

# Lapatinib alters the malignant phenotype of osteosarcoma cells via downregulation of the activity of the HER2-PI3K/AKT-FASN axis *in vitro*

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**Abstract.** Lapatinib, an inhibitor of human epidermal growth factor receptor 2 (HER2) phosphorylation, has been reported to inhibit several types of tumors such as HER2-overexpressing breast cancer. However, the effect of lapatinib on the malignant phenotype of human osteosarcoma (OS) cells and the potential molecular mechanisms remain unclear. To elucidate the effect of lapatinib on OS, two OS cell lines, U2-OS and MG-63, were utilized in the present study. Various concentrations of lapatinib were used to treat OS cells for different time durations. Cell proliferation was evaluated by MTT and colony formation assays. Flow cytometry (FCM) was used to evaluate cell apoptosis. Wound healing and Transwell invasion assays were performed to examine the migratory and invasive abilities of the cells. To investigate the possible molecular mechanisms involved, the expression of p-HER2, phosphatidylinositol 3-kinase (PI3K), p-AKT, AKT and fatty acid synthase (FASN) protein was detected by western blotting. MTT assays showed that lapatinib inhibited the proliferation of U2-OS and MG-63 cells in a dose- and time-dependent manner, and the rate of colony formation of the lapatinib-treated cells was significantly lower when compared to those cells not treated with lapatinib in both cell lines. FCM assay revealed a significantly higher apoptotic rate in the lapatinib-treated OS cells. Wound healing and Transwell invasion assays revealed that the migratory and invasive abilities of OS cells were significantly inhibited by lapatinib ( $P < 0.05$ ). Western

blotting showed that lapatinib suppressed the activity of HER2-PI3K/AKT-FASN in U2-OS and MG-63 cells *in vitro*. These results suggest that lapatinib may alter the malignant phenotype of OS cells via downregulation of the activity of the HER2-PI3K/AKT-FASN signaling pathway *in vitro*. Thus, lapatinib may be an effective chemotherapeutic agent for the treatment of osteosarcoma.

## Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumors in children and adolescents. With the advent of effective chemotherapy, the 5-year survival rate for patients treated with intensive multidrug chemotherapy and aggressive local control has been reported to be 55-80% (1-4). However, chemotherapy fails to eliminate all OS cells due to intrinsic or acquired drug resistance, which is the most common cause of tumor recurrence resulting in poor clinical outcomes (5). Therefore, novel reagents are urgently needed for the effective chemotherapy of OS.

Human epidermal growth factor receptor 2 (HER2) is a 185-kDa transmembrane receptor tyrosine kinase (RTK), belonging to the epidermal growth factor receptor (EGFR) family. Aberrant upregulation of HER2 is found in various types of cancers such as breast cancer (6), ovarian cancer (7) and OS (8-10). Patients with HER2-positive cancer have a high risk for diminished effectiveness of cancer treatments and poor clinical outcomes due to increased tumor cell metastasis (11). Ligand stimulation induces dimerization of the HER2 receptor (homodimer or heterodimer), which leads to self-phosphorylation on tyrosine residues localized to the C-terminal domain of HER2 receptors. Furthermore, the phosphorylated HER2 receptors activate a variety of downstream signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt (12), which plays an essential role in cell-extracellular matrix (ECM) and cell-cell adhesion in cell proliferation and survival. Recently, various studies have revealed that targeting HER2 is an important therapeutic strategy for treating OS (13,14).

Lapatinib is a small-molecule kinase inhibitor and a derivative of 4-anilinoquinoline (15) whose molecular formula

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is  $C_{29}H_{26}ClFN_4O_4S$ , and chemically it is *N*-[3-chloro-4-[(3-fluorophenyl)methoxy]phenyl]-6-[5-[(2-methylsulfonyl)ethylamino)methyl]-2-furyl]quinazolin-4-amine (16). Lapatinib potently and reversibly binds to the intracellular TK domains of EGFR and HER2, which leads to inhibition of substrate phosphorylation. This inhibition blocks downstream MAPK and PI3K/AKT proliferation and survival signaling pathways both *in vitro* and *in vivo* (17,18). Lapatinib was reported to effectively inhibit human tumor cell proliferation (19). In March 2007, the US Food and Drug Administration approved the use of lapatinib for the treatment of advanced breast cancer overexpressing HER2 (HER2<sup>+</sup>) (20).

