



## FULL LENGTH ARTICLE

# Alantolactone inhibits proliferation, metastasis and promotes apoptosis of human osteosarcoma cells by suppressing Wnt/ $\beta$ -catenin and MAPKs signaling pathways

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Wnt/ $\beta$ -catenin

**Abstract** Although there are many therapeutic strategies such as surgery and chemotherapy, the prognosis of osteosarcoma (OS) is still far from being satisfactory. It is urgent to develop more effective, tolerable and safe drugs for the treatment of OS. In the present study, we investigated the anti-OS activity of Alantolactone (ALT), a natural eucalyptone sesquiterpene lactone mainly exists in *Inula helenium*, and probed the possible mechanism involved. We demonstrated that ALT significantly inhibited cell proliferation of various human OS cell lines while had relative lower cytotoxicity against normal cells. Then, we validated that ALT reduced migration, decreased invasion possibly through reversing epithelial mesenchymal transition (EMT) process and suppressing Matrix metalloproteinases (MMPs). Moreover, we confirmed that ALT promoted apoptosis and arrested cell cycle at G2/M phase of human OS cells *in vitro*. In addition, we confirmed that ALT restrained tumor growth and metastasis of OS 143 cells in a xenograft model *in vivo*. Mechanistically, ALT inhibited the activity of Wnt/ $\beta$ -catenin and p38, ERK1/2 and JNK Mitogen Activated Protein Kinases (MAPKs) signal pathway. Notably, the combination of ALT and Wnt/ $\beta$ -catenin inhibitor, as well as the combination of ALT and MAPKs inhibitors resulted in a synergistically effect on inhibiting the proliferation, migration and invasion of OS cells. Collectively, our results validate the ALT may inhibit proliferation, metastasis and promotes apoptosis of human OS cells possibly through suppressing Wnt/ $\beta$ -Catenin and MAPKs signaling pathways.

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## Introduction

Osteosarcoma (OS) is a kind of malignant tumor originated from mesenchymal tissue, which occurs frequently in children and adolescents. OS holds the characteristics of extreme invasiveness and early systemic metastasis.<sup>1</sup> Despite of the development of limb salvage surgery, neo-adjuvant chemotherapy, molecular targeting and other therapies, the survival rate has not been significantly improved.<sup>2</sup> Chemotherapy plays an essential role in the treatment of OS. However, currently used anti-OS drugs such as doxorubicin, cisplatin, methotrexate, and isocyclophosphamide, are prone to develop drug resistance.<sup>3</sup> Moreover, these anti-OS drugs both have strong cytotoxicity against normal cells which may lead to several side effects including hemolysis, liver and kidney dysfunction, and even life-threatening complications.<sup>4</sup> Therefore, it is necessary to explore more effective and safe drugs for the treatment of OS.

Intensive studies have evidenced that Chinese medicine and natural products may be good resources for their preventive or therapeutic effects on various malignant tumors whereas lower cytotoxicity against normal cells. ALT is mainly extracted from the root of *Inula helenium* L. which is a well-known medicinal plant officially listed in some European pharmacopeias as *elecampane*.<sup>5</sup> It has been found that ALT possesses kinds of pharmacologic activities including anti-inflammatory, anti-bacterial, liver protecting and hypoglycemia prevention properties.<sup>6–8</sup> What's more, ALT was also shown to exert potent inhibitory activity against a variety of tumors such as colorectal cancer,<sup>9</sup> gastric cancer,<sup>10</sup> breast cancer,<sup>11</sup> pancreatic cancer,<sup>12</sup> liver hepatoma,<sup>13</sup> glioblastoma,<sup>14</sup> and chronic myelogenous leukemia (CML) sensitive or resistant to imatinib.<sup>15</sup> In this study, we thought to investigate the exact effect of ALT on human OS cells and to probe the possible underlying mechanism. We found that ALT inhibited proliferation, invasion and migration while enhanced apoptosis and arrested cell cycle at G2/M phase of human OS cells *in vitro*. Furthermore, we validated that ALT suppressed OS cell growth and metastasis in an *in vivo* xenograft model. Mechanistically, ALT was shown to inhibit Wnt/ $\beta$ -Catenin and p38, ERK1/2 and JNK MAPKs signaling. Notably, the combination of ALT and the inhibitor of Wnt/ $\beta$ -catenin, p38, ERK1/2 and JNK caused a synergistically inhibitory effect on the proliferation, migration and invasion of OS cells. To sum up, our results suggest that ALT may inhibit proliferation, metastasis and promote apoptosis of human OS cells possibly by suppressing Wnt/ $\beta$ -catenin and MAPK signaling pathways.

## Materials and methods

### Cell culture and reagents

Human OS cell lines 143B, MG63, U2OS and SaoS2 were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplied with 10% fetal bovine serum (Excell Bio, China), 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin (Gibco, Invitrogen, Thermo Fisher Scientific, Inc.) at 37 °C in a 5% CO<sub>2</sub> incubator. ALT (>98% drug purity) was purchased from Chengdu Herbpurify CO.,Ltd., Chengdu Bisite CO.,Ltd. and Chengdu PUSH Biotechnology CO., Ltd respectively and dissolved in Dimethylsulfide (DMSO) to a final concentration of 10 mM KYA1197K (Wnt/ $\beta$ -catenin inhibitor), SB203580 (p38 inhibitor), PD98059 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) were obtained from Selleckchem (Houston, TX, USA).

### Crystal violet staining

OS cells were seeded in a 24-well plate at a density of  $3 \times 10^4$  cells/well, and treated with different concentrations (0  $\mu$ M, 4  $\mu$ M, 6  $\mu$ M, 8  $\mu$ M and 10  $\mu$ M respectively) of ALT for 24h, 48h and 72h. At the scheduled time point, cells were subjected to crystal violet staining to visualize the cell viability. For quantification of crystal violet staining, the absorbance of each well was measured at 595 nm by a multifunctional enzyme labeling instrument.

### MTT

OS cells were cultured in a 96-well plate at a density of  $5 \times 10^2$ /well in the presence of various concentrations of ALT for 24h or 48h. Then, cells were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (20 $\mu$ L/well) for 2h. MTT solution was discarded and DMSO (150 $\mu$ L/well) was added to fully dissolve the MTT formazan. The cell plates were subsequently placed in a multifunctional enzyme labeling instrument to detect the absorbance at 490 nm. All assays were performed in triplicate.

### Colony formation assay

OS cells were seeded in a 6-well plate at a density of  $5 \times 10^2$ /plate and cultured in DMEM supplemented with different concentration of ALT for 1 week. Cells were washed with PBS, fixed with 4% paraformaldehyde for

30min, stained with crystal violet, and then the colony forming number was counted.

### Wound healing assay

OS cells were seeded in a 6-well plate and cultured for 24 h. A uniform wound was created by scratching cells with a 10  $\mu$ L pipette. Then, cells were washed with PBS and incubated with fresh DMEM containing different concentrations of ALT (0  $\mu$ M, 4  $\mu$ M, 8  $\mu$ M, 10  $\mu$ M). At the indicated time point, three different fields of each wound were randomly photographed using a light microscope.

