected the signal by the standard protocol, The expression of *miR-152* was detected using a Bulge-Loop™ miRNA qRT-PCR Primer Set (Ribobio, Guangzhou, China) according to the manufacturer's instructions. The GAPDH were used as internal control. The following primers were used:

PVT1-F: 5'-AAAACGGCAGCAGGAAATGT-3'
PVT1-R: 5'-GGAGTCATGGGTGTCAGACA-3'
has-miR-152-F: 5'- GCGCTCAGTGCATGACAGA 3'
has-miR-152-R:5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACCCAAGT -3'
c-MET F: 5'- CTGGGCACCGAAAGATAAACC-3'
c-MET R: 5'- GTGTTTCCGCGGTGAAGTTG-3'
GAPDH- F: 5'-CCAGGTGGTCTCCTCTGA-3'
GAPDH- R: 5'-GCTGTAGCCAAATCGTTGT-3'

2.5. Western blot

The cells were harvested and washed with 1 × PBS, and then we used 2 × SDS loading buffer to lyse cells. The lysates were boiled at 95℃ for 10 min. The solution was subject to centrifuge at 12,000 rpm for 1 min. About 50ug of total proteins was loaded onto SDS-PAGE gel and resolved at 120 V for 0.5–1 h. After that, the proteins were transferred to PVDF membrane at 300 mA for 2–3 h. The membrane was blocked with 5% non-fat milk in 1 × TBST for 1 h at room temperature, and then the membrane was incubated with proper primary antibodies at 4℃, overnight. The following day, the membrane was washed with 1 × TBST for 3 times, 10 min each time.The memberane was incubated with secondary antibody at room temperature for 1 h. Finally, the membrane was incubated with ECL and then exposed using Bio-Rad ChemiDoc Touch Imaging System. The following antibodies were used in this study: anti-PI3K (CST, USA), anti-p-PI3K (CST, USA), anti-AKT (CST, USA), anti-p-AKT (CST, USA), anti-caspase-3 (CST, USA), anti-c-MET (CST, USA), anti-cleaved caspase-3 (CST, USA), anti-GAPDH (Proteintech, USA), anti-actin (Proteintech, USA).

2.6. Flow cytometry

For apoptosis detection, cells (2 × 10⁵ cells) were digested by trypsin and then washed with 1 × PBS, next, the cells were subject to be washed with Annexin V-binding buffer followed by incubation with 5 μl annexin V-FITC and 5 μl propidium iodide for 15 min at room temperature in the dark. Cells were then washed with binding buffer and subject to flow cytometry analyses.

2.7. Soft agar assay

The base layer of soft agar contains complete DMEM media with 0.5% low-melting agarose (Invitrogen,USA); the top layer of soft agar contains complete DMEM media with 0.35% low-melting agarose and then was mixed with 5000 cells and plated over the base layer. After 3–4 weeks, the cell colonies were fixed and stained with 0.005% crystal violet for 1 h, visible colonies were counted manually.

2.8. Xenograft experiment

Female BALB/c nude mice (4 weeks-old) were maintained at the Medical Experimental Animal Center of our hospital. All animal experiments were approved by the Animal Care Committee of Second Affiliated Hospital of Xiangya Hospital, Central South University. For tumor growth research, 0.2 mL PBS containing 1 × 10⁶cells of MG63 transfected with *Lv-PVT1* or scramble control were injected into right armpit of each nude mice after using ethanol and iodine to sterilize the insertion area. One week after cells injection, the nude mice were

injected with GEM (50 mg/kg) by tail vein for twice every week, and saline injection was used as control. The tumor volumes were measured every week. Tumor volume was calculated by using the formula V (mm3) = length / 2 × width^2. Four weeks after treatment, the mice were sacrificed by injection with overdose chloral hydrate. Tumors were then excised and photographed and the tissues were used for qRT-PCR analysis.

2.9. Luciferase reporter assays

lncRNA PVT1 mutant was generated using site-directed mutagenesis. Wildtype and mutant *lncRNA PVT1* were cloned into psiCHECK-2 vector. Then, the sequence of the *miR-152* was cloned into the firefly luciferase-expressing vector pBV-luciferase plasmid. As for luciferase assay, the 293 cells were seeded for triplicates in 24-well plates at the day before transfection, and transfected with the *miR-152* reporter vector, *lncRNA PVT1* or *lncRNA PVT1* mutant. Then, the cells were harvested and lysed, and the luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega, USA). Three independent experiments were performed.

2.10. RNA immunoprecipitation (RIP)

The cells were washed with 1 × PBS and then extract the nuclei with nuclear isolation buffer: 1.28 M sucrose, 40 mM Tris-HCl pH 7.5, 20 mM MgCl$_2$, 4% Triton X-100. Then, resuspend nuclear pellet in freshly prepared RIP buffer: 150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 100 U/ml RNase inhibitor SUPERase•in™ (add freshly each time), Protease inhibitors (add freshly each time). Next, the resuspended nuclei were mechanically sheared chromatin using a dounce homogenizer with 15–20 strokes and the resulting solution was subject to centrifuge at 13,000 rpm for 10 min, 4℃. Add antibody to supernatant and incubate for 2 h at 4℃with gentle rotation and then add protein A/G beads to the mixture for additional 1 h. After that, wash the beads with RIP buffer for 2 times and PBS for once. Isolate RNAs in TRIzol reagent and finally elute RNA with Rnase-free water.

2.11. Statistical analysis

Each experiment was performed for three times, all values were presented as mean ± SD, comparisons of parameters were performed using the two-tailed unpaired student's t-test. *P < 0.05 was considered statistically significant.