However, the effect of lapatinib on the malignant potential of human OS cells and the potential molecular mechanisms are still unclear. To explore the possibility of developing lapatinib as a therapeutic agent and to clarify its potential molecular mechanisms, we investigated the effect and underlying molecular mechanisms of lapatinib in human OS cells. The present study was conducted by evaluating the effect of lapatinib on the proliferation, migration and invasion abilities of OS cell lines U2-OS and MG-63 cells and the involvement of the HER2-PI3K/AKT-FASN signaling pathway *in vitro*.

## Materials and methods

**Cell lines and cell culture.** The human OS cell lines, U2-OS and MG-63, were obtained from the American Type Culture Collection (Manassas, VA, USA) and routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) in a humidified 37°C incubator containing 5% CO<sub>2</sub>.

**Cell growth assay.** The U2-OS and MG-63 cells were cultured in 96-well tissue culture plates at a cell density of 5,000 cells/well, in DMEM containing 10% FBS and 2 mM glutamine. Following adherence overnight, the medium was replaced and the cells were incubated with different concentrations (5, 10, 20, 30 and 40  $\mu$ mol/l for U2-OS cells and 5, 10, 15, 20 and 25  $\mu$ mol/l for MG-63 cells) of lapatinib for 24, 48 and 72 h. Viable proliferating cells were detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, using 6-wells/time period. Cell viability was expressed as optical density (OD), which was detected by an enzyme-linked immunosorbent assay (ELISA) reader (MK3; Thermo, USA) at a 490-nm wavelength. The inhibitory rate of cell proliferation was calculated. Six independent experiments were performed over multiple days.

**Flow cytometry (FCM).** Human OS U2-OS and MG-63 cells were seeded at  $5 \times 10^5$  cells/ml into T25 culture flasks for 24 h. The cells were then treated with 0, 5, 10 and 15  $\mu$ mol/l lapatinib. Following incubation, the cells were trypsinized, washed with phosphate-buffered saline (PBS) and fixed overnight in ice-cold 70% ethanol. Subsequent to fixation, the cells were washed twice with 1% bovine serum albumin (BSA) in PBS, and resuspended in 1 ml DNA-binding propidium iodide (PI) solution (10 mg/ml in PBS, containing 0.05 mg/ml RNase A), incubated at room temperature in the dark for 15 min and analyzed with an EPICS XL flow cytometer (Beckman

Coulter, Miami, FL, USA). The number of apoptotic cells was measured with the control software of the flow cytometer. Six independent experiments were performed over multiple days.

**Colony formation assay.** Cells ( $2 \times 10^6/2$  ml/well) were seeded in tissue culture plastic dishes, and treated with lapatinib (15  $\mu$ mol/l for U2-OS and 10  $\mu$ mol/l for MG-63) for 2 weeks to form colonies. The formed colonies were stained with Giemsa, and the colonies containing >50 cells were counted under an inverted microscope. Six independent experiments were performed over multiple days.

**Invasion assay.** The invasiveness of the OS cells was measured using BD BioCoat™ BD Matrigel™ invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The medium in the lower chamber contained 15% FBS as a source of chemoattractant. The cells were suspended in serum-free medium containing lapatinib (15  $\mu$ mol/l for U2-OS and 10  $\mu$ mol/l for MG-63) and added to the upper chambers simultaneously ( $2 \times 10^3$  cells in 0.1 ml). The cells that passed through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex, Kobe, Japan), and images were captured. Cell invasion was quantified by direct microscopic visualization and counting. The invaded cells were counted in 5 randomly selected fields under an inverted microscope. The cells not treated with lapatinib were used as a normalization control. Six independent experiments were performed over multiple days.