### Transwell migration and invasion assay

Cell migration and invasion were assessed by Transwell chamber insert assay. For cell invasion assay, cells ( $5 \times 10^4$ /well) were suspended in serum-free medium containing different concentrations of ALT at 0  $\mu$ M, 4  $\mu$ M, 8  $\mu$ M and 10  $\mu$ M were placed in the upper chamber which was coated with 50  $\mu$ L matrigel. 200  $\mu$ L of medium with 10% FBS was placed in the lower chamber for 24 h. Cells adhering to the upper surface of the membrane were removed by a cotton swab. Intrusive cells (on the underside of the filter) were fixed with 4% paraformaldehyde, then stained by crystal violet solution, and photographed under light microscopy. Finally, the numbers of stained cells were counted under a microscope. For cell migration assay, all subsequent steps were performed in the same manner as described for cell invasion assay except the matrigel was removed.

### Hoechst 33258 staining

OS cells were seeded in a 24-well plate ( $2.5 \times 10^4$ /well) and cultured for 12h. Then, cells were treated with ALT at different concentrations for 24h. Then, cells were fixed with 4% paraformaldehyde, washed with PBS, and subjected to Hoechst 33258 staining. The apoptotic cells were observed and photographed under a fluorescence microscope, and the apoptosis rate was calculated.

### Flow cytometry analysis

OS cells were treated with different concentrations of ALT for 24h. Cells were harvested and suspended in PBS. For apoptosis analysis, cells were fixed with 75% ethanol solution, stained with Annexin V-FITC/PI dual-labeling staining kit and subjected to flow cytometry analysis to determine apoptosis rate. For cell cycle determination, cells were fixed in 70% ethanol overnight, stained with PI and subjected to flow cytometry analysis.

### Western Blot

OS cells were collected after ALT treatment, and were lysed in RIPA lysate containing protease inhibitor. The protein concentration was determined by bicinchoninic acid (BCA) assay. The proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with bovine serum

albumin, and then incubated with primary antibody, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Protein of interest was visualized by an ECL kit, and photographed by a gel imaging system.

### Establishment of OS xenograft model

143B cells were collected and resuspended with sterile PBS to a final cell density of  $2 \times 10^7$ /ml. 50  $\mu$ L cell suspension was injected into the proximal tibia of female athymic mice (4–6 weeks old). Then, animals were treated with different doses of ALT (5 mg/kg, 15 mg/kg and 25 mg/kg) or sodium carboxymethyl cellulose (CMC-Na) by intra-gastric administration once every 2 days. The tumor length and width were measured every 2 days after the first week. At 21 days after injection, the mice were sacrificed, the tumor and lung sample were collected for H&E staining and immunohistochemistry analysis.

### Immunohistochemistry

5  $\mu$ m thick sections was prepared from paraffin-embedded tumor tissue sample, followed by rehydration using a graded series of 100%, 90%, 80% and 70% ethanol. The sections were then reacted with primary antibody. After washing with PBS, sections were incubated with a secondary antibody conjugated with horseradish peroxidase. Protein of interest was visualized by DAB kit, and observed under a light microscope.

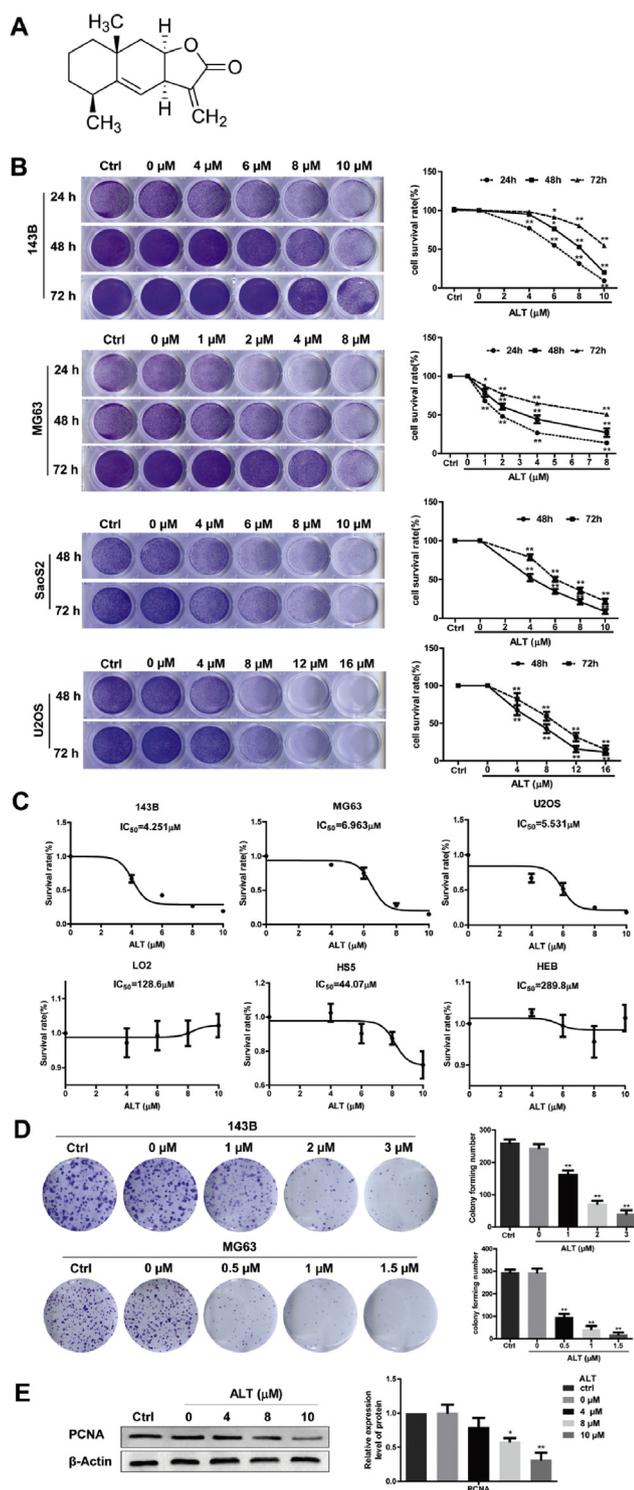
### Statistical analysis

Statistical analyses were performed using SPSS 22.0 software and Graph Pad Prism 5 software. The measurement data were expressed as the mean  $\pm$  standard error of the mean. The difference between groups was compared using t-tests. The difference among multiple groups was compared using one-way analysis of variance with Tukey's multiple comparison tests. Each experiment was performed at least 3 times. A value of  $P < 0.05$  was considered to indicate statistically significant differences.

