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ORIGINAL ARTICLE

Gefitinib causes growth arrest and inhibition of metastasis in human chondrosarcoma cells

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Summary

Purpose: *Chondrosarcomas are primary malignant cartilage-forming tumors of bone which are not responsive either to chemotherapy or radiation treatment and display potent capacity to invade locally and cause distant metastasis. Epidermal growth factor receptor (EGFR) pathway plays an important role in the development and progression of many cancers. However, the effect of EGFR inhibitor gefitinib on cell growth and metastasis in human chondrosarcoma cells is largely unknown.*

Methods: *Features of the protein expression of EGFR in 3 human chondrosarcoma cell lines JJ2012, SW1353 and OUMS27 were analyzed. The inhibitory effects of EGFR inhibitor gefitinib on cell proliferation, cell cycle and metastasis were assessed by using MTS, flow cytometry and migration assays, respectively. The expression of metastasis-related proteins was evaluated by western blotting.*

Results: *All the three human chondrosarcoma cell lines expressed EGFR protein. Gefitinib significantly inhibited the growth, induced cell cycle arrest and decreased the migration ability of human chondrosarcoma cells. In addition, gefitinib also reduced the expression of metastasis-related proteins, basic fibroblast growth factor (bFGF), matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinases-9 (MMP-9).*

Conclusions: *The discovery that gefitinib inhibited the proliferation and reduced the metastatic capacity of chondrosarcoma cells may help increase the understanding of the mechanism underlying human chondrosarcoma growth and metastasis. Thus, gefitinib may represent a promising agent for controlling chondrosarcoma proliferation and metastasis.*

Key words: *cell cycle, chondrosarcoma, EGFR, gefitinib, metastasis, proliferation*

Introduction

Chondrosarcoma, the second most common primary malignant bone tumor in adults, is a devastating disease with potent capacity to invade locally and cause distant metastasis, especially to the lungs [1,2]. Chondrosarcoma is notable for its lack of response to conventional types of chemotherapy and surgical resection remains the primary mode of therapy for this tumor [3,4]. There has been no progress in the clinical outcomes over the past 30 years [5,6]. Due to the absence of an effective adjuvant therapy, this malignancy has a poor prognosis with only 10-25% 5-year survival rate [5]. Therefore, there is an extremely urgent need to improve the understanding of the cellular and molecular factors that promote chondrosarcoma progression and to explore new therapeutic approaches to combat this tumor.

By targeting molecules critical to cancer development, targeted therapy was recently recognized as a promising strategy for cancer treatment [7,8]. Targeted therapy using EGFR-tyrosine kinase inhibitors (EGFR-TKI) is currently one of

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the most acceptable therapies [8,9]. EGFR has attracted increasing attention as a plausible target for cancer therapy and the knowledge pertaining to the importance of EGFR in cancer is unceasing in the past few years [10,11]. Abnormal EGFR activation is found in various cancer cells, making it a good target for therapeutic agents [12-14]. EGFR often plays an important role in the development and progression of many cancers such as lung, gastric, colon, pancreatic and neck cancers [11,15-17], rendering it to an attractive therapeutic target.

Gefitinib, a selective TKI of EGFR, has been successful in the treatment of certain cancers, especially lung cancer [18]. However, the role of gefitinib in the pathogenesis of human chondrosarcoma cells is not well understood.

The primary aim of the present study was to evaluate the antitumor effects of EGFR inhibitor gefitinib on human chondrosarcoma cells. The EGFR protein expression of three human chondrosarcoma cell lines and the effects of gefitinib on cell proliferation, cell cycle and metastasis were assessed. Another aim of this study was to clarify whether there is a direct relationship between gefitinib and the metastasis-related proteins' expression in human chondrosarcoma cells.

Methods

Cell culture

The human chondrosarcoma cell lines JJ012, SW1353 and OUMS27, and chondrocyte cell line LB-PVA were obtained from the American Type Culture Collection (VA,USA). Cells were cultured in Dulbecco's modified Eagle's medium/α-minimum essential medium or RPMI1640 supplemented with 10% fetal calf serum (Gibco, New York, USA), 2 Mm glutamine and 1% penicillin/streptomycin (100 U/ml) at 37˚C in a humidified atmosphere of 5% CO_2.