**Migration assay.** Cell migration was assessed by determining the ability of the cells to move into a cellular space in a two-dimensional wound healing assay *in vitro*. In brief, the cells were cultured in a 6-well tissue culture plastic dishes to  $5 \times 10^6$  cells/well, and subsequently treated with lapatinib (15  $\mu$ mol/l for U2-OS and 10  $\mu$ mol/l for MG-63) cells for 24 h. The cells were then denuded by dragging a rubber policeman (Fisher Scientific, Hampton, NH, USA) through the center of the plate well. The culture plates were rinsed with PBS, and fresh quiescent medium alone or with 10% BSA was added, in which the cells were incubated at 37°C for 24 h. The cells were photographed at 0 and 24 h, and the migrated distance was measured. The rate of migration was assessed from 5 randomly selected fields under an inverted microscope. The cells not treated with lapatinib were used as a normalization control. Six independent experiments were performed over multiple days.

**Western blot analysis.** U2-OS and MG-63 cells in the exponential growth phase were treated with lapatinib (15  $\mu$ mol/l for U2-OS and 10  $\mu$ mol/l for MG-63) for 24 h. The cells were then washed with cold PBS. Total protein from the cells was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer containing 60  $\mu$ g/ml phenylmethanesulfonyl fluoride (PMSF), and the protein concentration was determined using a Bradford assay. Equal amounts of protein were electrophoresed by 10% SDS-PAGE and transferred onto pure nitrocellulose blotting membranes (0.22- $\mu$ m pore size). The membranes were blocked with 5% Difco skim milk for 1 h at room temperature (RT), and then blocked with the primary antibody (rabbit anti-human P-HER2, AKT, P-AKT, FASN,

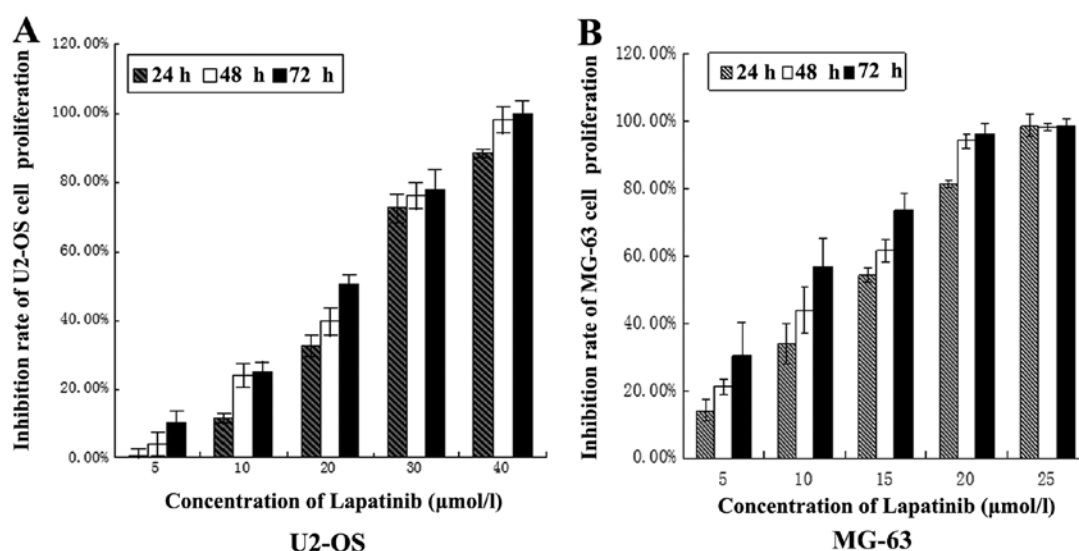


Figure 1. Effect of various concentrations of lapatinib on OS cell proliferation. The result showed that lapatinib inhibited (A) U2-OS and (B) MG-63 cell proliferation in a dose and time-dependent manner. OS, osteosarcoma.

mouse anti-human PI3K, GAPDH IgG, 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. The membranes were then washed prior to incubation with the appropriate peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse, 1:5,000; Santa Cruz Biotechnology, Inc.). The immune complexes were detected with a Pro-Light HRP kit (Tiangen, Beijing, China). All experiments were repeated 6 times over multiple days.