## Result

### ALT inhibits the proliferation of OS cells

ALT was a low molecular weight (232.32 Da) lactone compound, and its chemical structure was shown in [Figure 1A](#). We thought to investigate the effect of ALT on the proliferation of OS cells by crystal violet staining assay. We found that ALT effectively suppressed the proliferation of common-used OS cell lines 143B, MG63, SaoS2 and U2OS ([Fig. 1B](#)). Subsequently, we further confirmed by MTT that ALT effectively inhibited the proliferation of 143B, MG63 and U2OS cells with  $IC_{50}$  value of 4.251  $\mu$ M, 6.963  $\mu$ M and 5.531  $\mu$ M, respectively ([Fig. 1C](#)). We also tested the potency of ALT provided by other two different vendors and found these two sources of ALT inhibited the proliferation of 143B and MG63 OS cells as well ([Fig. S1](#)). What is most



**Figure 1** ALT inhibits OS cell proliferation *in vitro*. (A) Chemical structure and formula of ALT. (B) the effect of ALT on the proliferation of OS cells (crystal violet staining). (C) The effect of ALT on the proliferation of OS cells and normal cells (MTT). (D) The effect of ALT on the proliferation of OS cells (colony formation assay). (E) The effect of ALT on PCNA protein level of 143B OS cells (Western Blot). Data were presented as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control and the 0  $\mu\text{M}$  ALT group.

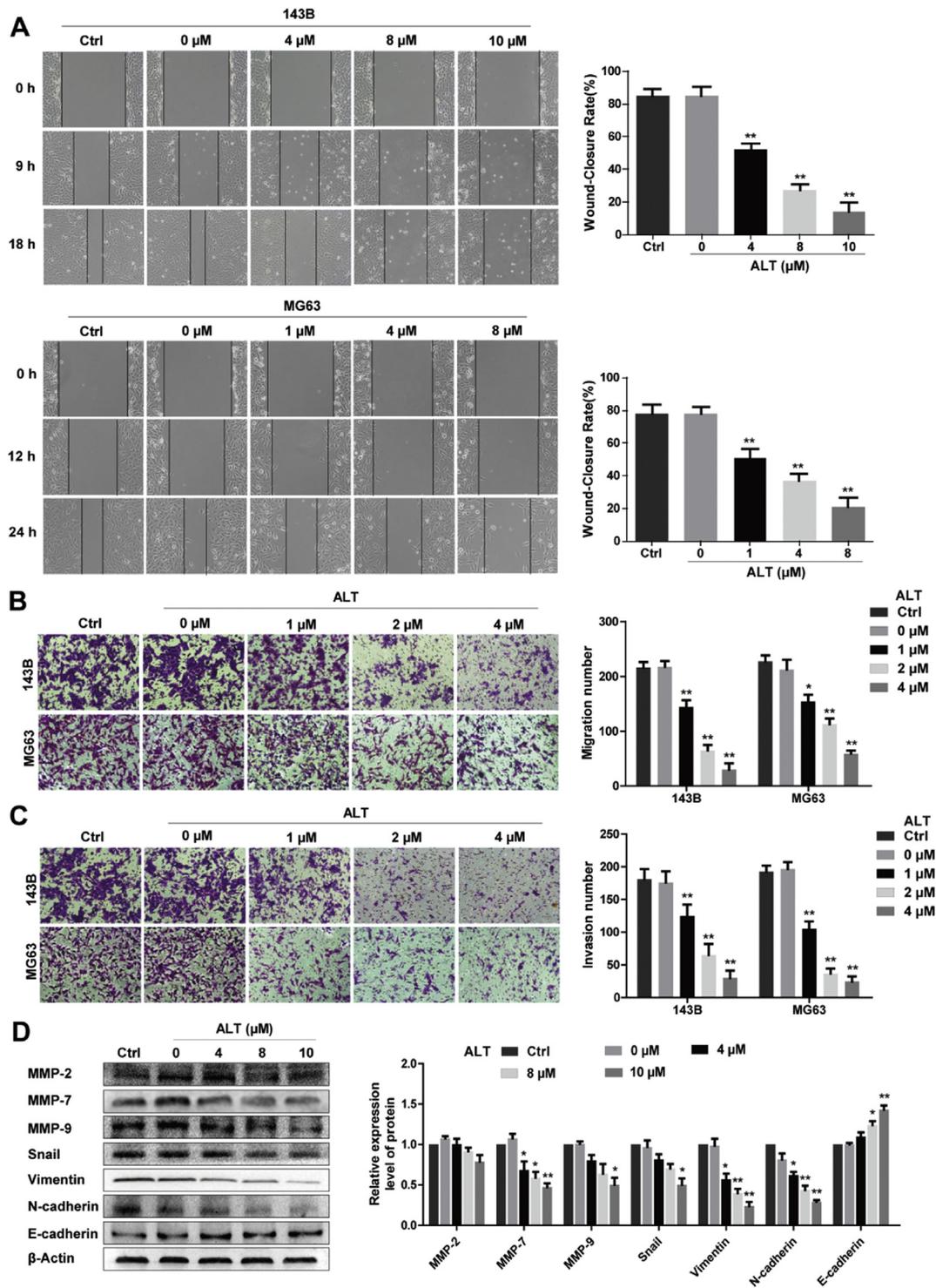
noteworthy, however, was that ALT had relative lower cytotoxicity against normal cells including human hepatocyte LO2 ( $\text{IC}_{50}$  was 128.6  $\mu\text{M}$ ), human bone marrow stromal cells HS5 ( $\text{IC}_{50}$  was 44.07  $\mu\text{M}$ ), and human brain glial cells HEB ( $\text{IC}_{50}$  was 289.8  $\mu\text{M}$ ). The inhibitory effect of ALT on OS cells proliferation was further validated by colony formation assay (Fig. 1D). Finally, we found that the protein level of PCNA, a well-established cell proliferation marker, was down-regulated upon ALT treatment (Fig. 1E). These results indicate that ALT may effectively inhibit the proliferation of human OS cells whereas have relative lower cytotoxicity against normal cells.

### ALT inhibits the migration and invasion of OS cells

We then decided to evaluate the effect of ALT on the migration and invasion of OS cells. By using wound healing assay, we found that ALT inhibited cell migration ability, resulting in a decreased wound healing rate (Fig. 2A). The inhibitory effect of ALT on OS cell migration was further confirmed by Transwell assay (Fig. 2B). Moreover, we found that ALT treatment significantly reduced the number of invaded cells cross the Matrigel-coated membrane (Fig. 2C). These results suggest that ALT may inhibit the migration and invasion of OS cells. Epithelial-mesenchymal transition (EMT) and the ECM degradation are known to play important roles in tumor cell migration and invasion. Therefore, we sought to determine if EMT-related molecules and ECM degradation markers were affected by ALT treatment. We found that the protein level of E-cadherin, a well-known epithelial marker, was remarkably elevated, while the protein level of N-cadherin, Vimentin and Snail which are critical mesenchymal markers, was reduced by ALT treatment. Matrix metalloproteinases (MMPs) can degrade almost all kinds of protein components in ECM, which is beneficial to tumor migration and invasion. We found by western blot that ALT suppressed the protein level of MMP-2, MMP-7, and MMP-9 which were important ECM degradation executers (Fig. 2D). These above results imply us that ALT may inhibit the migration and invasion of OS cells possibly through reversing EMT process and suppressing MMPs.