Reagents and antibodies

Gefitinib (J&K Chemical, New Jersey, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, MO USA) with a stock concentration of 1 mM and stored at –80˚C until use. The final concentration of DMSO was 0.1%. The antibodies anti-EGFR, anti-β–actin, anti-bFGF, anti-MMP-2 and anti-MMP-9 were all purchased from Santa Cruz Biotechnology (CA, USA).

MTS assay

The cells of the 3 lines were seeded into 96-well culture plates at a density of 2-5×10^3 cells per well in 200 µl of medium and allowed to attach overnight. Cell proliferation was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. After treated with gefitinib (0–25 µM) for 48 or 72 hrs, cells were washed with phosphate buffer saline (PBS) and 20 µl MTS (Sigma, MO, USA) were added to each well, then the well plates were incubated at 37 °C for 3 hrs. The absorbance of each well was determined at 490 nm using a microplate reader (BioRad, USA). All experiments were performed in triplicate.

Cell cycle analysis

After incubation in the absence or presence of gefitinib (5 µM) for 48 hrs, cells were washed with PBS and detached with trypsin at 37 °C, then centrifuged at 1000 rpm, fixed in 70% cold ethanol and kept at 4 °C overnight. Cells were stained for DNA content with propidium iodide, Trition X-100 and RNase A and kept at 37 °C for 30 min. The stained cell nuclei were measured using flow cytometry analysis (Becton Dickinson FACScan, CA, USA).

Migration assay

The migration activity was measured using two-chamber-Transwell (Corning, USA). The lower surface of the upper chamber plates was coated with the polycarbonate membrane with 8 µm pores. Chondrosarcoma cells JJ012 (1.2×10^4) and SW1353 (1.5×10^4) were added to the upper chamber in 200 µl of serum-free medium and incubated for 24 hrs at 37 °C in 5% CO_2, then the cells in the upper chamber were wiped off with a cotton swab. Cells that migrated through the pores onto the underside of the membranes were fixed in 3.7% formaldehyde for 5 min and stained with crystal violet in PBS for 15 min. Cells on the underside of the filters were examined and counted under a microscope (x100).

Western blotting

Cell lysates containing 30 µg of protein were separated via SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked for 2 hrs at ambient temperature in a blocking solution consisting of 5% non-fat milk and then probed with specific antibodies at 4 °C overnight. Subsequently, the blots were incubated with goat anti-rabbit or goat anti-mouse peroxidase-conjugated secondary antibody for 1 hr at room temperature. The blots were detected by enhanced chemiluminescence (Amersham Bioscience, Boston, MA, USA).

Statistics

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test. A p value < 0.05 was considered statistically significant.