**Statistical analysis.** Data are expressed as the means  $\pm$  SD. The differences in invasion and migration capabilities between the cells treated with and without lapatinib were evaluated with independent-sample t-tests. A value of  $P < 0.05$  was considered to indicate a statistically significant difference. All analyses were performed with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

## Results

**Effect of lapatinib on OS cell proliferation in vitro.** The effect of lapatinib on the growth of the U2-OS and MG-63 cell lines was investigated using MTT and colony formation assays. The results showed that the proliferation of U2-OS and MG-63 cells was inhibited by lapatinib in a dose- and time-dependent manner (Fig. 1). The  $IC_{50}$  values for lapatinib in U2-OS and MG-63 cells at 24 h were 22.150 and 11.646  $\mu$ mol/l, respectively. A concentration of 15  $\mu$ mol/l for U2-OS and 10  $\mu$ mol/l for MG-63 was chosen for treatment of the OS cells in the following assays. The colony formation rate of cells treated with lapatinib was obviously lower than the rate of the cells not treated by lapatinib (Fig. 2). These data indicate that lapatinib inhibits U2-OS and MG-63 cell proliferation *in vitro*.

**Lapatinib induces OS cell apoptosis.** FCM analysis was used to investigate the effect of lapatinib on the induction of apoptosis of U2-OS and MG-63 cells *in vitro*. Lapatinib at various concentrations was added to the U2-OS and MG-63 cells in the exponential growth phase for 24 h, and cell samples were

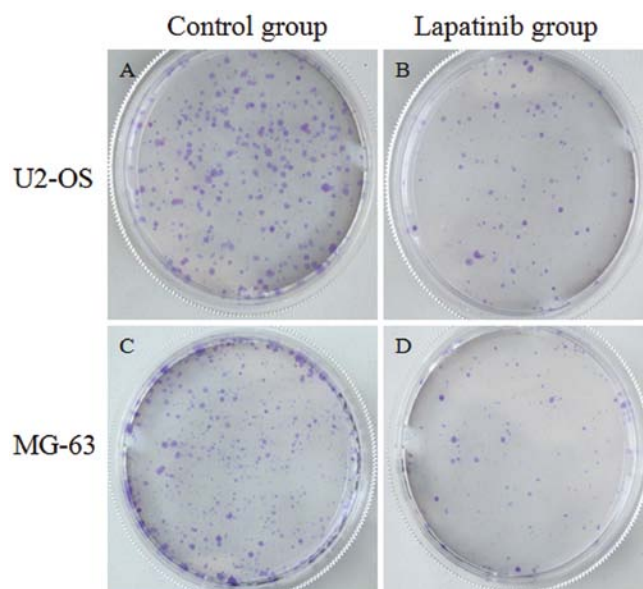


Figure 2. Effect of lapatinib on the colony formation of the OS cell lines. In the (A and D) U2-OS and (C and D) MG-63 cell lines, the colony formation rate of the cells treated with lapatinib (B and D) was obviously lower than that of the cells that were not treated (A and C). OS, osteosarcoma.

obtained and fixed for FCM analysis. The results revealed that apoptosis was induced by lapatinib in a dose-dependent manner (Fig. 3). This indicated that lapatinib induced U2-OS and MG-63 cell apoptosis *in vitro*.