3. Results

3.1. Expression of LncRNA PVT1 influences chemoresistance of osteosarcoma cells to gemcitabine

To investigate whether *lncRNA PVT1* played critical roles in chemoresistance of osteosarcoma to GEM, we detected the differential expression of *PVT1* in OS drug-sensitive cells (MG63) and drug-resistant cell (MG63/DOX) by qRT-PCR. The results showed that *lncRNA PVT1* level was upregulated about 3.3 folds in MG63/DOX cells compared to MG63 cells (Fig. 1A).In addition, we infected MG63 cells with lentivirus to construct cell lines that stably expressed the *lncRNA PVT1*. Meanwhile, we constructed and generated *lncRNA PVT1*-depleted MG63/DOX cell lines by siRNA against *lncRNA PVT1*. qRT-PCR analysis showed thatlncRNA PVT1 level in *lncRNA PVT1*-overexpressed MG63 cell was about 6.4-fold higher than control, whereas *lncRNA PVT1*-knockdown cells exhibited significant decrease in *lncRNA PVT1* expression level (Fig. 1B). Next, we examined whether *lncRNA PVT1* had a role in chemoresistance of the cells to GEM. First of all, we detected the expression of p-glycoprotein (p-gp), a multidrug resistance-related geneand found that p-glycoprotein level was higher in MG63/DOX cells

than in MG63 cells. In addition, knockdown of *lncRNA PVT1* in MG63/DOX cells (MG63/DOX-si-*PVT1*) led to lower level of p-glycoprotein than MG63/DOX cells, suggesting *lncRNA PVT1* might play a role in chemoresistance of MG63/DOX cells (Fig. 1C). More importantly, the results revealed that the cells displayed relatively higher cell viability when overexpressed *lncRNA PVT1*; however, *lncRNA PVT1* loss led to lower cell viability in the presence of GEM compared to control cells (Fig. 1D). Furthermore, we observed that IC50 value of gemcitabine increased by around 2-fold in *lncRNA PVT1*-overexpressed cells. Conversely, IC50 value was markedly decreased upon *lncRNA PVT1* deficiency MG63/DOX cells compared with control cells (Fig. 1E). On the other hand, colony formation assay were showed that *lncRNA PVT1* overexpression resulted in more potent growth rate of cells treated with various concentrations of GEM. In contrast, *lncRNA PVT1* knockdown attenuated the chemoresistance of cells to GEM (Fig. 1F). Thus, our experiments indicate that *lncRNA PVT1* promotes chemoresistance of MG63 to GEM.

3.2. LncRNA PVT1 influences gemcitabine-reduced apoptosis in OS cell lines

To further study the mechanism of *lncRNA PVT1* on cell chemoresistance, we performed flow cytometric analysis of apoptosis. As shown in Fig. 2A, after exposure to 0, or 2.5 μg/ml GEM for 24 h, MG63 cells transfected *Lv-PVT1* exhibited a significantly decreased apoptosis index compare with negative controls. Expectedly, *lncRNA PVT1* knockdown led to contrary effect of MG63/DOX cells on GEM-induced apoptosis when exposed to GEM (0, 200 μg/ml) (Fig. 2A). Mechanistically, we detected whether caspase-3 activation, a key factor for apoptosis execution, was affected by *lncRNA PVT1*. The western blot analysis showed that cleaved caspase-3 was clearly probed when the cells was exposed to GEM and *lncRNA PVT1* greatly attenuated this activation (Fig. 2B and C). Taken together, *lncRNA PVT1* played positive roles in drug resistance of osteosarcoma cell to GEM.

3.3. LncRNA PVT1 attenuated GEM-induced tumor growth inhibition of osteosarcoma cells in vivo

Furthermore, we used a nude mouse xenograft model to studied the function of lncRNA *PVT1* to confer chemoresistance in OS. Lv-*PVT1* MG63 cells were subcutaneously implanted into nude mice. One week after implantation, the mice were injected with GEM by tail vein for three weeks. We found that GEM significantly inhibited the tumor growth. Howerer, *lncRNA PVT1* overexpression significantly attenuated GEM-induced tumor growth inhibition in nude mice, which was evaluated by increasing tumor volume and weight (Fig. 3A–C). On four weeks after implantation, all mice were sacrificed by injection with overdose chloral hydrate. The tumor tissues were obtained and used for qRT-PCR analysis. As shown in Fig. 3D, PVT1 expression was significantly increased in tumor tissues formed from Lv-PVT1 + GEM group than those from scramble + GEM and scramble group (Fig. 3D). These results suggested that PVT1 increased the chemoresistance of MG63 cells to GEM *in vivo*.