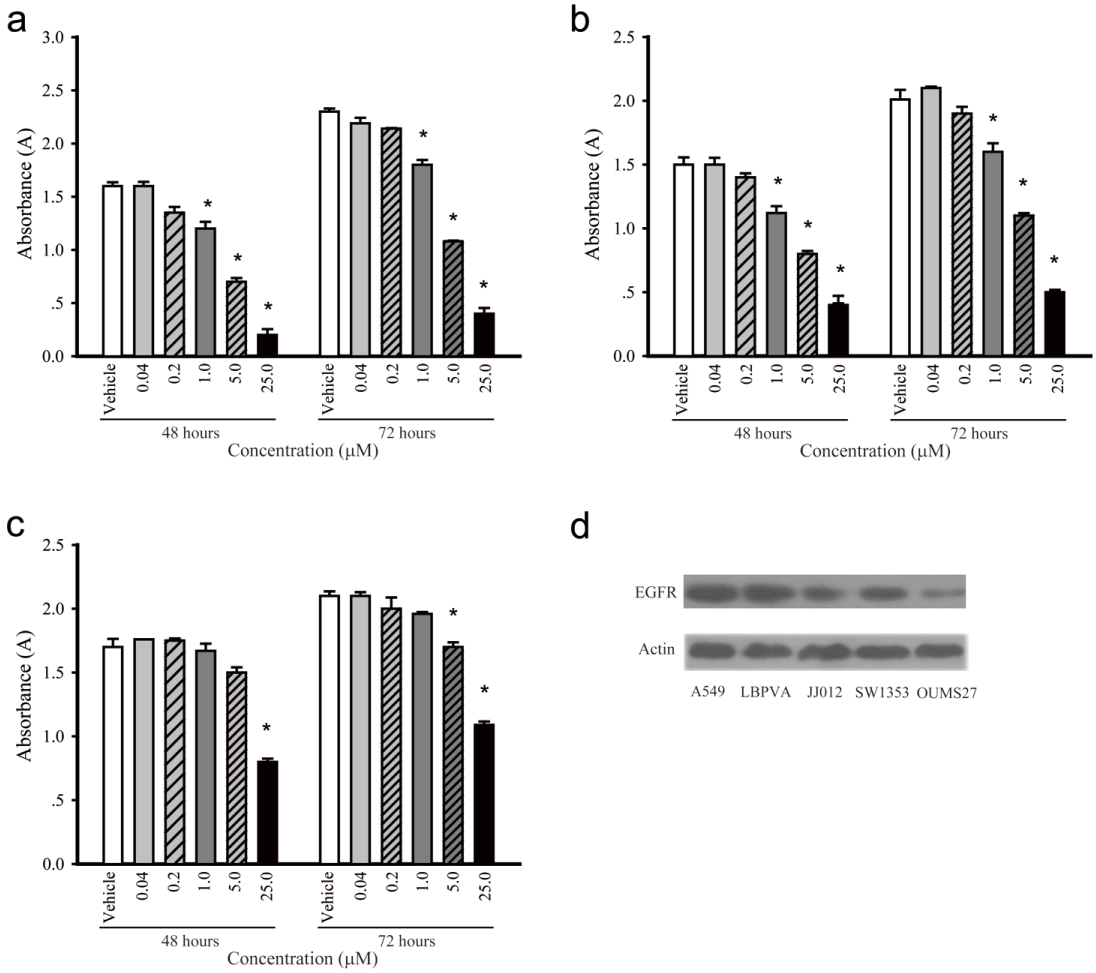

Figure 1. Gefitinib inhibits growth of human chondrosarcoma cells. Gefitinib significantly inhibited the growth of SaOS2 **(a)** and U2OS **(b)** cells in a dose-dependent manner. Gefitinib had no significant inhibitory effect on the growth of OUMS27 cells **(c)**. **(d)** EGFR expression of chondrosarcoma cell lines in basal culture conditions. Data are presented as mean±SEM. * p<0.01, compared with vehicle-treated chondrosarcoma cells.

Results

Gefitinib inhibits the growth of chondrosarcoma cell lines

In order to determine whether EGFR inhibition affects the proliferation of human chondrosarcoma cells, JJ012, OUMS27 and SW1353 cells were exposed to gefitinib at concentrations ranging from 0 to 25 μM for 48 and 72 hrs. The growth of the three chondrosarcoma cells lines was evaluated by MTS. As shown in Figure 1a, gefitinib caused a dose-dependent inhibition of cell growth in JJ012 cells after treatment for both 48 and 72 hrs. Similarly, the proliferation of SW1353 cells was also significantly inhibited by gefitinib treated for 48 and 72 hrs (Figure 1b). However, no dose-dependent inhibitory effect of gefitinib on OUMS27 cells was observed (Figure 1c). For the three chondrosarcoma cell lines tested, EGFR protein expression was observed at higher levels in JJ012 and SW1353 cells (Figure 1d). However, OUMS27 cells had significantly lower EGFR expression compared with JJ012 and SW1353 cells (Figure 1d).

Effects of gefitinib on the cell cycle of human chondrosarcoma cells

To monitor the alterations in the cell cycle distribution by gefitinib treatment in human chondrosarcoma cells, flow cytometry analysis was performed. Chondrosarcoma cells were treated with 5 μM gefitinib for 48 hrs. The JJ012 and SW1353 cells appeared to arrest at G2/M phase. As shown in Figure 2a, the proportion of the JJ012 cells at G2/M phase was significantly increased and the proportion of the JJ012 cells at G1 phase was decreased compared with the vehicle-treated cells. Our results also showed that gefitinib led to increased proportion of SW1353 cells at