**Lapatinib inhibits OS cell invasion and migration in vitro.** To examine the effect of lapatinib on OS cell migration and invasion, the migration and invasion capabilities were assessed with the wound healing and Transwell invasion assays. As shown in Fig. 5, the migration rate of cells treated with lapatinib was  $32.70 \pm 3.00\%$  in the U2-OS cells and  $30.65 \pm 6.15\%$  in the MG-63 cells, compared with  $94.52 \pm 4.76\%$  and  $91.83 \pm 2.32\%$  in the cells not treated with lapatinib. In the Transwell invasion

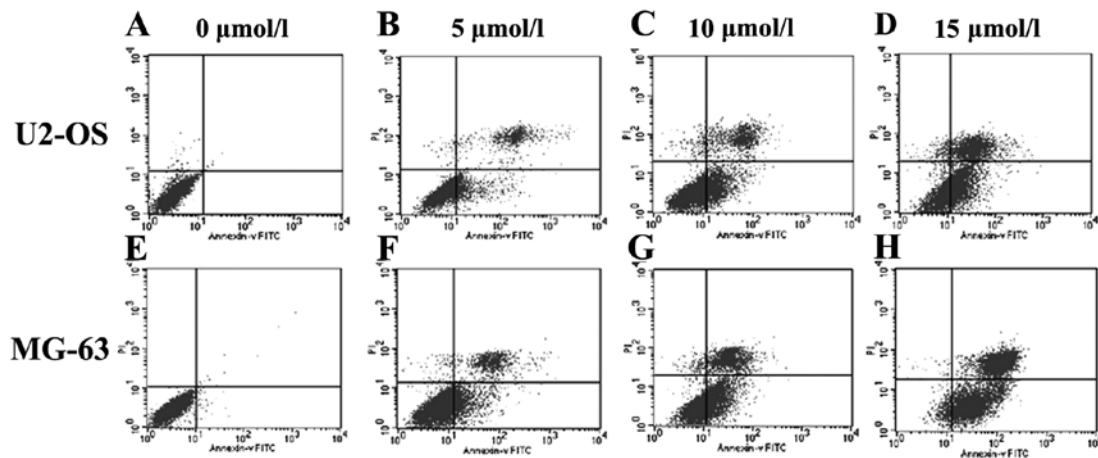


Figure 3. Flow cytometric (FCM) analysis of OS cells treated with various concentrations of lapatinib. (A and E) Few apoptotic cells were noted in the control group. Yet, the percentages of apoptotic cells in the OS cells treated with 0, 5, 10 and 15  $\mu\text{mol/l}$  of lapatinib were (B) 20.55, (C) 34.18 and (D) 62.22% in the U2-OS cells, respectively, and (F) 27.72, (G) 57.15 and (H) 94.40% in the MG-63 cells, respectively. The result revealed that lapatinib induced OS cell apoptosis in a dose-dependent manner. OS, osteosarcoma.