3.4. LncRNA PVT1 directly targeted miR-152

LncRNA can function as a competing endogenous RNA (ceRNA) and overexpression of it may reverse the negative regulation between miRNAs and their target genes. A large body of evidences demonstrate that *miR-152* is involved in cancer progression and drug resistance [21], in addition, *lncRNA PVT1* has been reported to inhibit expression of *miR-152*, prompting us to hypothesize that *lncRNA PVT1* might function throught interaction with *miR-152* in osteosarcoma cell. First, the bioinformatic analyses revealed that *lncRNA PVT1* exhibited sequence complementary to *miR-152*, suggesting *lncRNA PVT1* was capable of recognizing and binding to *miR-152* (Fig. 4A). Then, we performed dual
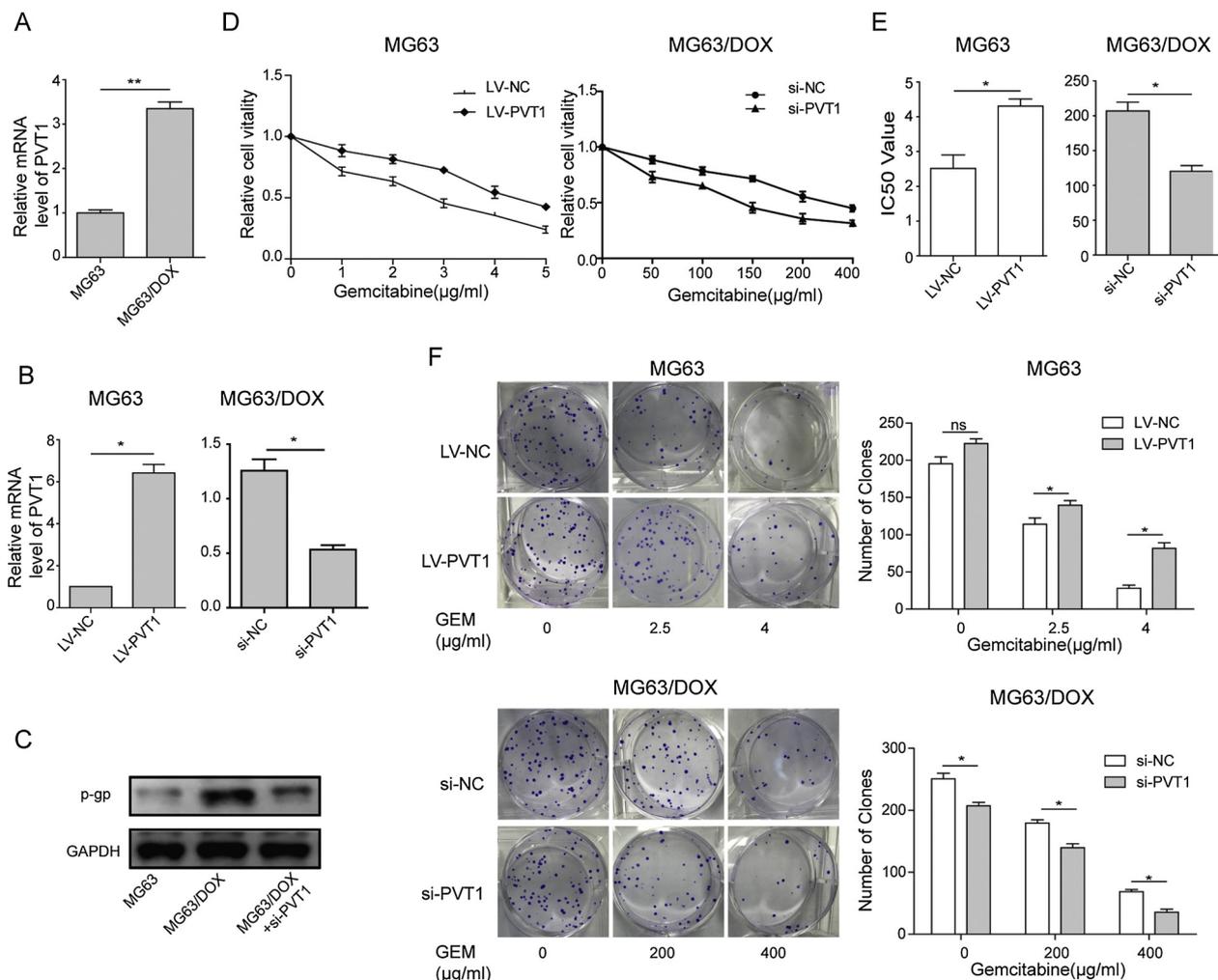
Fig. 1. *LncRNA PVT1* influences cell vitality induced by gemcitabine in osteosarcoma cells. (A) qRT-PCR analysis was performed to detected the expression of *lncRNA PVT1* in MG63 and MG63/DOX cells. U6 was used as internal control, all data were presented as mean ± SD (*P < 0.05, **P < 0.01). (B) qRT-PCR analysis was performed to detected the expression of lncRNA *PVT1* after transfection of MG63 with LV-*PVT1* (or negatively control, LV-NC) and of MG63/DOX cells with si-*PVT1*(or negatively control, si-NC). GAPDH was used as internal control, all data were presented as mean ± SD (N = 3, *P < 0.05, **P < 0.01). (C) Western blot analysis of p-glycoprotein (p-gp) protein level in MG63 cell, MG63/DOX cell, MG63/DOX cell transfected with si-*PVT1*. GAPDH was used as internal control. (D) Cell viability assay of MG63 and MG63/DOX cells, transfected with the indicated plasmids, treated with various concentration of GEM by MTT assay. The data were presented as mean ± SD (*P < 0.05). (E) IC50 value for GEM in MG63 cells transfected with *LV-PVT1* and MG63/DOX cells transfected with si-*PVT1*. (F) Soft agar assay of MG63 and MG63/DOX cells, treated with different concentration of GEM, transfected with the indicated plasmids (left) and statistical analysis of the number of visible colonies (right). All data were presented as mean ± SD (N = 3, *P < 0.05, **P < 0.01).

luciferase reporter assay to confirm *lncRNA PVT1* directly interacted with *miR-152*. The results showed that *lncRNA PVT1* diminished the luciferase activity when the cells were co-transfected with *miR-152* and wildtype *lncRNA PVT1*, not mutant form of *lncRNA PVT1* (Fig. 4B). As Ago2 was a crucial component of RNA-induced silence complex (RISC), we carried out RIP to further determine *lncRNA PVT1* associated with *miR-152* within Ago2-containing complex. We found that *lncRNA PVT1* and *miR-152* were both detected in Ago2-containing complex, indicating that *lncRNA PVT1* and *miR-152* existed in RISC (Fig. 4C). Next, we detected *miR-152* level by qRT-PCR method to examine whether *lncRNA PVT1* regulated the level of *miR-152*. The data revealed that *lncRNA PVT1* greatly lowered the level of *miR-152* when the MG63 cell was transfected with *lncRNA PVT1* vector (Fig. 4D). Finally, qRT-PCR showed that *miR-152* level was significantly reduced in MG63/DOX cells (Fig. 4E).