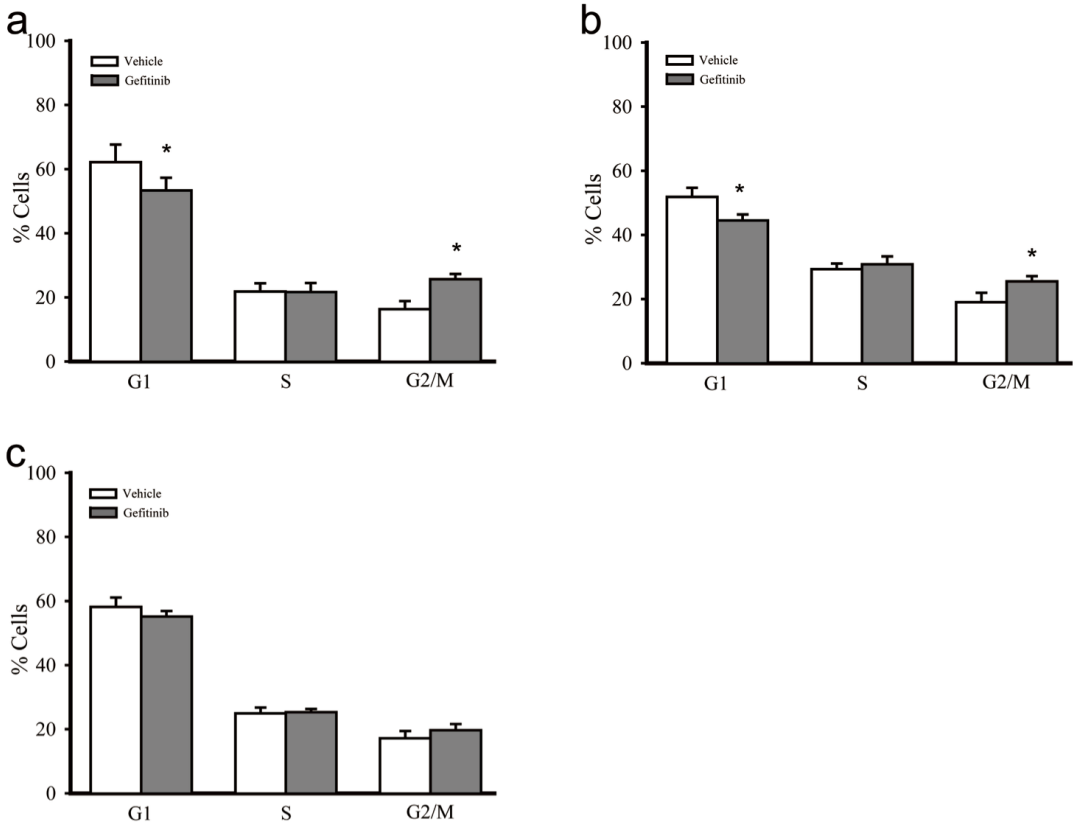

Figure 2. Gefitinib leads to cell cycle arrest in chondrosarcoma cells. Treatment of cells with gefitinib induced an accumulation of JJ012 **(a)** and SW1353 **(b)** cells in the G2/M phase of the cell cycle. Gefitinib had no significant effect on the cell cycle distribution of OUMS27 cells **(c)**. Data are presented as mean±SEM. *p<0.01, compared with vehicle-treated chondrosarcoma cells.

G2/M phase and decreased proportion of cells at G1 phase (Figure 2b). However, no significant influence of gefitinib on cell cycle distribution of OUMS27 cells was observed (Figure 2c).

Effects of gefitinib on the migration ability of human chondrosarcoma cells

We next examined whether EGFR inhibition by gefitinib is involved in the regulation of cell migration and can influence the metastatic phenotype in chondrosarcoma cells. The migration assay was carried out and the migrating cell number was counted. As shown in Figure 3a, pretreatment of JJ012 cells with gefitinib (5 μM) significantly reduced the cell migration compared with the vehicle control group. Gefitinib also significantly decreased SW1353 cell migration in comparison with the vehicle control group (Figure 3b). However, gefitinib had no significant effect on the migration ability of OUMS27 cells treated with gefitinib (data not shown). These data suggest that gefitinib may play a critical role in the migration of human chondrosarcoma cells lines JJ012 and SW1353.

Effects of gefitinib on the expression of metastasis-related proteins

To further investigate the mechanism by which gefitinib influences the metastasis inhibition of human chondrosarcoma cells, western blotting analysis was performed and the expression of metastasis-related proteins was evaluated. JJ012 and SW1353 cells treated with gefitinib for up to 48 hrs were analyzed by western blotting. We found that the expression of bFGF was significantly reduced in JJ012 and SW1353 cells treated with gefitinb compared with the vehicle-treated cells (Figure 4a). Meanwhile, the results showed that the protein expression of MMP-2 and MMP-3 were also decreased in JJ012 and SW1353 cells treated with gefitinib compared with the vehicle-treated cells (Figures 4b and 4c). However, gefitinib had no significant effect on these three