### ALT promotes apoptosis and induces cycle arrest at G2/M phase of OS cell

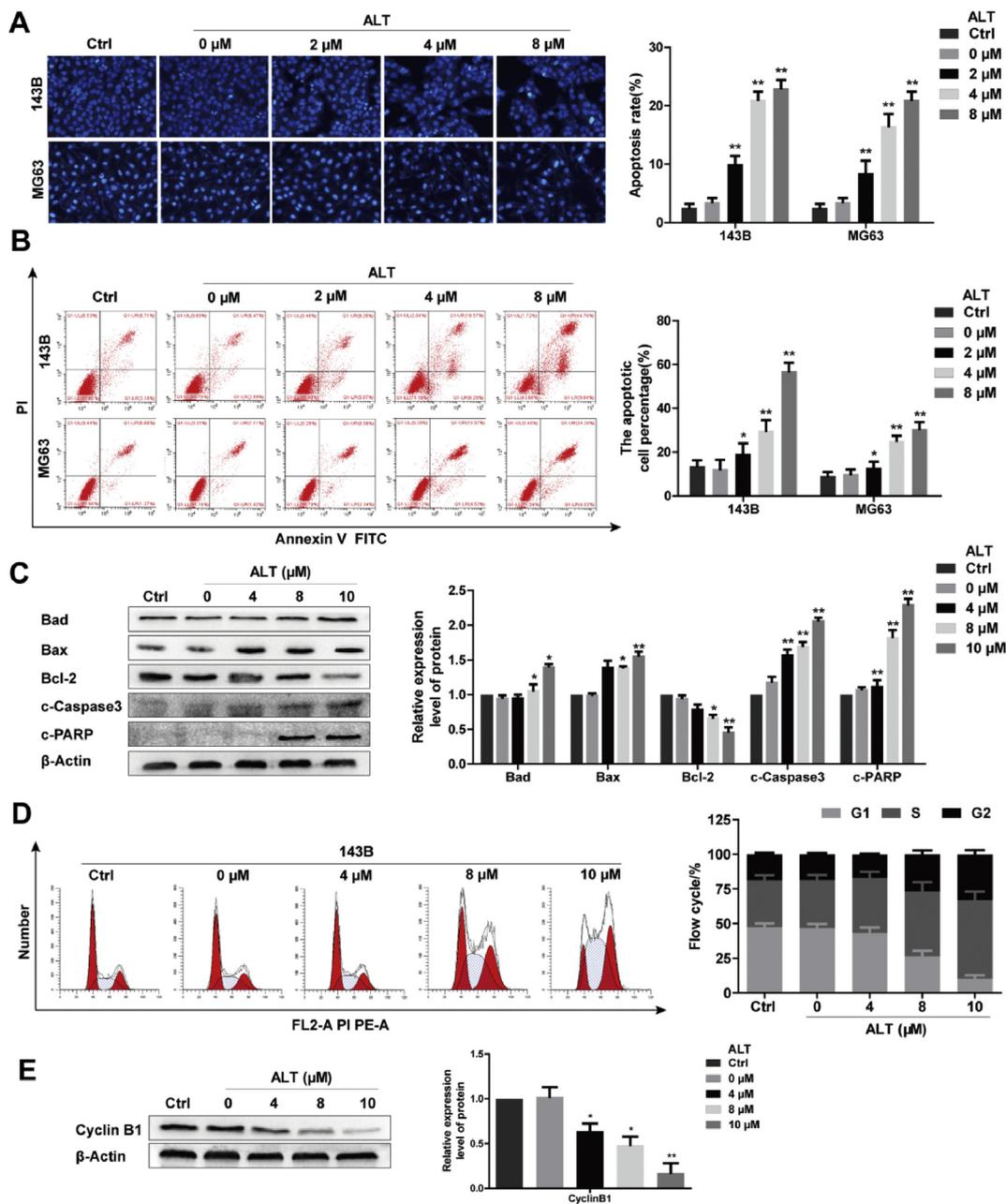
We thought to determine if ALT affected the apoptosis of OS cells. We found by Hoechst 33258 staining assay that ALT increased nuclear condensation, fragmentation and chromatin shrinkage of OS cells (Fig. 3A), implying that ALT may induce apoptosis of OS cells. We then employed flow cytometry analysis and further confirmed that the apoptotic rate of 143B and MG63 OS cells was significantly increased by ALT treatment (Fig. 3B). Besides, the protein level of anti-apoptotic factor Bcl-2 was decreased while the protein level of pro-apoptotic factors, Bax and Bad, were increased upon ALT treatment (Fig. 3C). Moreover, the level of cleaved caspase-3 and cleaved PARP, which were classical markers of apoptosis activation, were both increased by ALT stimulation (Fig. 3C). In summary, these results indicate that ALT may promote apoptosis of OS cells.



**Figure 2** ALT inhibits the migration and invasion of OS cells. (A) The effect of ALT on the migration of OS cells (Wound healing assay,  $\times 200$ ). (B) The effect of ALT on the migration of OS cells (Transwell assay,  $\times 200$ ). (C) The effect of ALT on the invasion of OS cells (Matrigel-coated transwell assay,  $\times 200$ ). (D) The effect of ALT on the protein level of MMP-2, MMP-7, MMP-9, Snail, Vimentin, N-Cadherin and E-Cadherin (Western Blot). Data were presented as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control and the 0  $\mu$ M ALT group.

To monitor the alteration of cell cycle, we conducted flow cytometry analysis and found that G2/M phase of OS cells was increased by ALT treatment (Fig. 3D). We then employed western blot to detect the protein level of cyclin

B1 which was an essential manipulator of G2/M transition, and found that the cyclin B1 protein level was decreased by ALT treatment accordingly (Fig. 3E). These results suggest that ALT may induce cycle arrest at G2/M phase of OS cell.

