

Dihydroartemisinin inhibits tumor growth of human osteosarcoma cells by suppressing Wnt/ β -catenin signaling

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Abstract. Osteosarcoma (OS) is the most common type of bone cancer. Even with early diagnosis and aggressive treatment, the prognosis for OS is poor. In the present study, we investigated the proliferation and invasion inhibitory effect of dihydroartemisinin (DHA) on human OS cells and the possible molecular mechanisms involved. We demonstrated that DHA can inhibit proliferation, decrease migration, reduce invasion and induce apoptosis in human OS cells. Using an *in vivo* tumor animal model, we confirmed that DHA can prevent OS formation and maintain intact bone structure in athymic mice. In addition, we examined the possible molecular mechanisms mediating the function of DHA. We found that the total protein levels and transcriptional activity of β -catenin in OS cells are reduced by DHA treatment, and this may result from the increased catalytic activity of glycogen synthase kinase 3 β (GSK3 β). Moreover, the inhibitory effect of DHA on OS cells is reversed by overexpression of β -catenin, but is further enhanced by knockdown of β -catenin, respectively. Collectively, our results reveal that DHA can inhibit tumor growth of OS cells by inactivating Wnt/ β -catenin signaling. Therefore, DHA is a promising chemotherapy agent in the treatment of human OS.

Introduction

Osteosarcoma (OS) is one of the most common types of non-hematologic malignant bone cancer in humans, particularly in children and adolescents (1,2). Although other types of cancer can eventually penetrate into parts of the skeleton, OS is one

of the few that actually initiate in bones and sometimes metastasize elsewhere, usually to the lungs (1-4). Approximately 80% of OS patients have metastatic disease at the time of diagnosis, and yet 10-15% of these lesions are detectable with current radiographic imaging modalities (4). The main clinical approach for OS patient is comprised of surgery with wide excision of the primary tumor and chemotherapy, a treatment which often proves difficult for patients due to the systemic toxicities of chemotherapy agents (5,6). Chemotherapy drugs may cause hearing loss, anemia, abnormal bleeding, kidney and liver damage. Nevertheless, chemotherapy plays a major role in the treatment of OS currently (7-9). The effectiveness of chemotherapy in the prognosis of OS patients cannot be further improved simply by increasing the dose of chemotherapeutic drugs. Therefore, there is a great clinical need to explore new agents for the treatment of OS.

It is currently accepted that herbal and natural products are valuable resources for anticancer drugs (10,11). Artemisinin (ART) is a natural product originally isolated from the plant *Artemisia annua* L., an herbal drug that has been used in traditional Chinese medicine for centuries (12,13). Dihydroartemisinin (DHA) is a derivative of ART and is a potent anti-malarial drug with only mild adverse effects. Recent studies have shown that ART derivatives including DHA also have profound effects against human tumors including breast, pancreas, prostate and liver cancer with fewer toxic effects (14-18). However, the exact molecular mechanisms by which DHA exerts its anticancer effects remain to be fully investigated.

Here, we investigated the antitumor effect of DHA on human OS cells, as well as the possible molecular basis underlying the function. Our results strongly indicate that DHA can inhibit tumor growth of OS cells both *in vitro* and *in vivo*. The inhibitory effect of DHA on OS cells can be mediated by inactivating Wnt/ β -catenin signaling, which may be processed in part through increasing catalytic activity of glycogen synthase kinase 3 β (GSK3 β).

Materials and methods

Cell culture and drug preparations. OS cell lines 143B, U2OS, SaoS2 and MG63 were obtained from the American

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Type Culture Collection, and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μ g/ml of streptomycin at 37°C in 5% CO₂. DHA was purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide (DMSO) for *in vitro* test, or prepared with 0.5% carboxymethyl cellulose sodium (CMC-Na) as suspension for *in vivo* experiments. Antibodies were from Santa Cruz Biotechnology, Inc., and Cell Signaling Technology. All other reagents were purchased from Sigma-Aldrich, unless otherwise indicated.

Crystal violet assay. OS cells were seeded in 24-well plates and treated with different concentrations of DHA or DMSO as control. At the scheduled time points, cells were stained with crystal violet to visualize the cell viability. For quantification, crystal violet in the stained cells was extracted with 1 ml 20% acetic acid at room temperature for 20 min with shaking. A total of 100 μ l were removed and added to 1 ml ddH₂O. Absorbance at 570 nm was measured (19). Each assay was performed in triplicate.

MTT proliferation assay. 143B cells were seeded in 96-well plates and treated with different concentrations of DHA. Cell proliferation was assessed by MTT assay at the scheduled time points. Optical absorbance was measured at 570 nm using a 96-well microplate reader. All experiments were performed in triplicate.

Colony formation assays. One day prior to DHA treatments, 200 cells were seeded into 6-well plates in complete DMEM. The cells were then treated with variable concentrations of DHA and cultured in complete DMEM for up to 14 days. Colonies were visualized by crystal violet staining and counted. The colony forming rate was obtained by the following calculation: (colony number/seeded cell number) x100%. All experiments were performed in triplicate.