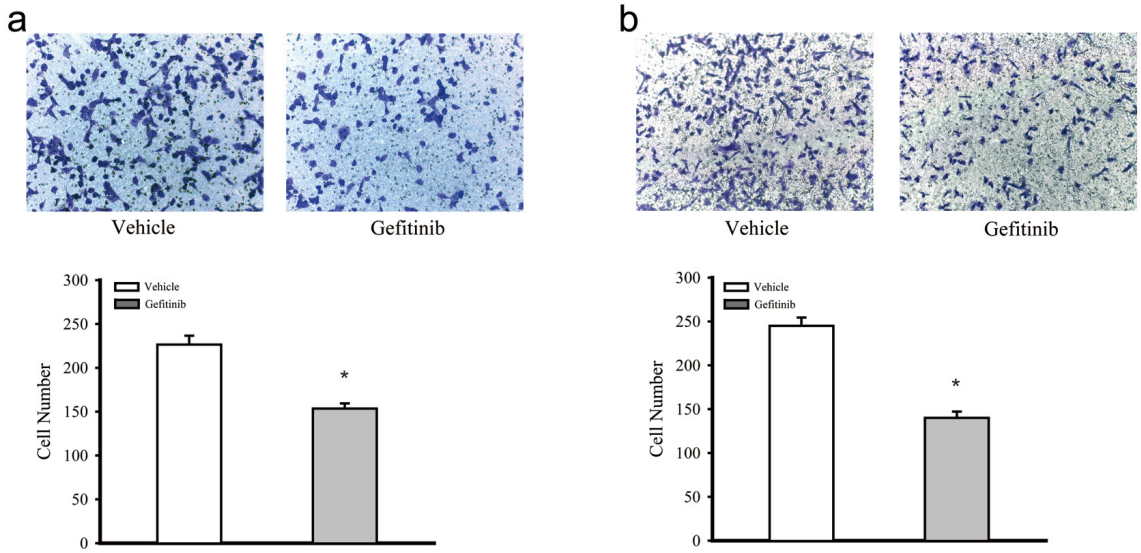

Figure 3. Gefitinib inhibits the migration ability of human chondrosarcoma cells. Gefitinib treatment resulted in a qualitative decrease in the number of migrating JJ012 **(a)** and SW1353 **(b)** cells. The migrating cells were visualized by microscopy (x100). Data are presented as mean±SEM. * p<0.01, compared with vehicle-treated chondrosarcoma cells.