3.5. MiR-152 played an important role in lncRNA PVT1- mediated chemoresistance of OS cells to GEM

To determine whether *miR-152* was implicated in *lncRNA PVT1*-promoting chemoresistance of OS cells to GEM, we generated *lncRNA PVT1*-overexpressed alone or*lncRNA PVT1* (Lv-*PVT1*) and *miR-152* both overexpressed MG63 cells (Lv-*PVT1*+*miR-152* mimics), as well as *lncRNA PVT1* knockdown alone (si-*PVT1*) or *lncRNA PVT1* and *miR-152* both depleted MG63/DOX cells (si-*PVT1*+*miR-152* inhibitor). Next, we examined if *miR-152* affected *lncRNA PVT1* overexpression or deficiency-caused IC50 value change of cells exposed to GEM. The results demonstrated that *lncRNA PVT1* altered IC50 value by about 2.4 folds compared to control cells, whereas *miR-152* reverted the IC50 value of *lncRNA PVT1*-expressing MG63 to level comparable to control cells (Fig. 5A). Conversely, knockdown of miR-152 by inhibitor remarkably rescued the reduction of IC50 value by *lncRNA PVT1* depletion (Fig. 5B). Colony formation assay revealed that *miR-152* remarkably impaired the stimulatory role of *lncRNA PVT1* in anchorage-independent growth of MG63 cell. Similarly, *miR-152* inhibitor had the ability to rescue anchorage-independent growth of MG63/DOX cell blocked by *lncRNA PVT1* depletion (Fig. 5C). Besides, we conducted flow cytometry analysis to detect apoptosis of these stable cell lines. As shown in Fig. 5D, *lncRNA PVT1* inhibited the apoptosis of MG63 and loss of *lncRNA PVT1* resulted in higher apoptosis rate of cells. More
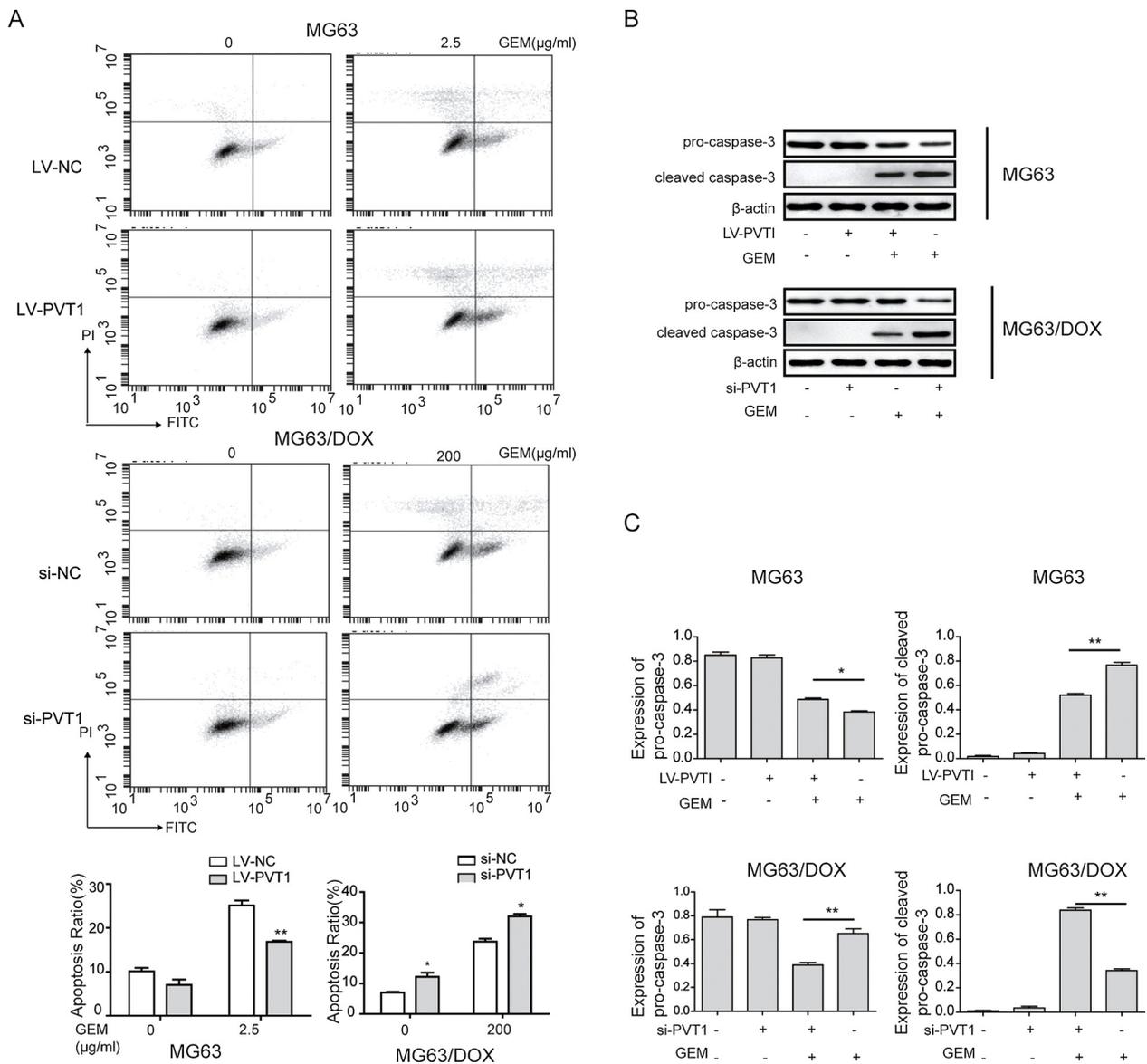
Fig. 2. *lncRNA PVT1* inhibited GEM-induced apoptosis of MG63 through blocking caspase-3 activation. (A) Flow cytometry analyses showing apoptosis of MG63 and MG63/DOX cells transfected with the indicated plasmids in the presence of GEM (left) and statistical analysis of the apoptosis rate of cells (right). (B) Westernblot analysis of activated caspase-3 caused by *lncRNA PVT1* and GEM treatment. (C) Signal intensity of caspase-3 was measured by Image J software.