Transwell migration and invasion assay. The migration and invasion assay was performed as previously described (20,21). For the migration assay, 143B cells were seeded at 2x10⁵/well in the upper chamber of type I-collagen-coated 24-well culture inserts. For the invasion assay, the upper side of the insert was coated with extracellular matrix (ECM) gel. DHA was added to the lower chamber. After 24 h, the cells were dried for 5 min, fixed with dehydrated alcohol, and stained with hematoxylin-eosin. The transmembrane cells were counted. All experiments were performed in triplicate.

Hoechst apoptosis staining. 143B cells were plated in 24-well plates and treated with different concentrations of DHA or DMSO. After maintaining at 37°C in 5% CO₂ for 48 h, cells were collected and subjected to Hoechst 33258 staining. The apoptotic cells were visualized under fluorescence microscopy as previously reported (22). Each assay was carried out in triplicate.

Western blot assay. 143B cells were plated in 24-well plates and treated with different concentrations of DHA or DMSO. The cells were then collected and lysed in Laemmli buffer. Cleared total cell lysate was denatured by boiling and loaded onto a

4-20% gradient SDS-PAGE. Following electrophoretic separation, proteins were transferred to an Immobilon-P membrane. The membrane was blocked with SuperBlock blocking buffer and probed with the primary antibody, followed by incubation with a secondary antibody-conjugated with horseradish peroxidase. The proteins of interest were detected by using SuperSignal West Pico Chemiluminescent Substrate kit. Each assay was carried out in triplicate.

Luciferase reporter assay. 143B cells were seeded in T-25 flask and transfected with 3 μ g of Top-luc luciferase reporter plasmids. Twelve hours later, trypsinized cells were seeded to 24-well plates and were then treated with DHA or DMSO. At the scheduled time points, cells were lysed and subjected to luciferase assays using luciferase assay kit. Luciferase activity was normalized by total cellular protein concentrations of the samples. Each assay was performed in triplicate.

Establishment of orthotopic OS tumor animal model. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University. Athymic mice (female, 4-6 weeks old, 5/group) were ordered from the Animal Centre of Chongqing Medical University (Chongqing, China). 143B cells were collected and resuspended in cold (4°C) phosphate buffer solution (PBS) to a final density of 2x10⁷ cells/ml. Cells in 50 μ l of cold PBS were injected into the proximal tibia of athymic mice. Animals were then treated with either different doses of DHA (5, 10 and 20 mg/kg) or solvent by intragastric administration, once a day until the 37th day. The tumor size was measured every day from the 1st week. Animals were sacrificed at 37 days after injection. The tumor samples were retrieved for histological evaluation.

Statistical analysis. Data presented are the results of at least 2 independent experiments performed in triplicate. Microsoft Excel was employed to calculate the standard deviations. The differences were analyzed using the Student's t-test. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

DHA inhibits the proliferation of OS cells. We first employed crystal violet staining to test the proliferation inhibitory effect of DHA on human OS cells. The results showed that DHA inhibited the proliferation of 143B OS cells effectively in a concentration-dependent manner, even at the minimum concentration of 2.5 μ M (Fig. 1A and B). Similar results were obtained in other commonly-used OS cell lines, including U2OS, SaoS2 and MG63 (Fig. 1C and D). Moreover, the inhibitory effect of DHA on 143B cell proliferation was further confirmed by MTT assay (Fig. 1E) and colony formation assay (Fig. 1F and G). Proliferating cell nuclear antigen (PCNA) was a simple but potent marker for the assessment of OS growth (23). Therefore, we also tested the level of PCNA with western blotting. The results showed that the level of PCNA in 143B cells was decreased by DHA as well (Fig. 1H). Collectively, these results suggest that DHA effectively inhibits the proliferation of OS cells.