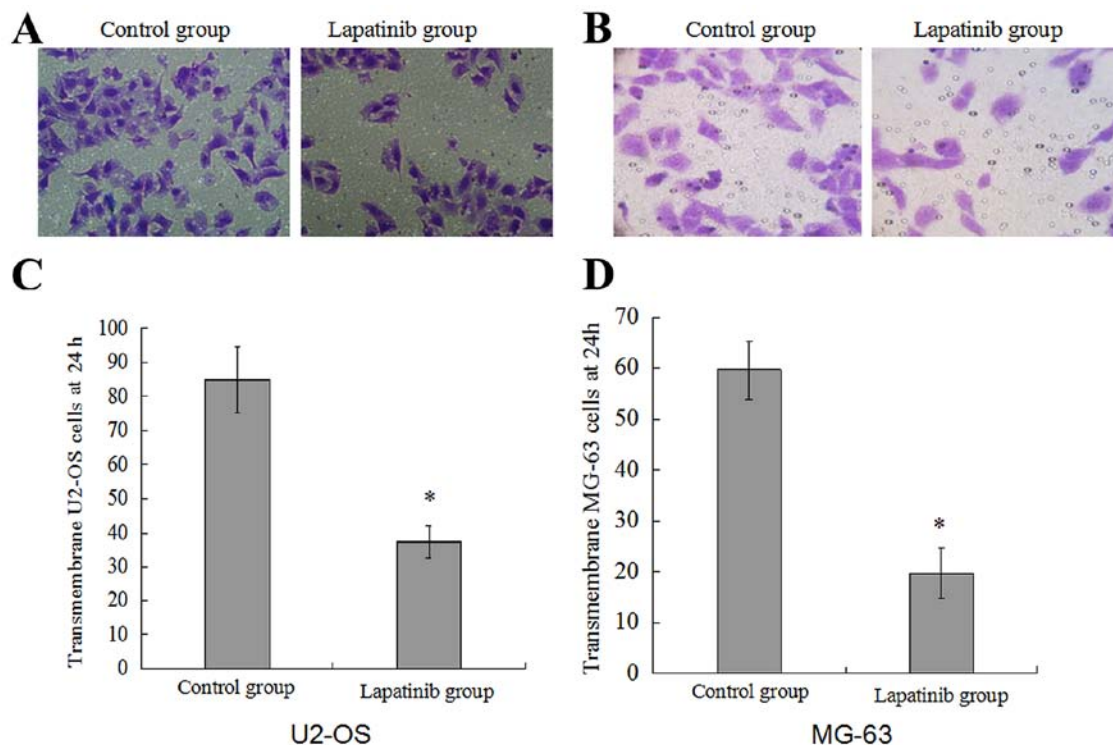


Figure 4. Transwell invasion assay was used to evaluate the effect of lapatinib on OS cell invasion. In the (A and C) U2-OS and (B and D) MG-63 OS cell lines, the number of transmembrane cells in the lapatinib-treated group was significantly fewer than the number of the cells not treated with lapatinib at 24 h. Columns, mean (n=6); bars, SD; \*P<0.05 vs. control group. OS, osteosarcoma.

assay (Fig. 4), the invasion of the cells treated with lapatinib was significantly inhibited compared with that in the untreated cells (P<0.05). This suggests that lapatinib suppresses U2-OS and MG-63 cell migration and invasion *in vitro*.

**Lapatinib suppresses the activity of the HER2-PI3K/AKT-FASN signaling pathway.** To investigate the effect of lapatinib on the activity of the HER2-PI3K/AKT-FASN signaling pathway, the protein expression levels of p-HER2, PI3K, p-AKT, AKT and FASN were detected. The results showed that the protein expression levels of p-HER2, PI3K, p-AKT

and FASN except for AKT were significantly decreased in the cells treated with lapatinib when compared with these levels in the untreated cells (Fig. 6). This suggests that lapatinib suppresses the activity of HER2-PI3K/AKT-FASN in U2-OS and MG-63 cells *in vitro*.

## Discussion

Lapatinib (originally known as GW572016) is a 4-anilinoquinoline derivative, which was reported to induce cell apoptosis and inhibit cell proliferation, migration and invasion in various