importantly, *miR-152* greatly rescued the consequence of *lncRNA PVT1* in osteosarcoma cells. To summarize, *miR-152* was essential for *lncRNA PVT1*-promoting resistance of osteosarcoma cell to GEM.

3.6. MiR-152 attenuated lncRNA PVT1-induced drug-resistance via c-MET/PI3K/AKT pathway activation in OS cells

Some studies reported that c-MET was the direct target of *miR-152* in various types of tumors, so we sought to test whether *miR-152* had the ability to regulate c-MET expression in OS cells [24,25]. To validate, qRT-PCR and western blot assay were performed to evaluate the effect of miR-152 on c-MET expression in MG63 and MG63/DOX cells. Results showed that both the mRNA and protein levels of c-MET in miR-152 overexpression group were significantly decreased compared with negative control. In contrast, c-MET mRNA and protein levels were markedly increased in miR-152 inhibitor group (Fig. 6A and B). In addition, the expression of c-MET and phosphorated form of PI3K and AKT levels were much stronger in *lncRNA PVT1*-overexpressed cells; however, total PI3K and AKT levels were not changed in MG63

(Fig. 6C). By contrast, *lncRNA PVT1* loss led to marked decrease in c-MET, p-PI3K and p-AKT levels, not total PI3K and AKT in MG63/DOX (Fig. 6D). Furthermore, co-transfected with *miR-152* mimics abrogated the *lncRNA PVT1*-triggered activation of PI3K/AKT (Fig. 6C). On the contrary, miR-152 knockdown by inhibitor greatly restored lncRNA *PVT1* null-induced inactivation of PI3K/AKT pathway in MG63/DOX cells (Fig. 6D). Moreover, to further confirmed the role of c-MET in *lncRNA PVT1* and *miR-152* regulating PI3K/AKT pathway, we utilized c-MET inhibitor crizotinib (10 nM) to treat MG63cells. The results demonstrated that crizotinib inhibited the effect of *PVT1* and miR-152 on c-MET/PI3K/AKT expression (Fig. 6E). In summary, lncRNA *PVT1* enhanced chemoresistance of OS cells to GEM, in large part, through inhibition of miR-152 and activation of c-MET/PI3K/AKT pathway.

4. Discussion

In our study, the results suggest that *lncRNA PVT1* promotes chemoresistance of osteosarcoma cell by regulating *miR-152*/c-MET/PI3K/AKT pathway. Specifically, *lncRNA PVT1* significantly promotes cell
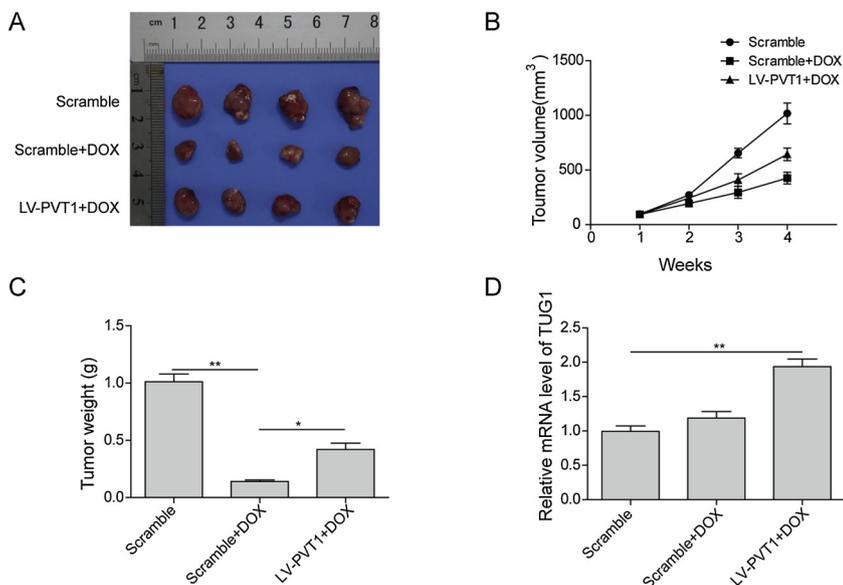
Fig. 3. *LncRNA PVT1* enhanced chemoresistance of osteosarcoma to gemcitabine in vivo.

(A) MG63 cells transfected with *Lv-PVT1* or scramble control were injected into right armpit of each nude mice. One week after cells injection, the nude mice were injected with GEM (50 mg/kg) by tail vein for twice every week, and saline injection was used as control. Representative images of excised xenograft tumors from nude mice. (B) Growth curve of tumor volumes. (C) Tumor weights were determined. (D) Tumor levels of *PVT1* measured by qRT-PCR. Data were presented as mean ± standard deviation. *P < 0.05, **P < 0.01.

viability, colony formation and inhibits apoptosis of osteosarcoma cell line MG63. In addition, bioinformatic prediction and dual luciferase reporter assay confirmed that *lncRNA PVT1* bound *miR-152* and decrease level of *miR-152* which could rescue the effect of *lncRNA PVT1* on MG63 cell. Finally, westernblot analyses indicate that *lncRNA PVT1* enhances chemoresistance of osteosarcoma cells by activation of PI3K/AKT pathway via c-MET. These similar results were also confirmed in *lncRNA PVT1*-depleted MG63/DOX cells. Together, our findings highlight the contributory role of *lncRNA PVT1* in chemoresistance of osteosarcoma cell.