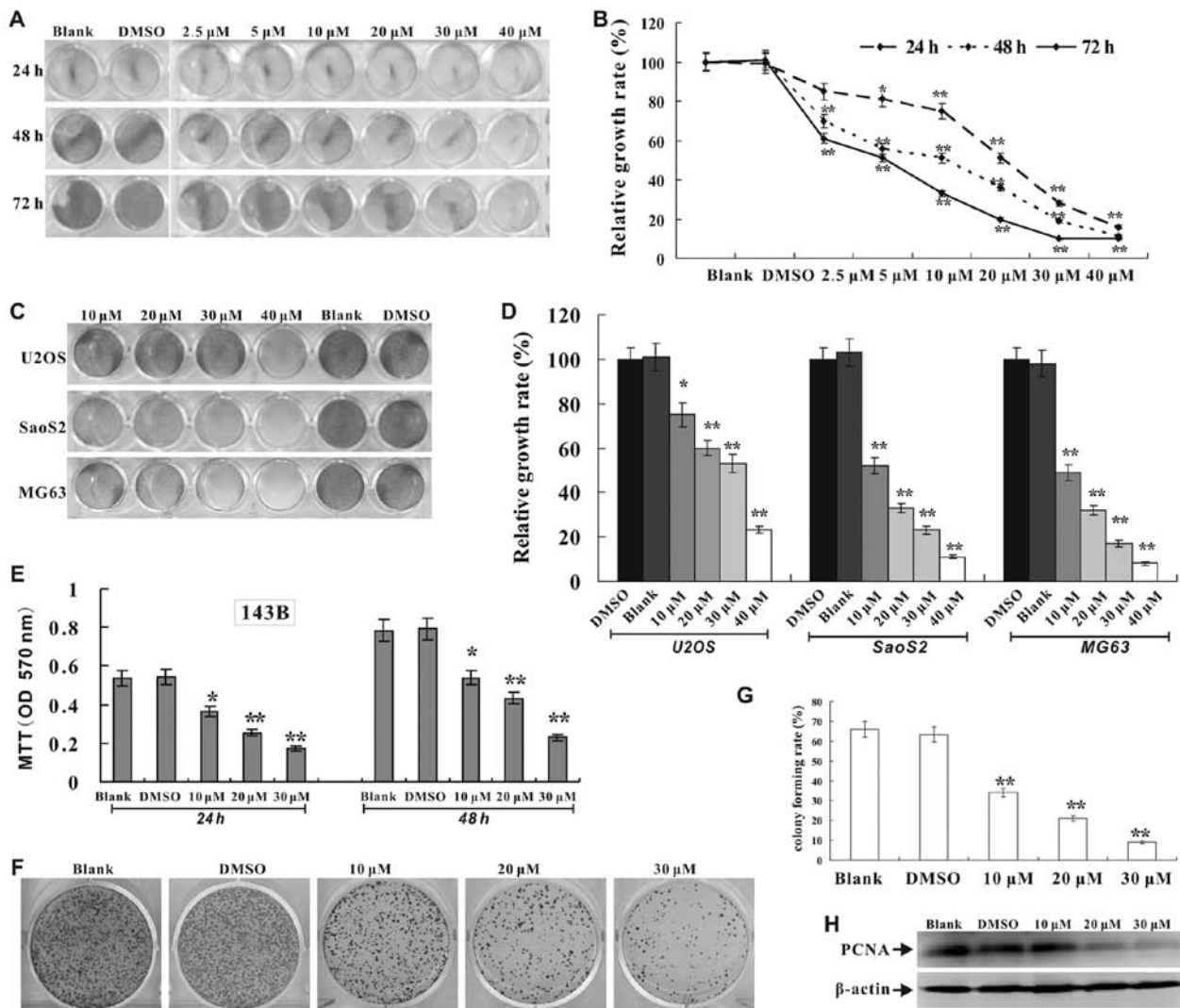


Figure 1. DHA inhibits the proliferation of human OS cells. (A) The crystal violet staining results in 143B OS cells. 143B cells were seeded in 24-well plates and treated with DHA at the indicated concentrations. At 24, 48 and 72 h after treatment, the cells were stained with crystal violet. (B) Quantitative results of crystal violet staining in 143B cells. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (C) The crystal violet staining results in U2OS, SaoS2 and MG63 OS cells. U2OS, SaoS2 and MG63 OS cells were seeded in 24-well plates and treated with DHA at the indicated concentrations. At 72 h after treatment, the cells were stained with crystal violet. (D) Quantitative results of crystal violet staining in U2OS, SaoS2 and MG63 OS cells. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (E) MTT results in 143B cells. 143B cells were seeded in 96-well plates and treated with DHA as the indicated concentrations. At 24 and 48 h after treatment, the cells were subjected to MTT assay. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (F) The colony formation results (crystal violet staining) in 143B cells treated with DHA at the indicated concentrations. At 14 days after treatment, the formed colonies were subjected to crystal violet staining. (G) Quantitative results of colony formation rates (%) in 143B cells. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (H) Western blot assay for the PCNA protein level in 143B cells. Cells were seeded in 6-well plates and treated with the indicated concentrations of DHA or DMSO for 24 h and were then harvested for western blot assay. DHA, dihydroartemisinin; OS, osteosarcoma; DMSO, dimethyl sulfoxide; PCNA, proliferating cell nuclear antigen.

DHA inhibits the migration and invasion of OS cells. Cell migration and invasion play a crucial role in the process of tumor metastasis. Herein, transwell assay without/with ECM was used to assess the alteration in cellular migration and invasion induced by DHA in OS cells. As shown in Fig. 2A and B, DHA inhibited the number of migrated OS cells in a dose-dependent manner. We also found that when 143B cells were treated with DHA, the number of invaded cells across the ECM-coated membranes decreased accordingly (Fig. 2C and D). Moreover, as illustrated in Fig. 2E, DHA reduced the protein level of matrix metalloproteinase 9 (MMP9), vascular endothelial growth factor (VEGF) and cyclooxygenases 2 (COX2), which have been proved to highly associate with migration and inva-

sion in cancer metastasis (24-26). These results demonstrate that DHA effectively attenuates the migrating and invasiveness phenotype of OS cell *in vitro*. This function may at least partly result from the reduction of MMP9, VEGF and COX2 proteins in OS cells.