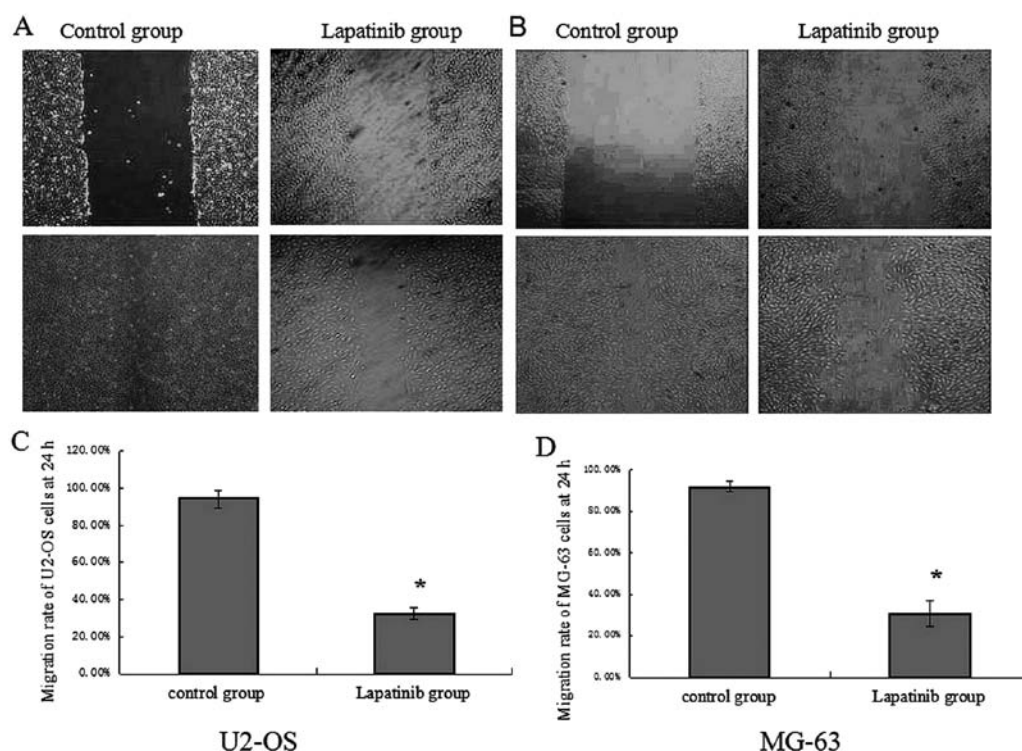


Figure 5. Wound healing assay was performed to investigate the effect of lapatinib on OS cell migration. In the (A and C) U2-OS and (B and D) MG-63 OS cell lines, the migration rate in the cells treated with lapatinib was significantly lower than that in the untreated cells at 24 h. Columns, mean (n=6); bars, SD; \*P<0.05 vs. control group. OS, osteosarcoma.

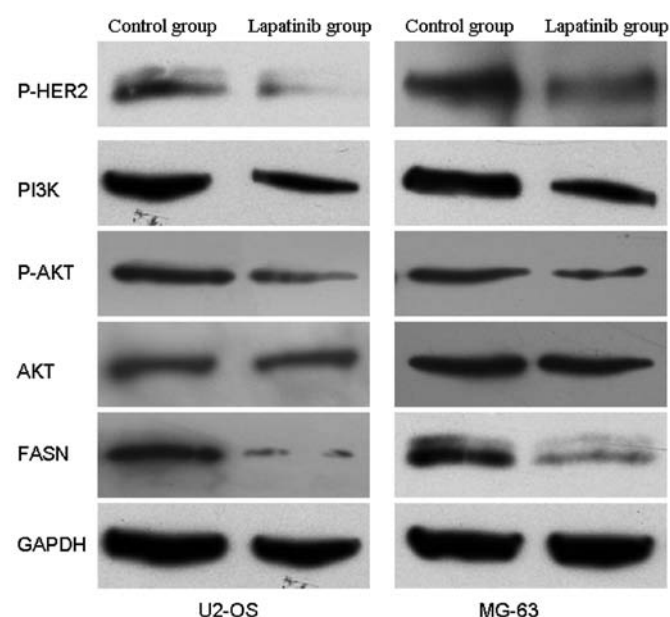


Figure 6. Western blot analysis of the expression levels of p-HER2, PI3K, p-AKT, AKT and FASN. Results showed that that expression levels of all proteins except for AKT were significantly inhibited by lapatinib. This suggests that lapatinib suppresses the activity of the HER2-PI3K/AKT-FASN pathway in U2-OS and MG-63 cells *in vitro*. HER2, human epidermal growth factor receptor 2; PI3K, phosphatidylinositol 3-kinase; FASN, fatty acid synthase.