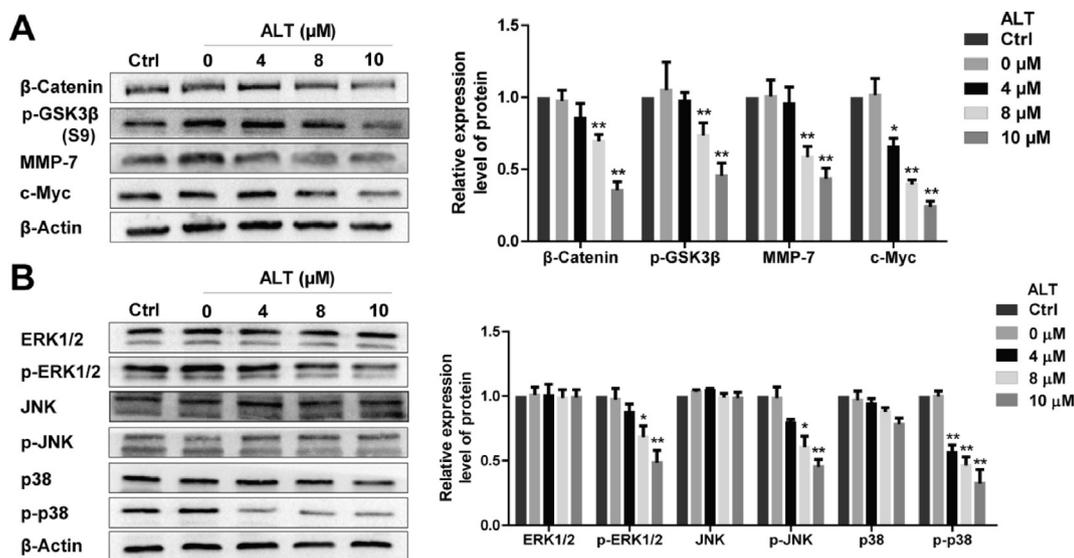


**Figure 3** ALT promotes OS cell apoptosis and induces cycle arrest at G2/M phase. (A) The effect of ALT on OS cell apoptosis (Hoechst 33258 staining). (B) The effect of ALT on OS cell apoptosis (flow cytometry). (C) The effect of ALT on the protein level of Bcl-2, Bax, Bad, Cleaved PARP, and Cleaved Caspase 3 (Western Blot). (D) The effect of ALT on the cell cycle of OS cell (flowing cytometry). (E) The effect of ALT on the protein level of cyclin B1 (Western Blot). Data were presented as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control and the 0  $\mu$ M ALT group.

### ALT suppresses Wnt/ $\beta$ -catenin and MAPKs signal pathway in OS cells

We sought to decipher the possible molecular mechanisms involved in the effect of ALT on OS cells. Abnormal activation of Wnt/ $\beta$ -catenin and MAPKs signal pathway was closely related to the proliferation, migration, invasion and apoptosis of OS.<sup>16–21</sup> Therefore, we decided to testify if ALT affected the activity of Wnt/ $\beta$ -catenin and MAPKs signal pathway in OS cells. We found that ALT remarkably

reduced the protein level of  $\beta$ -catenin and its downstream target molecule c-Myc (Fig. 4A). Furthermore, we demonstrated that ALT suppressed the phosphorylation of serine 9 residue in GSK3 $\beta$ , an inactive of GSK3 $\beta$  that negatively regulates  $\beta$ -catenin degradation (Fig. 4A). These findings indicated us that ALT may increase GSK3 $\beta$  catalytic activity through inhibiting GSK3 $\beta$  phosphorylation at serine 9, and subsequently result in the reduction of  $\beta$ -catenin protein. We also analyzed the alteration of MAPKs signaling pathway, and found that ALT inhibited the phosphorylation



**Figure 4** ALT suppresses Wnt/ $\beta$ -catenin and MAPKs signal pathway in OS cells. (A) The effect of ALT on the activation of Wnt/ $\beta$ -catenin signal pathway (Western Blot). (B) The effect of ALT on the activation of MAPKs signal pathway (Western Blot). Data were presented as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control and the 0  $\mu\text{M}$  ALT group.

of p38, ERK1/2 and JNK without altering the total amounts of these proteins obviously (Fig. 4B). These above results suggest that ALT may block Wnt/ $\beta$ -catenin and MAPKs signal pathway activation in OS cells.

### The combination of ALT and inhibitors of Wnt/ $\beta$ -catenin and MAPKs synergistically inhibits the proliferation, migration and invasion of OS cells

As ALT was shown to inhibit Wnt/ $\beta$ -catenin and MAPKs signal pathway. We therefore sought to test if the combination of ALT and inhibitors of Wnt/ $\beta$ -catenin and MAPKs may obtain synergistically inhibitory effect on OS cells. We validated by crystal violet staining that the combination of ALT (6  $\mu\text{M}$ ) with Wnt/ $\beta$ -catenin inhibitor KYA1197K (30  $\mu\text{M}$ ), P38 inhibitor SB203580 (30  $\mu\text{M}$ ), ERK1/2 inhibitor PD98059 (40  $\mu\text{M}$ ), and JNK inhibitor SP600125 (40  $\mu\text{M}$ ) respectively both caused synergistical effect on inhibiting the proliferation (Fig. 5A, B), migration (Fig. 5C) and invasion (Fig. 5D) of 143B OS cells. These results suggest that ALT may potentiate the anticancer activity of the other agents targeting different signaling pathways in the treatment of human OS cells.

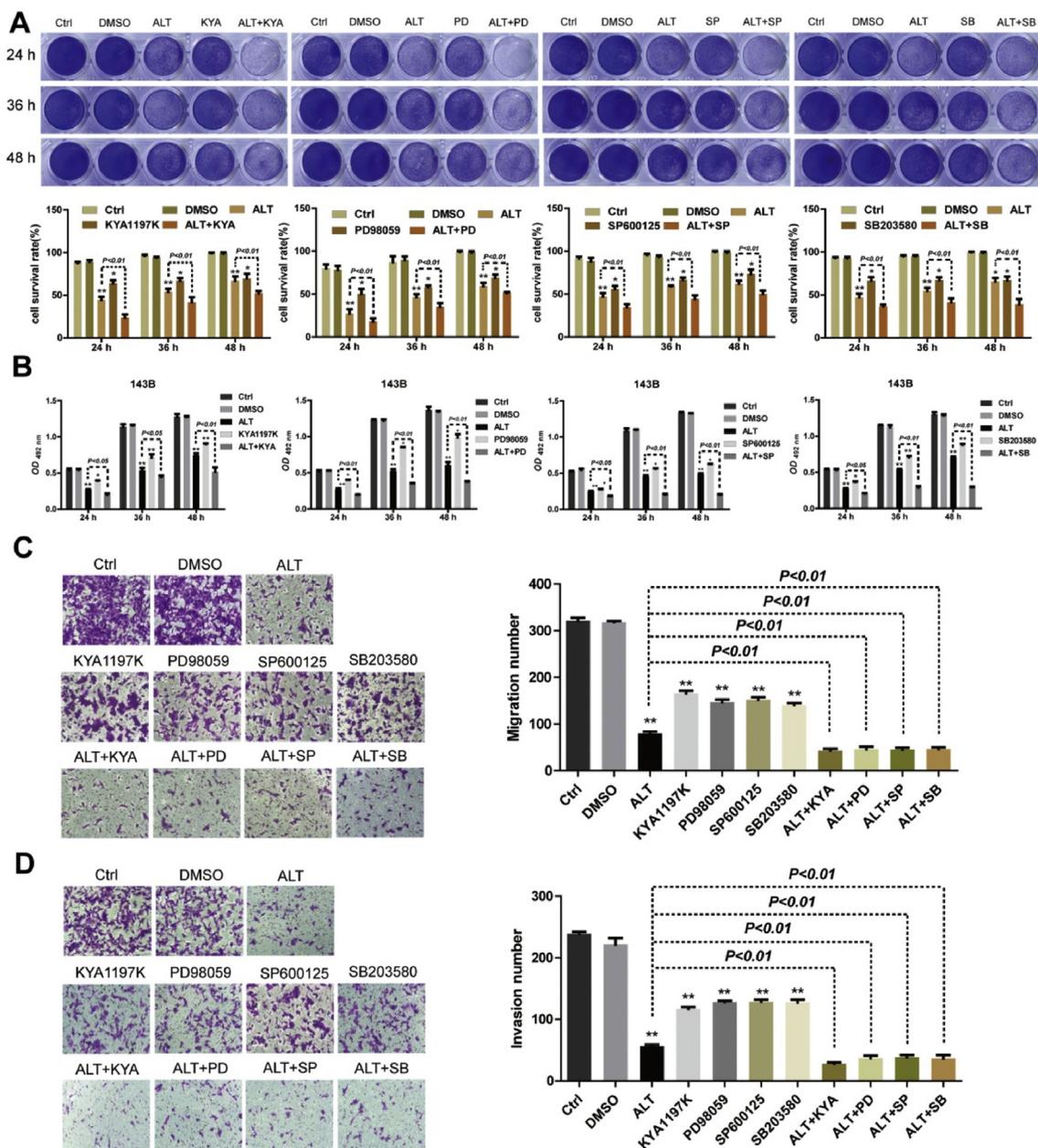
### ALT inhibits OS cells xenograft growth and metastasis *in vivo*