DHA induces apoptosis in OS cells. We next investigated whether DHA induces OS cells to undergo apoptosis with Hoechst 33258 staining assay. The result showed that the apoptotic cells in the DHA-treated group were more pronounced than those of the blank or DMSO group (Fig. 3). Furthermore, we found that the level of cleaved caspase 3, a well-established central executor in cell apoptosis, was increased by DHA in

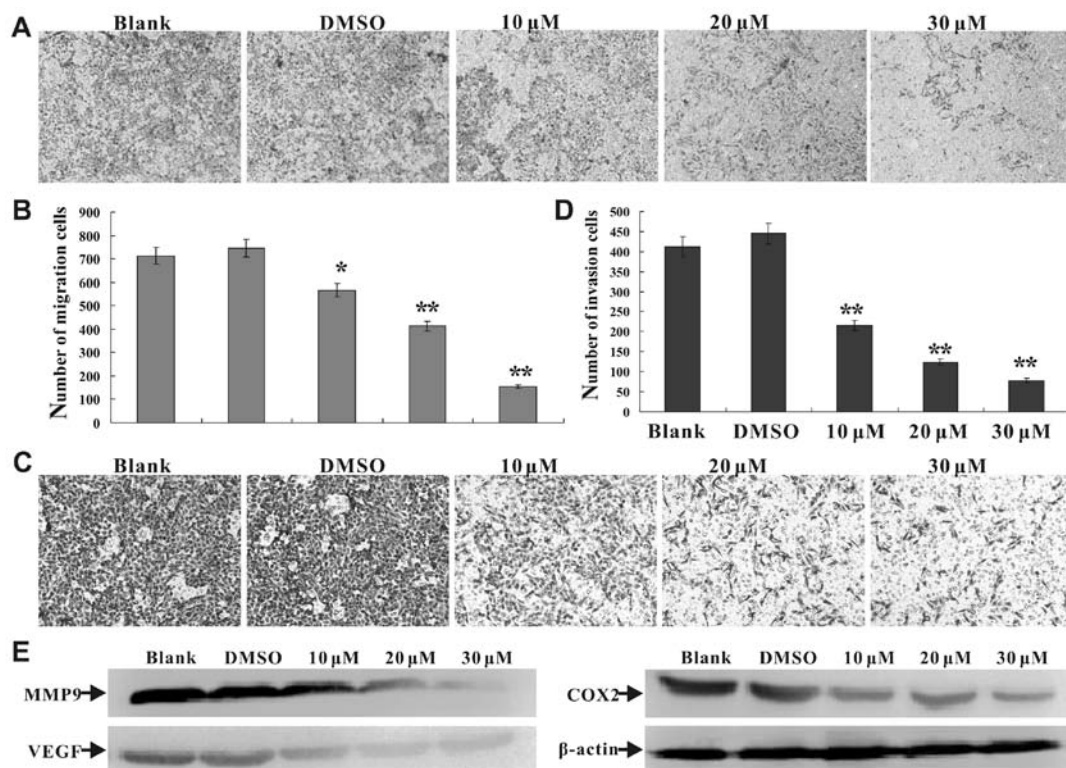


Figure 2. DHA inhibits migration and invasion of OS cells. (A) The transwell migration assay (without EMC) in 143B cells treated with DHA at the indicated concentrations. Migrated cells were fixed, stained and microphotographed. Representative results are shown. Magnification, $\times 100$. (B) The quantitative results of transwell migration assay in 143B cells. Migrated cells were counted as the average number of migrated cells per 5-10 high power fields. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (C) The transwell invasion assay (with EMC) in 143B cells treated with DHA at the indicated concentrations. Invaded cells were fixed, stained and microphotographed. Representative results are shown. Magnification, $\times 100$. (D) The quantitative results of transwell invasion in 143B cells. Invaded cells were counted as the average number of migrated cells per 5-10 high power fields. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (E) Western blot assay for the MMP9, VEGF and COX2 proteins in 143B cells. Cells were seeded in 6-well plates and treated with the indicated concentrations of DHA or DMSO for 24 h, then harvested for western blot assay. DHA, dihydroartemisinin; OS, osteosarcoma; DMSO, dimethyl sulfoxide; MMP9 metalloproteinase 9; VEGF, vascular endothelial growth factor; COX2, cyclooxygenases 2.

a concentration-dependent manner. DHA also disturbed the balance between proapoptotic protein Bad and anti-apoptotic protein Bcl2, led to an increase in Bad but a reduction in Bcl2 protein (Fig. 2C). The data indicate that DHA is capable of inducing and promoting apoptosis, a characteristic shared by most of the currently used chemotherapy agents (27,28).

DHA inhibits tumor growth of OS cells *in vivo*. The above data demonstrate that DHA inhibited tumor growth of OS cells *in vitro*. We sought to confirm these findings in an orthotopic OS tumor animal model *in vivo*. 143B cells were implanted into the proximal tibia of athymic mice under DHA treatment, and tumor size was monitored. As shown in Fig. 4A, the DHA treatment group exhibited significantly decreased tumor size, compared with the control group. At 37 days, the animals were euthanized and the tumor masses were retrieved (Fig. 4B). The H&E staining results showed that sample in the control group was more aggressive than that of the DHA-treated groups, the OS cells invaded and replaced almost all the bone at primary site (Fig. 4C). However, the samples retrieved from the DHA group displayed relative intact bone structure and less tumor cells, particularly in the high dose group (Fig. 4C). These results confirm that DHA inhibits OS tumor growth *in vivo*.