Many studies have shown that *lncRNA PVT1* act as oncogenes to affect tumorigenesis, metastasis, prognosis or diagnosis. For example, *LncRNA PVT1* regulates prostate cancer cell growth by inducing the methylation of *miR-146a* [26]. Moreover, there are studies have reported that *lncRNA PVT1* is linked to chemoresistance of various types of cancer cells. Shen et al. [12] found that *LncRNA PVT1* could decrease *miR-195* expression via enhancing histone H3K27me3 and also via

direct sponging of *miR-195*, which can modulate responses of the cancer cells to PTX via regulating EMT. In addition, Overexpression of long non-coding RNA *PVT1* in gastric cancer cells promotes the development of multidrug resistance. However, little is known about whether *lncRNA PVT1* has a role in chemoresistance of osteosarcoma cell. Our study demonstrates that overexpression *lncRNA PVT1* increased cell proliferation and IC50 value treatment with gemcitabine in MG63 cells. Meanwhile, *lncRNA PVT1* influences gemcitabine-reduced apoptosis in OS cell lines. Therefore, we revealed that *lncRNA PVT1* play a vital role in chemoresistance of osteosarcoma cell to GEM, which deepens our understanding of the role of *lncRNA PVT1* in osteosarcoma progression and treatment.

It is suggested that lncRNA may act as ceRNA to suppress the biological functions of miRNAs [27]. Similar to those reports, *lncRNA PVT1* directly interacts with EZH2 and binds the promoter region of miR-195 in cervical cancer cells to increase H3K27me3 level, which may be one possible mechanism underlying *lncRNA PVT1* negatively regulating
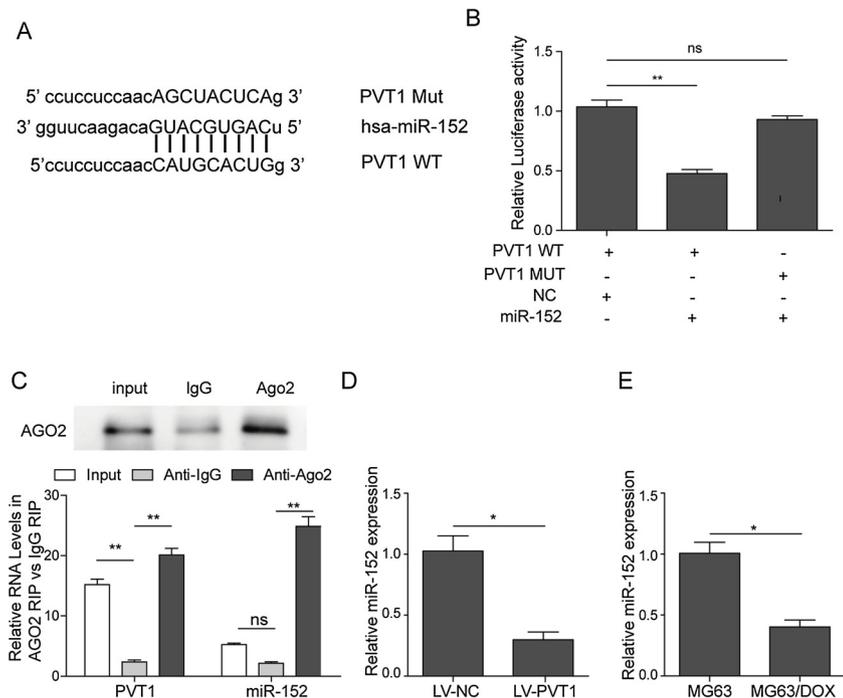