Lastly, we tested the *in vivo* anti-OS activity of ALT by employing a xenograft tumor model of human 143B OS cells. We demonstrated that the ALT treatment significantly retarded tumor size (Fig. 6A) and tumor volume (Fig. 6B) without affecting body weight obviously (Fig. 6C). We then found by H&E staining that ALT reduced the proliferation and malignance of OS cells, resulting in a decreased nucleo/plasm ratio, and an increased nuclear shrinkage as well as nuclear fragmentation (Fig. 6D).

Although no grossly apparent metastasis was observed in the lungs harvested from the control and ALT treatment group (Fig. 6A), H&E staining of the retrieved lung samples revealed that ALT treatment resulted in a significantly decreased incidence of pulmonary micro-metastasis (Fig. 6E). In addition, immunohistochemical staining results showed that the protein level of PCNA, Bcl-2, Vimentin,  $\beta$ -catenin, and p-p38 in tumors sample was both decreased by ALT (Fig. 6F). These results further confirm ALT may restrain tumor growth and suppress micro-metastasis of OS cells *in vivo*.

### Discussion

OS is the most common bone tumor characterized by a high potency for lung metastasis, with 10%–20% having detectable metastases at diagnosis.<sup>22</sup> Despite of the development of limb salvage surgery, neo-adjuvant chemotherapy and molecular targeting therapy, the survival rate of OS still warrants further improvement. Currently used drugs for the treatment of OS are prone to drug resistance and have severe cytotoxicity against normal cells, which make the treatment effects unsatisfactory.<sup>23,24</sup> ALT is a sesquiterpene lactone mainly existed in the root of *Inula helenium* L. In addition to its anti-inflammatory, anti-bacterial, liver protecting and hypoglycemia prevention activities,<sup>5,7,8</sup> ALT possesses remarkable effects on inhibiting various type of cancers.<sup>25–27</sup> In the current study, we explored the exact effect of ALT on human OS cells and probed the underlying molecular mechanism. ALT significantly inhibited proliferation, reduced migration, decreased invasiveness and triggered G2/M arrest in human OS cells. ALT was shown to induce apoptosis in OS cells and effectively inhibited xenograft tumor growth of OS cells. Furthermore, ALT inhibited the activation of Wnt/ $\beta$ -catenin and MAPKs signal pathway, and exhibited synergistic anticancer activity with inhibitor of Wnt/ $\beta$ -catenin and MAPKs in 143B OS cells. It is