DHA suppresses Wnt/ β -catenin signaling in OS cells. We found that DHA inhibits tumor growth of OS cells both *in vitro* and *in vivo*. Next, we sought to examine the possible mechanism behind the anticancer activity of DHA in OS cells. Western blot results showed that p-P53, P53 and MDM2 were not altered under DHA treatment, indicating that the level of these molecules is possibly not related to the anticancer effects of DHA in OS cells (Fig. 5A). However, total β -catenin protein was effectively reduced by DHA, as were the β -catenin targets C-myc and cyclin D1 (Fig. 5A). Furthermore, we found that DHA was able to inhibit reporter activity of TOP-Luc, which contains TCF/LEF-responsive elements and reflects β -catenin transcriptional activity (Fig. 5B). These results indicate that DHA suppresses Wnt/ β -catenin signaling in OS cells. To investigate how DHA reduces the β -catenin protein in OS cells, we employed western blot assay to detect the activation status of GSK3 β in OS cells. We found that DHA increased GSK3 β activity by suppressing phosphorylation of GSK3 β at serine 9 (Ser 9), which has been proved to be an inactive form of GSK3 β (Fig. 5A). Notably, we observed that the level of DVL proteins, a main switchboard of Wnt/ β -catenin signaling, is decreased by treatment of DHA as well (Fig. 5A). These findings suggest that downregulation of β -catenin protein in OS by DHA resulted from upregulation of GSK3 β activity and destabilize the β -catenin.

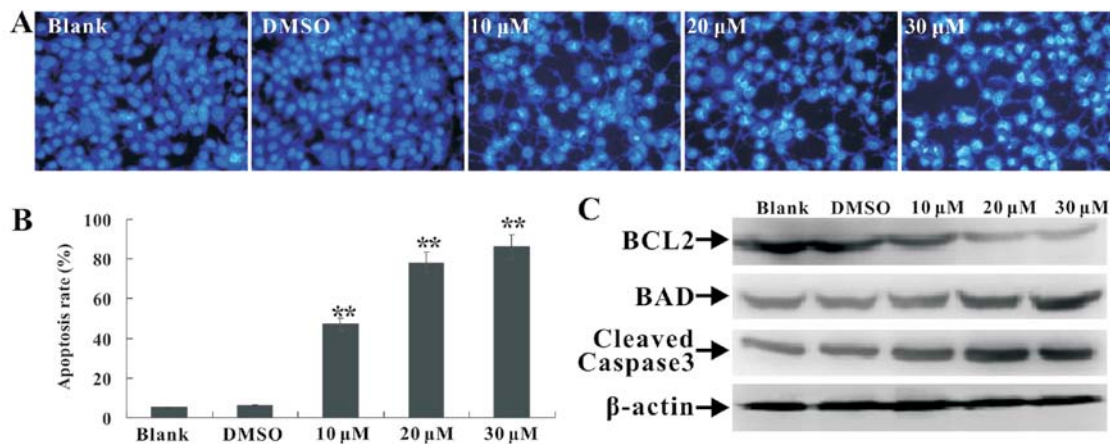


Figure 3. DHA induces apoptosis in human OS cells. (A) The Hoechst 33258 staining results in 143B cells. Cells were seeded in 24-well plates and treated with the indicated concentrations of DHA for 24 h, then harvested and stained with Hoechst 33258. Representative results are shown. Magnification, $\times 100$. (B) The quantitative results of Hoechst 33258 staining results. The assay was performed in triplicate. * $p < 0.05$ vs. blank and DMSO; ** $p < 0.01$ vs. blank and DMSO. (C) Western blot assay for Bad, Bcl2 and cleaved caspase 3 proteins in 143B cells. Cells were seeded in 6-well plates and treated with the indicated concentrations of DHA for 24 h, then harvested for western blot assay. DHA, dihydroartemisinin; OS, osteosarcoma; DMSO, dimethyl sulfoxide.

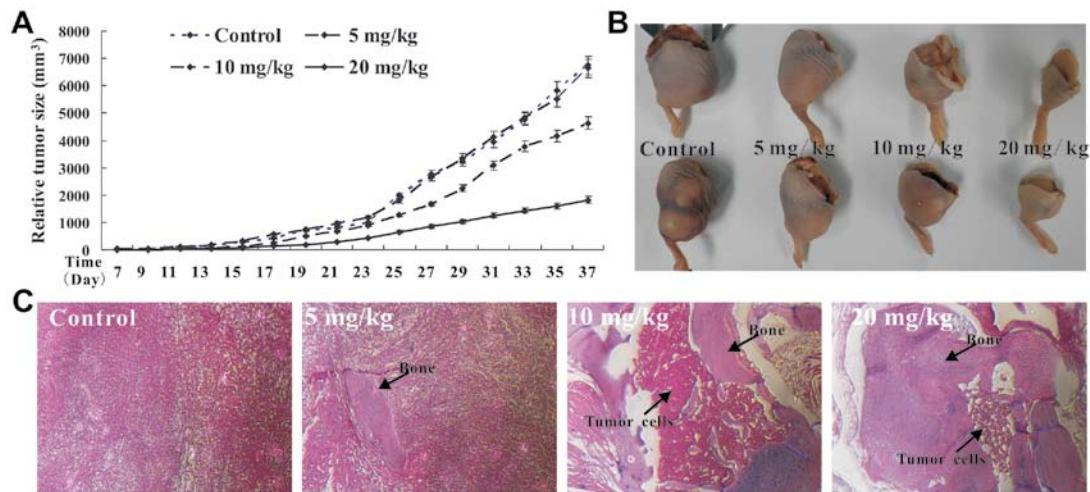


Figure 4. DHA inhibits OS tumor growth *in vivo*. (A) The change of tumor size with time. (B) Retrieved tumor samples from orthotopic OS tumor animal model. (C) H&E staining results of the retrieved tumor sample. Representative results are shown. Magnification, $\times 200$. DHA, dihydroartemisinin; OS, osteosarcoma; H&E; hematoxylin and eosin.