types of tumors (21,22). It was widely used for chemotherapeutic treatment alone or in combination with other anticancer

drugs (23). Recently, studies have shown that lapatinib is not active against EGFR-positive/HER2-negative disease (24,25). However, the effect of lapatinib on the malignant phenotype of osteosarcoma (OS) is still uncertain. Morris *et al* (13) suggested that targeting HER2 should be considered for the treatment of patients with osteogenic sarcoma. Therefore, to examine the effect of lapatinib on OS cell apoptosis, proliferation, migration and invasion, OS cell lines U2-OS and MG-63 were selected for study. The cell proliferation was evaluated with MTT and colony formation assays, and cell migration and invasion were assessed by wound healing and Transwell invasion assays. We found that lapatinib inhibited the proliferation of U2-OS and MG-63 cells in a dose- and time-dependent manner, and the rate of colony formation of lapatinib-treated cells was significantly lower than that in cells not treated with lapatinib. In the wound healing and Transwell invasion assays, the results revealed that the migratory and invasive capabilities were inhibited by lapatinib. These results indicate that the malignant phenotype of OS cells may be inhibited by lapatinib *in vitro*. Lapatinib may be an effectively agent for chemotherapy in the treatment of OS. However, further studies are necessary to unveil the potential molecular mechanisms of the inhibition of the malignant phenotype of OS by lapatinib.

Recently, studies have shown that target metabolic pathways such as glycolysis and lipid metabolism may represent a promising therapeutic strategy in cancer therapy (26). Fatty acid synthase (FASN), a lipogenic multi-enzyme complex, is an enzyme crucial for endogenous lipogenesis in mammals and is responsible for catalyzing the synthesis of long-chain fatty acids. The metabolic products of the FASN complex are rapidly consumed by actively dividing cells. Recent data



demonstrate that FASN expression is important for tumor growth and survival, suggesting that FASN is a metabolic oncogene. It is more pronounced in OS and correlates with pulmonary metastasis (27). Importantly, we demonstrated that inhibition of FASN with pharmacological inhibitors or siRNA leads to a significant antitumor effect in OS (28). HER2 overexpression increases the translation of FASN by altering the activity of the mTOR and PI3K/AKT signaling pathway in breast cancer cell (29), and inhibition of the HER2/PI3K/AKT signaling pathway leads to blockade of FASN in colorectal cancer cells (30). However, various studies have demonstrated that inhibiting FASN caused a marked decrease in the active forms of the HER2 protein (31,32). These findings suggest that the HER2 oncogene possibly established a positive bidirectional relationship with FASN, strictly ensuring a hyperactive *de novo* fatty acid biogenesis.

Lapatinib is a small-molecule, reversible inhibitor of HER2 TKS (33). In murine xenograft models, lapatinib inhibited autophosphorylation of HER2, as well as downstream MAPK/Erk1/2 and PI3K/Akt pathways (18,34). To investigate the possible involvement of the inhibition of the phosphorylation of HER2 by the PI3K/Akt/FASN signaling pathway in OS, the levels of phosphorylated HER2 in the OS cell lines U2-OS and MG-63 were downregulated by lapatinib. Our results demonstrated that the inhibition of phosphorylation of HER2 by lapatinib markedly reduced the expression of PI3K, p-AKT and FASN protein in U2-OS and MG-63 cells. This implies that HER2 effectively regulates the activity of the 'PI3K/Akt/FASN' signaling pathway in OS cells.

In conclusion, our findings suggest that lapatinib alters the cell malignant phenotype of OS cells via downregulation of the activity of the HER2-PI3K/AKT-FASN signaling pathway *in vitro*, and lapatinib may be an effective chemotherapeutic agent for OS. However, the tumor microenvironment plays an important role in tumor progression, invasion and cell migration. Thus, further experiments *in vivo* are necessary to ascertain whether lapatinib represents a new chemotherapeutic agent for the treatment of OS.

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