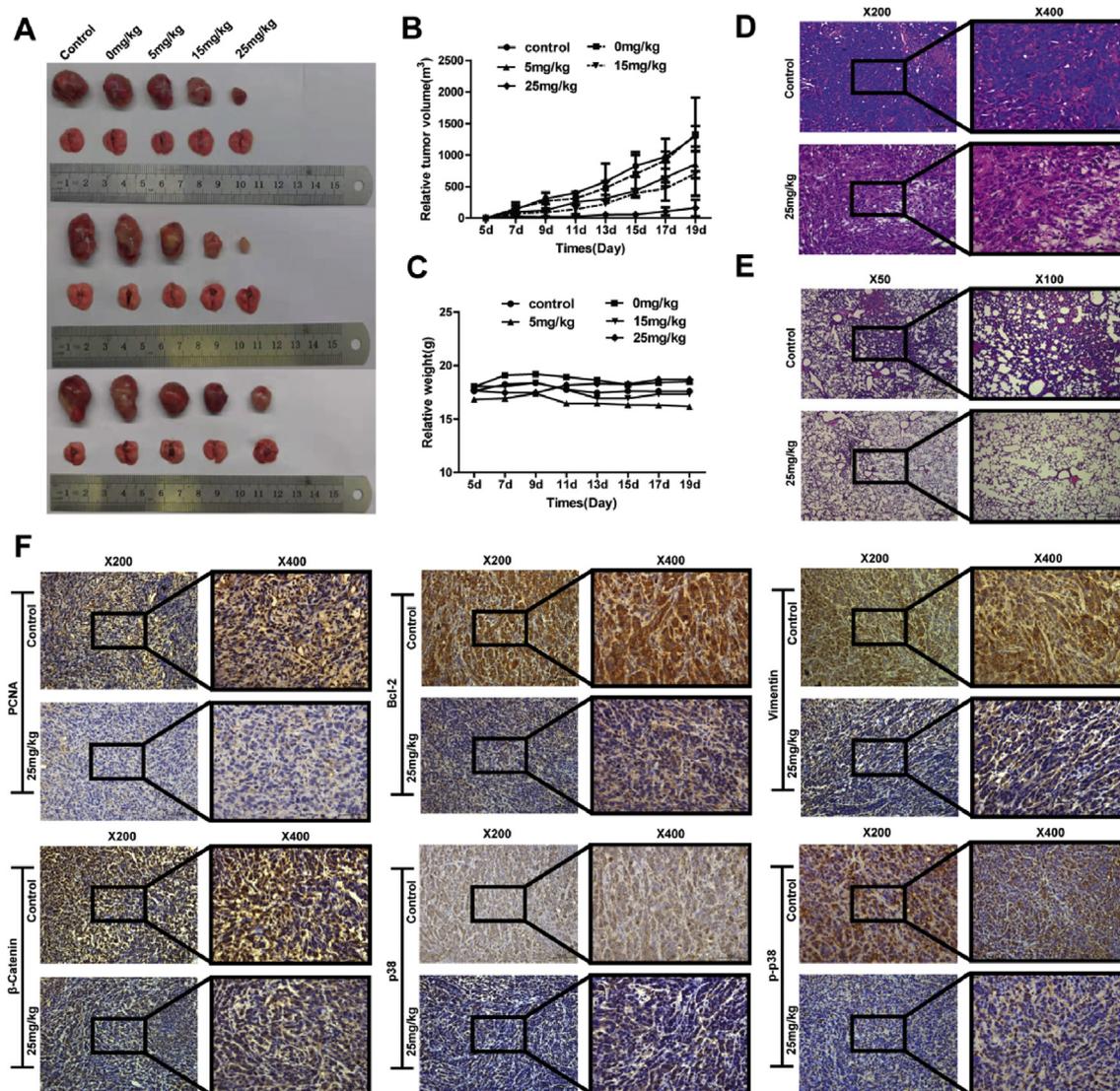


**Figure 5** ALT and signaling pathway inhibitors suppressed proliferation, migration, and invasion of OS cells. (A) The effect of the combination of ALT (6  $\mu$ M) and KYA1197K, SB203580, PD98059, SP600125 on the proliferation of 143B OS cells (crystal violet staining). (B) The effect of the combination of ALT (6  $\mu$ M) and KYA1197K, SB203580, PD98059, SP600125 on the proliferation of 143B OS cells (MTT). (C) The effect of the combination of ALT (6  $\mu$ M) and KYA1197K, SB203580, PD98059, SP600125 on the migration of 143B OS cells (Transwell assay,  $\times$  200). (D) The effect of the combination of ALT (6  $\mu$ M) and KYA1197K, SB203580, PD98059, SP600125 on the invasion of 143B OS cells (Matrigel-coated Transwell assay,  $\times$  200). Data are presented as the mean  $\pm$  standard deviation. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. the control and the 0  $\mu$ M ALT group.

noteworthy that although ALT exerted strong growth-inhibitory effect against OS cells, it displayed relative lower toxicity against human normal cells LO2, H55 and HEB. These results imply us that ALT is probably a safe and effective drug candidate for the treatment of OS.

Cell metastasis refers to the spread of cancer cells from primary site to other distant sites. Patients with aggressive and metastatic tumors may develop relative resistance to chemotherapy.<sup>28,29</sup> Cancer metastasis accounts for more

than 90% of cancer mortality.<sup>30</sup> Therefore, one of the anti-tumor strategies is to prevent malignant cell migration and invasion. By scratch wound healing assay and transwell assay, we demonstrated that ALT suppressed cell migration and invasion of OS cells. EMT is an important process related to malignant tumor progression and distant metastasis. It is characterized by the loss of epithelial features and the gain of mesenchymal characteristics.<sup>31,32</sup> Here, we validated that ALT increased the protein level



**Figure 6** ALT inhibits OS cells growth and metastasis *in vivo*. (A) Representation of retrieved OS tumor sample and lung of xenograft mice model. (B) The tumor volumes of retrieved sample. (C) The weight changes of nude mice in xenograft mice model. (D) H&E staining of retrieved OS sample. (E) Pulmonary micro-metastasis of xenograft OS model (H&E staining). (F) The protein level of PCNA, Bcl-2, Vimentin,  $\beta$ -catenin, p38 and p-p38 of retrieved tumor sample. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control and the 0  $\mu$ M ALT group.

of epithelial marker E-cadherin, whereas reduced the protein level of interstitial markers N-cadherin, Vimentin, and Snail, indicating a reversed EMT process. MMPs can hydrolyze almost all kinds of protein components including collagen laminin and fibronectin in ECM, destroy the histological barrier of tumor cell invasion, and play a key role in tumor metastasis.<sup>33,34</sup> We found that MMP-2, MMP-7, MMP-9 were both down-regulated by ALT treatment. These findings suggest us that ALT may inhibit OS cell migration and invasion possibly through reversing the EMT process and suppressing MMPs function.

Apoptosis refers to the spontaneous and orderly death of cells controlled by a series of genes in order to maintain the stability of internal environment.<sup>35</sup> Although the detailed mechanism of apoptosis is relatively ambiguous, caspase family has been documented to play an essential role in the

process of apoptosis.<sup>36</sup> In fact, the process of apoptosis is a cascade hydrolysis reaction accomplished by caspase. Caspase is a family containing at least 14 proteases, and caspase3 is the major executor in the process of apoptosis. When cells receive apoptosis promoting stimulation, caspase3 will be activated by being sheared into active form known as cleaved caspase3, acting as a proteolytic enzyme to induce apoptosis. PARP is the substrate of caspase3 and plays essential important role in apoptosis.<sup>37–39</sup> Therefore, cleaved caspase3 and PARP cleavage are considered to be important indicators of apoptosis. Bcl-2 family proteins are critical regulators of cell apoptosis. In Bcl-2 family, Bcl-2 is anti-apoptotic factor whereas Bad and Bax are pro-apoptotic factors.<sup>40</sup> In our current study, we found that ALT significantly increased apoptotic rate of OS cells. We further verified that the protein level of Bad, Bax, cleaved

caspase3, cleaved PARP was up-regulated by ALT treatment whereas Bcl-2 level was down-regulated. In short, these results indicate that ALT may promote OS cell apoptosis. In 143B cell xenograft model, we also confirmed that ALT inhibited Bcl-2 protein level by immunohistochemistry staining. Collectively, these results imply us that may induce apoptosis of OS cells.

The canonical Wnt/ $\beta$ -catenin pathway has been widely proved to do benefits to cell proliferation, migration and invasion.<sup>41,42</sup> Abnormal activation of Wnt/ $\beta$ -catenin signal is usually detected in OS, and is associated with the malignant proliferation and metastasis.<sup>43,44</sup> When cytoplasmic  $\beta$ -catenin is phosphorylated by GSK-3 $\beta$ , it will be discriminated by the E3 ubiquitin ligase  $\beta$ -TrCP for degradation eventually.<sup>45</sup> Therefore, GSK-3 $\beta$  is the key molecule to determine the total level of Wnt/ $\beta$ -catenin. The activity of GSK-3 $\beta$  is heavily dependent on itself phosphorylation. Phosphorylation of GSK-3 $\beta$  at tyrosine 216 will enhance its catalytic activity to phosphorylate  $\beta$ -catenin, however, phosphorylation of GSK-3 $\beta$  at site serine 9 reduce its catalytic activity.<sup>46,47</sup> In the present study, we found that ALT inhibited classical Wnt/ $\beta$ -catenin signal, leading to a decrease in  $\beta$ -catenin as well as its downstream target c-Myc. In addition, ALT inhibited the phosphorylation of GSK-3 $\beta$  at site serine 9. We therefore speculated that ALT may increase GSK-3 $\beta$  catalytic activity by inhibiting phosphorylating GSK-3 $\beta$  at site serine 9 through which to down-regulate  $\beta$ -catenin protein. In addition, Western Blot results showed that ALT significantly inhibited MAPKs pathway which has been proved to plays a key role in the development of OS,<sup>48–50</sup> causing a reduction in the phosphorylation of p38, ERK1/2, and JNK. To sum up, ALT may inhibit OS cells by affecting the typical Wnt/ $\beta$ -catenin and MAPKs signal pathways.