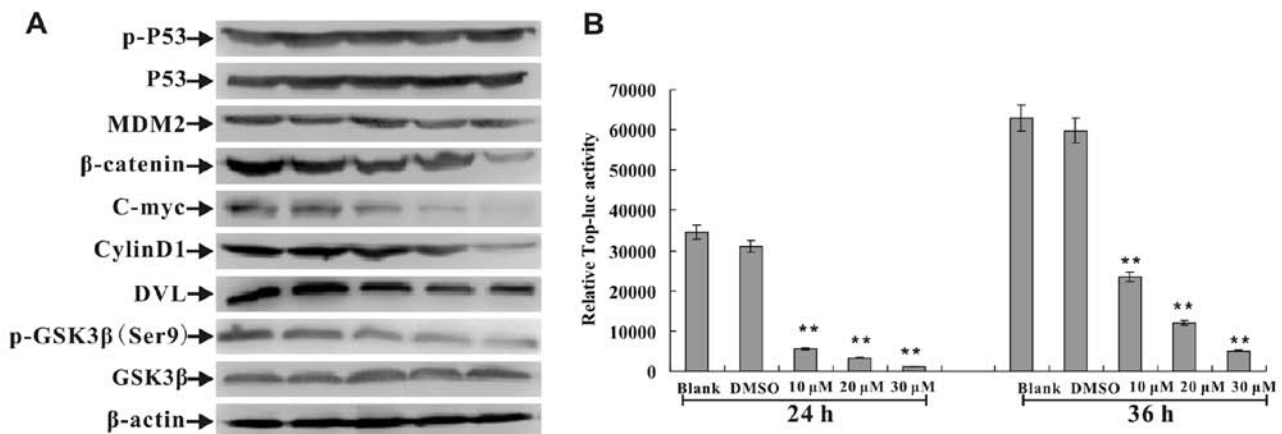


Figure 5. DHA suppresses Wnt/ β -catenin signaling in human OS cells. (A) Western blot assay for p-P53, P53, MDM2, β -catenin, c-myc, cyclin D1, DVL, p-GSK3 β (Ser 9) and GSK3 β in 143B cells. Cells were seeded in 6-well plates and treated with the indicated concentrations of DHA for 24 h and were then harvested for western blot assay. (B) β -catenin-controlled TOP-Luc reporter results in 143B cells. Cells were seeded in 6-well plates and were treated with the indicated concentrations of DHA for 24 or 36 h, then harvested for luciferase activity test. The assay was performed in triplicate. ** $p < 0.01$ vs. blank and DMSO. DHA, dihydroartemisinin; OS, osteosarcoma; GSK3 β , glycogen synthase kinase 3 β ; Ser 9, serine 9; DMSO, dimethyl sulfoxide.