Fig. 4. *lncRNA PVT1* directly targeted *miR-152*. (A) The putative binding site of *miR-152* and *LncRNA PVT1* is shown. (B) Luciferase reporter assay showing wildtype *PVT1* binds *miR-152*, not mutant *PVT1*. The relative luciferase activity was measured and the data were presented as mean ± SD (**P < 0.01). (C) RIP assay showing *lncRNA PVT1* co-exists with *miR-152* in Ago2-containing protein complex in MG63 cell. *lncRNA PVT1* and *miR-152* level were measured by qRT-PCR. IgG was used as negative control. Ago2 protein level was examined by westernblot. (D) qRT-PCR analysis of *miR-152* was examined in MG63 cells transfected with the indicated plasmids. (E) The expression of *miR-152* in MG63 and MG63/DOX cells were detected by qRT-PCR. GAPDH and U6 were used as internal control, all data were presented as mean ± SD (*P < 0.05, **P < 0.01).
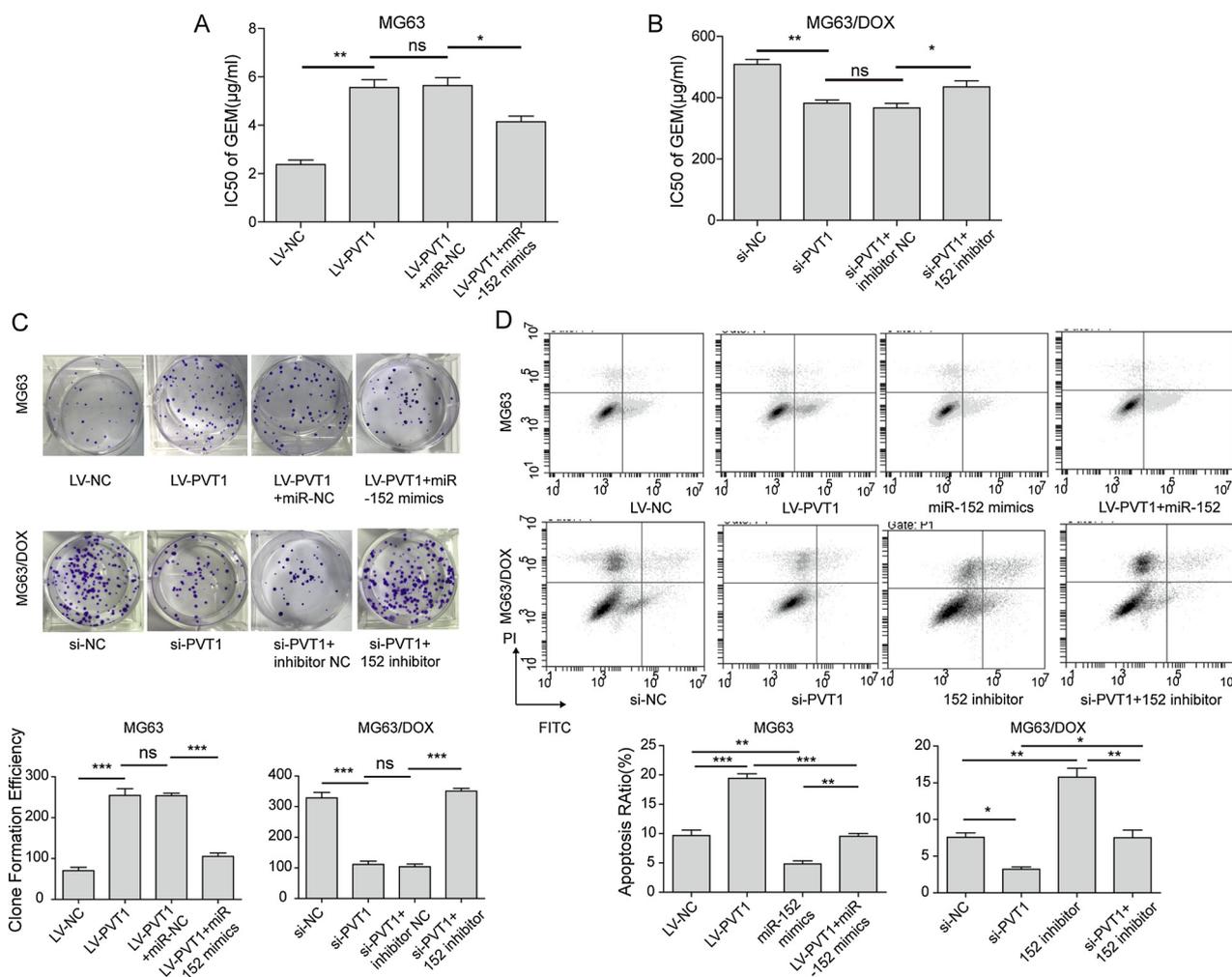
Fig. 5. *MiR-152* affected *lncRNA PVT1*-induced chemoresistance of MG63 cell to GEM. (A) IC50 value of MG63 cells, treated with GEM, transfected with the indicated plasmids, all data were presented as mean ± SD (*P < 0.05). (B) IC50 value of MG63/DOX cells, treated with GEM, transfected with the indicated plasmids, all data were presented as mean ± SD (*P < 0.05). (C) Soft agar assay of MG63 and MG63/DOX cells, treated with different concentration of GEM, transfected with the indicated plasmids (left) and statistical analysis of the number of visible colonies (right). (D) Flow cytometry analyses showing apoptosis of MG63 cells transfected with the indicated plasmids in the presence of GEM (left) and statistical analysis of the apoptosis rate of cells (right). All data were presented as mean ± SD (*P < 0.05).

microRNA expression [12]. Another study has shown that *lncRNA PVT1* is shown to act as a sponge to inhibit *miR-152* in gastric cancer cells [28]. Our dual luciferase reporter assay and RIP assay data showed that *lncRNA PVT1* directly targeted miR-152, which might be one reason for low expression of *miR-152* in osteosarcoma. Then, basing on this experimental results, we further confirmed the function of *miR-152* in *lncRNA PVT1* mediating chemotherapy resistance in os cells. In this study, we observed that *miR-152* overexpression could significantly attenuate *lncRNA PVT1*-induced resistance of osteosarcoma and *miR-152* knockdown strikingly block *lncRNA PVT1* null-mediated osteosarcoma phenotypes.

It has been shown that *miR-152* directly target 3'-UTR of c-MET and downregulate its expression in leiomyosarcoma and oral squamous cell carcinoma cells [24,29]. In this study, we confirmed c-MET is indispensible for the role of *lncRNA PVT1* and *miR-152* in chemoresistance of OS cells to GEM. c-MET, a receptor for hepatocyte growth factor (HGF), has been reported to promote tumorigenicity in a variety of cancers [30]. It has been reported that cMET blockade by crizotinib (CRZ) enhanced schwannoma radiosensitivity by enhancing DNA damage, and CRZ treatment combined with low-dose radiation was as effective as high-dose radiation. c-Met was also reported to activate sensitizes osteosarcoma cells to cisplatin via suppression of the PI3K–Akt signaling

[25].

Some studies have implicated PI3K/AKT pathway into emergence of tumor resistance, e.g., PI3K/AKT pathway has been established to be involved in miR-130b-mediated chemoresistance and proliferation of breast cancer [31]. In addition, down-regulation of *miR-497* contributes to cell growth and cisplatin resistance through PI3K/AKT pathway in osteosarcoma. Our data indicate that *miR-152* has the ability to attenuate the role of *lncRNAPVT1* in chemoresistance of osteosarcoma cell through inactivating PI3K/AKT pathway. But, the question of how miR-152 inhibits activation of PI3K/AKT pathway remains to be solved.