The combination of anti-tumor drugs has been proved to a more effective and safe treatment strategy that can reduce the dosage of chemotherapy drugs, enhance the susceptibility of tumor cells, decrease drug-related side effects and prevent the occurrence of drug resistant.<sup>51–55</sup> Wang et al reported that the combination of capsaicin (CAP) with a low dosage of cisplatin (CDDP), one of the gold standard drugs in OS treatment, significantly inhibited OS tumor growth in the xenograft model without significant nephrotoxicity.<sup>56</sup> A study by Naruse et al found that the cytotoxic effects of CDDP and doxorubicin (DXR) against OS cells were enhanced synergistically in the presence of meloxicam, a COX-2 inhibitor, and were partially due to an increase in apoptosis.<sup>57</sup> As ALT inhibited Wnt/ $\beta$ -catenin and MAPKs signal pathway activation, we speculated that ALT may synergize with inhibitors of Wnt/ $\beta$ -catenin and MAPKs in suppressing OS cells proliferation and metastasis. Notably, we demonstrated that the combination of ALT and inhibitors of Wnt/ $\beta$ -catenin and MAPKs synergistically inhibited the proliferation, migration and invasion of OS cells. Therefore, the combination of ALT and Wnt/ $\beta$ -catenin and MAPKs inhibitors will

be expected to develop a more effective strategy to treat OS.

In brief, we demonstrate that ALT may exert anti-OS activity through suppressing Wnt/ $\beta$ -catenin and MAPK signaling pathways. Besides, ALT was probably a safer drug candidate for OS treatment as the cytotoxicity of ALT against normal cells is relative lower. Our findings might shed light on the comprehending regarding the mechanisms of ALT anti-OS activity, and imply the clinical potential of ALT to become an efficacious therapeutic agent for the treatment of OS.

## Conflict of interests

The authors declare that they have no competing interests.

## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Author contributions

Y.J.L. contributed to the conception the study. M.C.Y. performed the experiments and drafted the manuscript. H.X.Y. provided the standard protocols of experiments. L.L.Z. and K.H.H. helped perform the analysis with constructive discussions. P.Z., H.C.Y., Q.M.W., M.C.Y. and Y.J.L. performed the data analyses. All authors have read and approved the final version of this manuscript.

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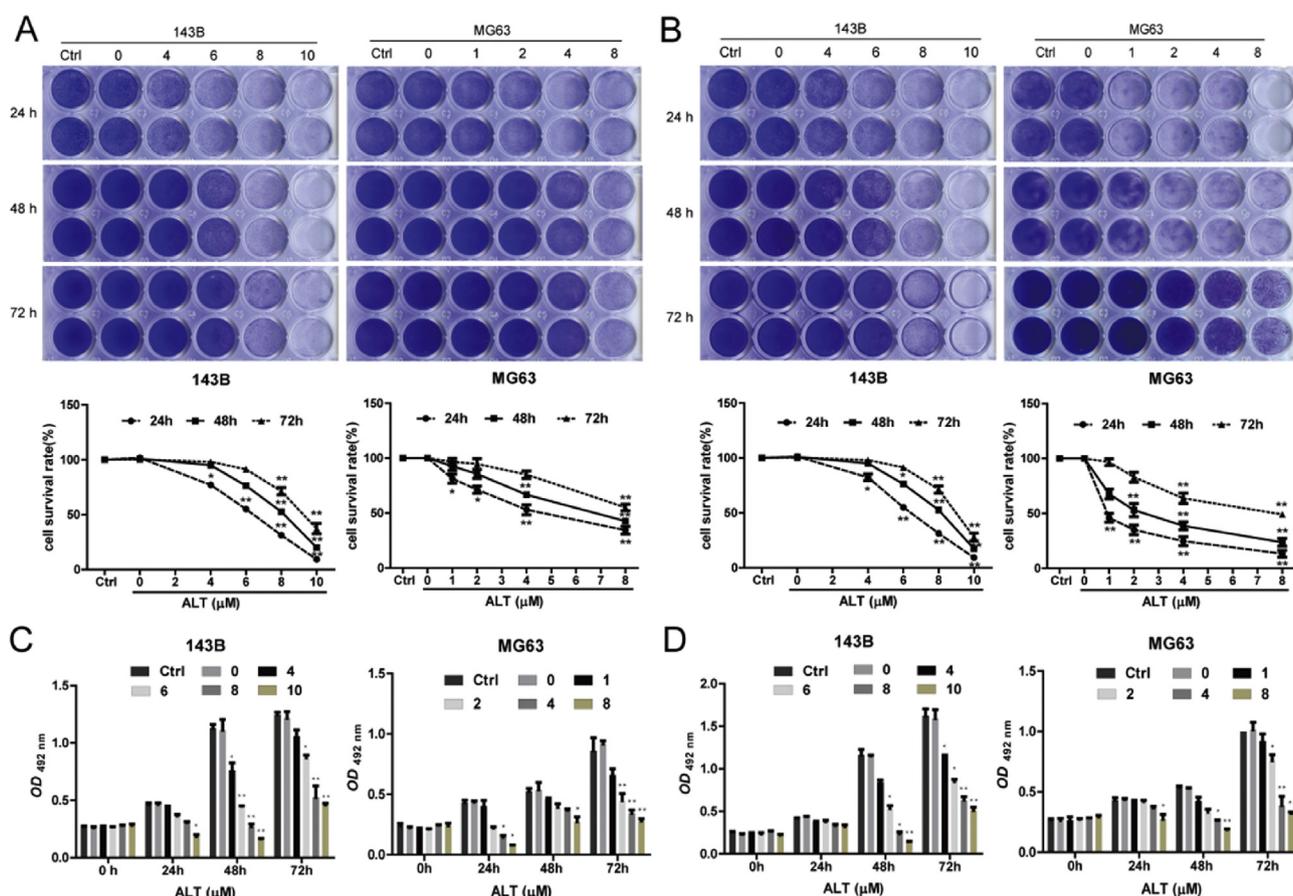
## Ethics approval and consent to participate

The animal experiments were conducted in accordance with the regulations of the Institutional Animal Care and Use Committee with the approval of the Ethics Committee of Chongqing Medical University.

## Patient consent for publication

Not applicable.

## Appendix



**Figure. S1** ALT inhibits OS cell proliferation *in vitro*. (A) The effect of ALT (obtained from Chengdu Bisite CO.,LTD) on the proliferation of OS cells (crystal violet staining). (B) The effect of ALT (obtained from Chengdu PUSH Biotechnology CO.,LTD) on the proliferation of OS cells (crystal violet staining). (C) The effect of ALT (obtained from Chengdu Bisite CO.,LTD) on the proliferation of OS cells (MTT). (D) The effect of ALT (obtained from Chengdu PUSH Biotechnology CO.,LTD) on the proliferation of OS cells (MTT). \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control and the 0  $\mu\text{M}$  ALT group.

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