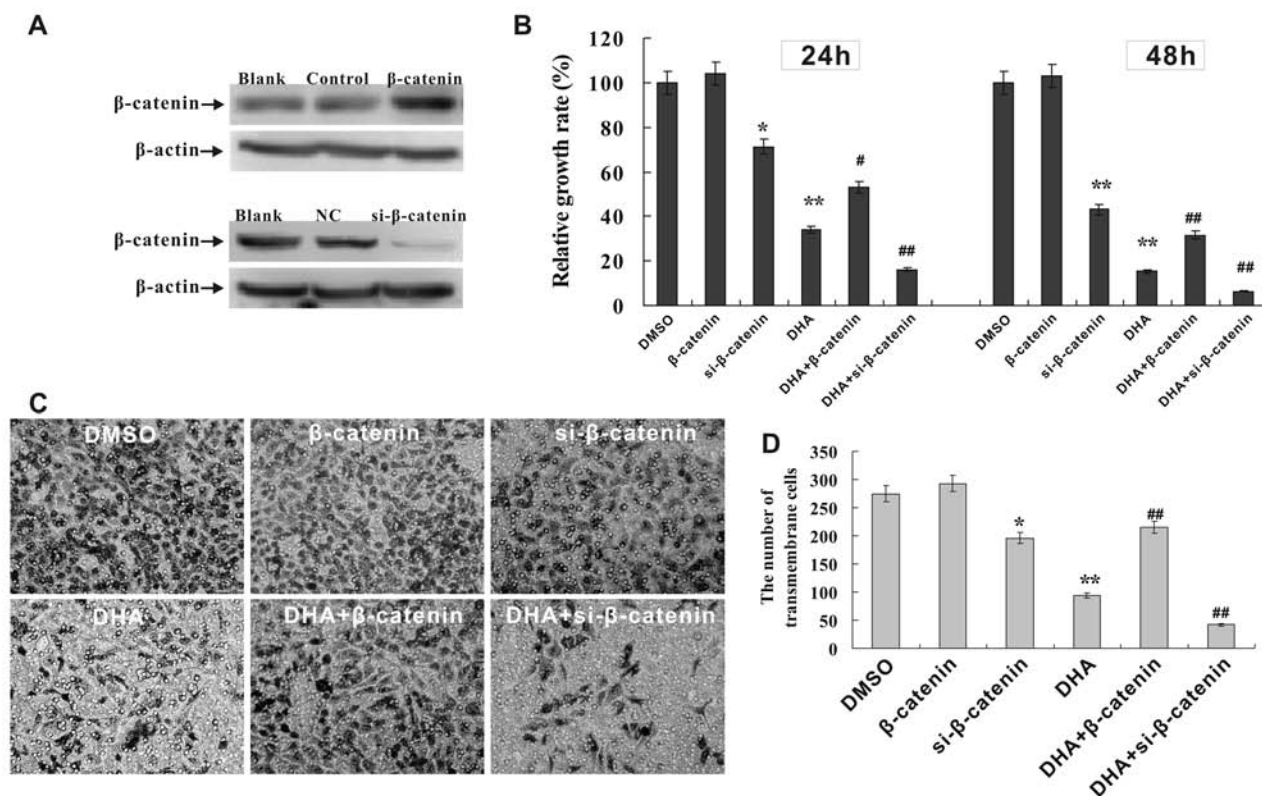


Figure 6. The function of β -catenin in the inhibitory effects of DHA on OS cells. (A) Western blot assay for exogenous expression and knockdown of β -catenin in 143B cells. (B) The quantitative results of 143B growth rate (crystal violet staining). Cells were seeded in 24-well plates and treated with DHA and with/without exogenous expressed β -catenin or knockdown of β -catenin. At 24 h after treatment, the cells were stained with crystal violet and growth rate was quantified. The assay was performed in triplicate. * $p < 0.05$ vs. DMSO; ** $p < 0.01$ vs. DMSO; # $p < 0.05$ vs. DHA; ## $p < 0.01$ vs. DHA. (C) The transwell invasion results (with EMC) in 143B cells. Cells were seeded in 24-well plates and treated with DHA and with/without exogenous expressed β -catenin or knockdown of β -catenin. Invaded cells were fixed, stained and microphotographed. Representative results are shown. Magnification, $\times 100$. (D) The quantitative transwell invasion results in 143B cells. Invaded cells were counted as the average number of migrated cells per 5-10 high power fields. The assay was performed in triplicate. * $p < 0.05$ vs. DMSO; ** $p < 0.01$ vs. DMSO; ## $p < 0.01$ vs. DHA. DHA, dihydroartemisinin; OS, osteosarcoma; DMSO, dimethyl sulfoxide.

To validate the role of β -catenin in the inhibitory effect of DHA on OS cells, the overexpression of exogenous β -catenin or knockdown of β -catenin in OS cells was achieved respectively (Fig. 6A). The proliferation of 143B cells was suppressed by knockdown of β -catenin, although it was not altered by exogenous β -catenin (Fig. 6B). Markedly, we found that exogenous expression of β -catenin attenuated the growth inhibitory function of DHA, whereas knockdown of β -catenin enhanced this function in 143B cells, suggesting that decrease of β -catenin protein plays an important role in the anti-proliferation function of DHA in OS cells (Fig. 6B). In the transwell invasion assay, we observed that the invasion suppressive effect of DHA was neutralized by exogenous expression, but augmented by β -catenin knockdown (Fig. 6C and D). Collectively, the above data indicate that the inhibitory effect of DHA on OS cells may result from the inactivation of Wnt/ β -catenin signaling.

Discussion

OS is a common primary bone cancer with considerable morbidity and mortality. The current clinical strategies for OS include surgical resection, radiotherapy and chemotherapy. However, OS patients still have a poor prognosis under these aggressive treatments (29). Thus, there is an urgent need to develop more effective therapies for OS treatment. Herbal

and natural products have been proved to be highly suitable sources for anticancer drugs (10,11,14-18). DHA, a derivative of ART, was initially used as an anti-malarial. However, recent studies indicated that DHA is capable of improving immunity, preventing inflammation, regulating cell differentiation and inhibiting cancer growth with low host toxicity (30-34). The anticancer effect of DHA is partly associated with iron homeostasis (33). Moreover, DHA is shown to activate p38 and ERK1/2, indicating these signal molecules are relevant to the anticancer effects of DHA (34). Despite these findings, the molecular mechanism through which DHA inhibits tumor growth remains largely unclear and warrants extensive studies. In this study, we investigated the anticancer effect of DHA on human OS cells. We found that DHA prevents cell proliferation in OS cells effectively. Of note, however, the inhibitory effect of DHA on OS cells is possibly mediated by suppressing the Wnt/ β -catenin signaling through activation of GSK3 β and subsequent reduction of β -catenin protein.