In summary, we demonstrate that *lncRNA PVT1* has a role in chemoresistance of osteosarcoma cell to GEM, which through directly targeting and downregulating *miR-152* to activate c-MET/PI3K/AKT pathway. These findings will enrich our knowledge of how *lncRNA PVT1* contributes to chemoresistance of osteosarcoma cell and help develop clinically effective strategies for diagnose and treatment of osteosarcoma. For one thing, high level of *lncRNA PVT1* and low level of *miR-152* could be used as diagnostic markers for indicating enhanced chemoresistance of osteosarcomas to GEM. For another, we could develop inhibitors for targeting *lncRNA PVT1* or *miR-152* mimics to improve sensitivity of osteosarcoma cells to GEM.
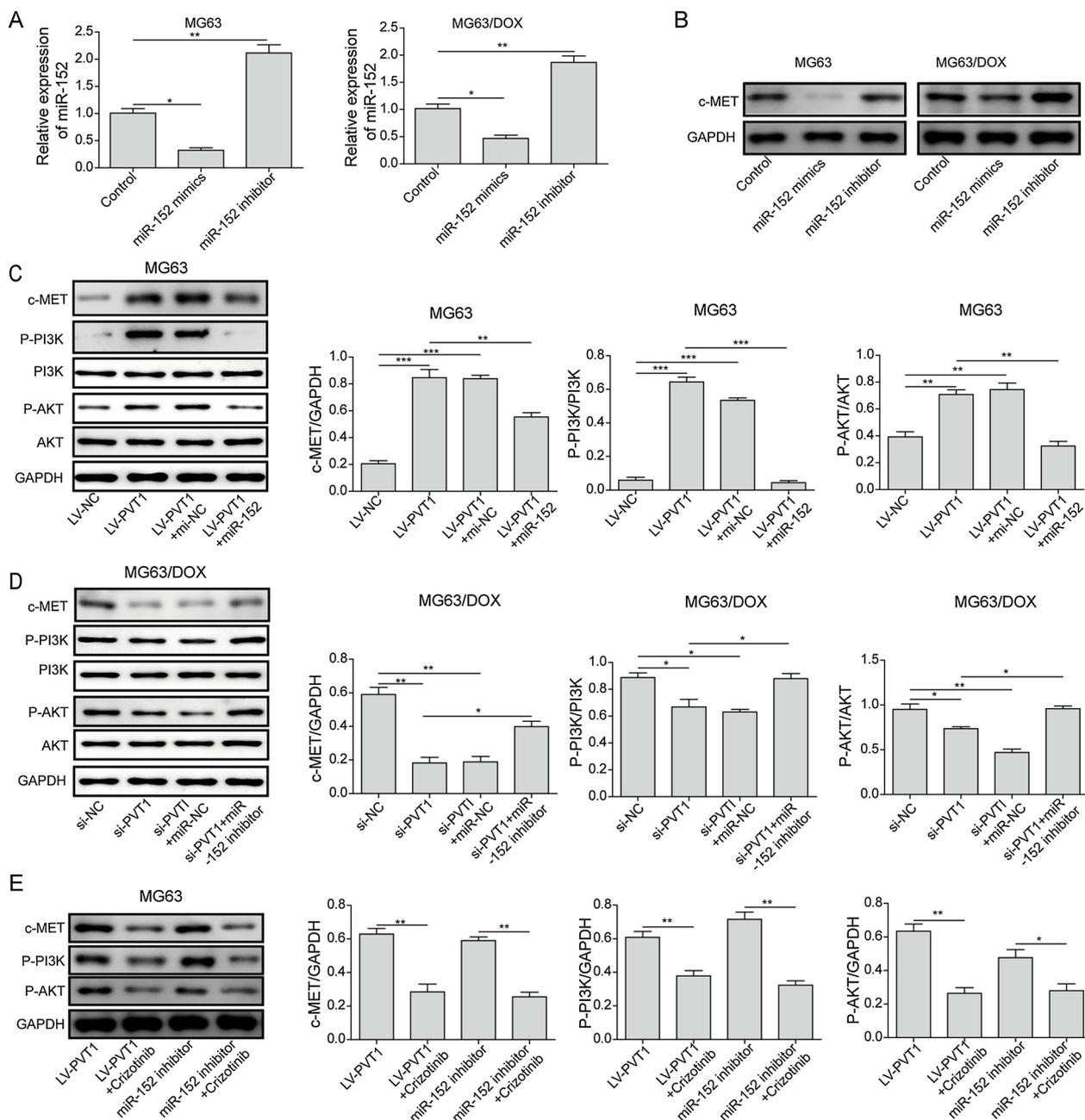
Fig. 6. *MiR-152* abolished *lncRNA PVT1*-induced c-MET/PI3K/AKT pathway activation. (A–B) qRT-PCR (A) and western blot assay (B) was performed to evaluate the effect of miR-152 on c-MET expression in MG63 and MG63/DOX cells transfected with miR-152 mimics or inhibitor. (C)Western blot analysis of c-MET, PI3K, p-PI3K, AKT and p-AKT protein level in MG63 cells transfected with the indicated plasmids (upper) and statistical analysis of protein amount as signal intensity. GAPDH was used as internal control. (D) Western blot analysis of c-MET, p-PI3K and p-AKT protein level in MG63 cells transfected with the indicated plasmids (upper) and treated with 10 nM Crizotinib, and statistical analysis of protein amount as signal intensity. GAPDH was used as internal control. All data were presented as mean ± SD (*P < 0.05).

Conflict of interest

We declare no conflict of interest.

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