We found that DHA can reduce metastatic phenotype, as well as expression of MMP9, VEGF and COX2 of OS cells *in vitro*. MMP9, member of the MMP family, is capable of degrading extracellular matrix and is involved in the metastasis of cancer (35,36). VEGF is a key angiogenic factor that can stimulate vasculogenesis and angiogenesis (37-39). Cancer that expresses VEGF is prone to invade and metastasize (38,39),

and downregulation of VEGF can attenuate the invasiveness phenotype of malignant tumor (40,41). COX2 is known to be closely associated with tumor growth and metastasis in several types of human tumors (42,43,25). COX2 is unexpressed under normal conditions in most cells, but is upregulated in several types of cancer. The overexpression of COX2 is associated with increased angiogenesis and aggressive invasion in cancer (43,25). In human OS, COX2 is directly associated with OS cell migration and invasion (25,44). Therefore, inhibition of MMP9, VEGF and COX2 expression by DHA may be an important mechanism responsible for the decreased metastatic phenotype in OS cells.

Induction of apoptosis is a common mechanism underlying the anticancer effects of synthetic and natural drugs. Hoechst 33258 staining assay shows that DHA can induce apoptosis in OS cells. Cleaved caspase 3, the activated form of caspase 3, is regarded as a critical effector in the sequential activation of caspases, and plays a central role in the execution-phase of mitochondrial apoptosis pathway. Our results indicated that DHA can increase the level of cleaved caspase 3 in OS cells, suggesting that the mitochondrial apoptosis pathway is likely involved in DHA-induced apoptosis. Bad and Bcl2 belong to the Bcl2 family members that are critical regulators of apoptosis. However, the role of Bad and Bcl2 in cell apoptosis is different. Bad is a well-established proapoptotic factor, whereas Bcl2 is an antiapoptotic molecule known for its ability to inhibit the release of cytochrome *c* from mitochondria (45). DHA can reduce the levels of Bcl2 in OS cell lines, whereas it increases Bad protein levels. Therefore, disturbance of the balance between proapoptotic protein (Bad) and antiapoptotic protein (Bcl2) by DHA provides relative evidence to demonstrate the DHA-induced apoptosis in OS cells. Induction of apoptosis of human OS cells by DHA may be an important mechanism responsible for the inhibitory effect on OS cell growth.

It has been reported that the abnormal activation of Wnt/ β -catenin signaling accounts for tumor initiation of human OS (45-49). Our results showed that DHA can reduce the total protein level of β -catenin. Overexpression of exogenous β -catenin alone in OS cells cannot further enhance proliferation and invasion of OS cells. In the present study, we hypothesized that the β -catenin protein in OS cells is already high enough, therefore exogenous β -catenin is not necessary to further increase malignant phenotype of OS cells. However, overexpression of exogenous β -catenin can neutralize the function of DHA, and knockdown of β -catenin can enhance the growth inhibitory effect of DHA. Therefore, we propose that the effect of DHA on OS cells may, in part, result from targeting Wnt/ β -catenin signaling.

The Wnt/ β -catenin signaling is a canonical Wnt pathway that causes an accumulation of β -catenin and its eventual translocation into the nucleus. The β -catenin in the nucleus interacts with TCF/LEF family to regulate downstream gene expression (50). Wnt/ β -catenin pathway plays important roles in embryogenesis and tumorigenesis. Without Wnt ligands, free β -catenin is rapidly degraded through proteasome machinery promoted by a destruction complex assembled with APC, Axin and GSK3 β . In the presence of Wnt ligands, this destruction complex function becomes disrupted and the β -catenin protein becomes stable in the cytoplasm and

translocates to the nucleus (50). Following translocation to the nucleus, β -catenin binds to T-cell factors (TCF) and activates the transcription of specific target genes, including C-myc and cyclin D1 (50). GSK3 β is a master protein in the Wnt/ β -catenin pathway by facilitating phosphorylation and promoting degradation of β -catenin (50). Catalytic kinase activity of GSK3 β is controlled through differential phosphorylation of serine/threonine residues. Phosphorylation at tyrosine 216 in GSK3 β enhances the enzymatic activity of GSK3 β , while phosphorylation of serine 9 significantly decreases catalytic activity (51). We found that phosphorylation of serine 9 residue in GSK3 β is decreased by DHA treatment, which lead to an increased catalytic activity of GSK3 β . This result indicates that the decrease of β -catenin protein in OS cells may result from the degradation initialized by GSK3 β . Our results show that DHA can downregulate DVL proteins in OS cells. DVL proteins are upstream controllers of APC/Axin/GSK3 β complex, and are positive mediators of the Wnt/GSK3 β pathway. It has been shown that DVL proteins are frequently upregulated and are significantly associated with abnormal elevated β -catenin protein in several types of cancer (52-54). Moreover, targeted inhibition of DVL proteins decreased β -catenin protein, reduced TCF-dependent transcription and inhibited cell growth in human cancer (55,56). Although further analysis should be conducted, the decreased DVL proteins by DHA are possibly related to the anticancer effects of DHA by disturbing Wnt/ β -catenin signaling in OS cells as well.

In conclusion, the present study suggests that DHA can be used as an effective chemotherapy agent for human OS. The proliferation and invasion inhibitory effects of DHA on OS cells may result from inactivating Wnt/ β -catenin signaling through increasing GSK3 β activity and thereby promoting the degradation of β -catenin. Future studies will be directed at elucidating the exact mechanism of DHA on the degradation of the β -catenin protein, as well as at combining DHA with other traditional chemotherapy drugs to develop a novel therapy strategy for human OS cancer.

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