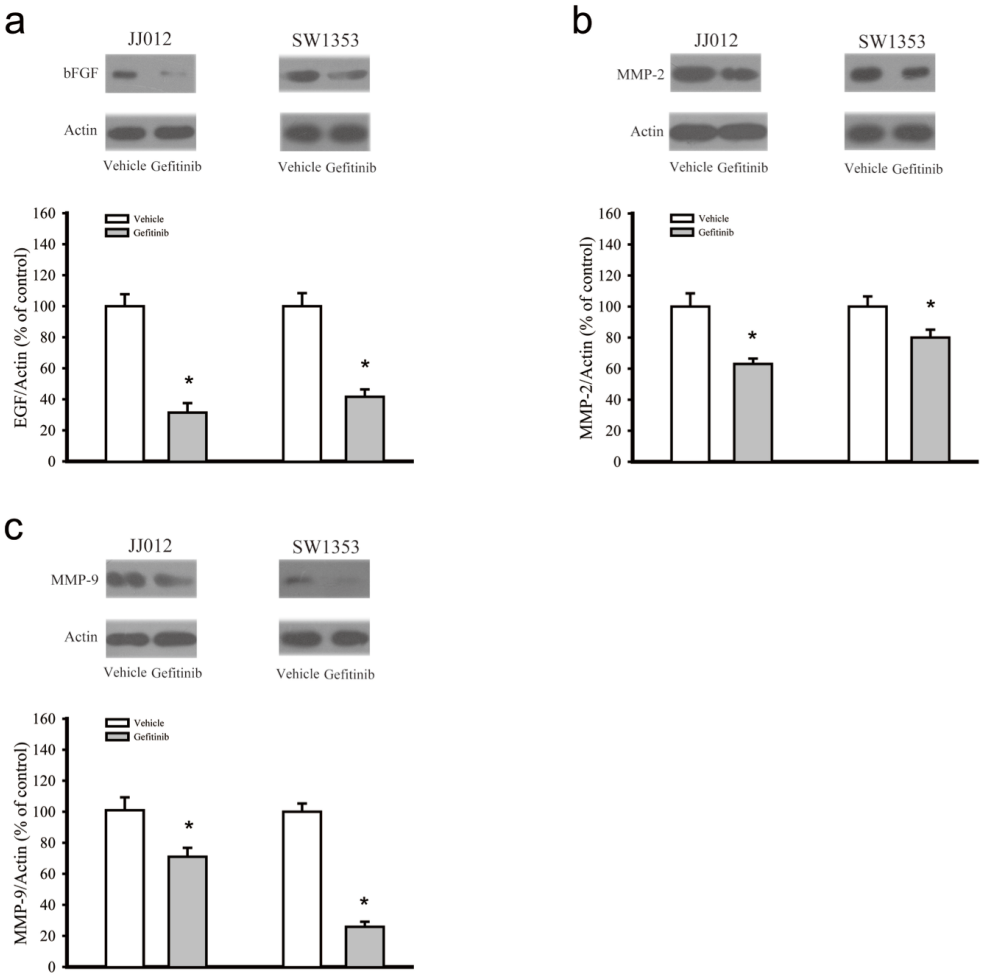

Figure 4. Western blotting analysis for the expression of the metastasis related proteins. Gefitinib significantly decreased the expression of bFGF **(a)**, MMP-2 **(b)** and MMP-9 **(c)** in human chondrosarcoma cells. Data are presented as mean±SEM. * p<0.01, compared with vehicle-treated chondrosarcoma cells.

proteins' expression in OUMS27 cells (data not shown). Our results indicated that gefitinib modulated the metastasis-related proteins' expression.

Discussion

Chondrosarcoma is a malignant tumor characterized by the formation of cartilage, lack of effective therapy, predilection for metastasis and poor prognosis [19-21]. As no effective systemic therapy exists for this neoplasm there is urgent need to search for new therapeutic agents. The EGFR inhibitor gefitinib has been shown to exert potent antitumor effects [7,22]. However, the effects of gefitinib in human chondrosarcoma cells are largely unknown. We sought to identify the effects of the EGFR inhibition by gefitinib that might contribute to the proliferation and metastasis of human chondrosarcoma cells.

The growth inhibitory activity of gefitinib has been shown for a large range of cancer cells [18,23-25]. To evaluate the role of gefitinib in the growth of human chondrosarcoma cells, three human chondrosarcoma cell lines were analyzed for EGFR protein expression by western blotting firstly. We found that EGFR protein was expressed in all the three human chondrosarcoma cell lines. OUMS27 cells had lower EGFR expression, which resulted to lack of significant effects of gefitinib on the cell proliferation and cell cycle arrest. EGFR expression may be associated with the cell's response to gefitinib. The effect of gefitinib on human chondrosarcoma cells was evaluated using MTS and flow cytometry assays. MTS analysis showed that gefitinib had a dose-dependent antiproliferative effect on the cell growth of JJ012 and SW1353 cells. In addition, cell cycle analysis revealed that gefitinib led the cells to G2/M phase arrest with decreased percentage of JJ012 and SW1353 cells in the G1 phase. Our results indicated that the inhibitory effects of gefitinib on the chondrosarcoma cells maybe via the cell cycle arrest.

As one of the main biological characteristics of cancer cell, tumor metastasis is highly complex and chondrosarcoma is notable for its propensity for developing lung metastases [1,26]. Therefore, a better understanding of the metastatic pathway is needed. In this study, we found that EGFR inhibition by gefitinib suppressed metastasis of human chondrosarcoma cell lines JJ012 and SW1353 cells. Previous studies reported that MMP-2 and MMP-9 are implicated in tumor metastasis and overexpression of MMPs in higher levels might be associated with tumor invasion and metastasis [27-30]. bFGF was also found to promote tumor angiogenesis and induce cancer cell metastasis [31-33]. Our research showed that gefitinib significantly reduced the expression of metastasis-related proteins, bFGF, MMP-2 and MMP-9 in chondrosarcoma cells. We speculate that the reduced migration ability of chondrosarcoma cells by gefitinib may be achieved through downregulation of bFGF, MMP-2 and MMP-9 expression.

The present study demonstrated that gefitinib blocked cell proliferation, induced cell cycle arrest, prevented cell migration and reduced the expression of metastasis-related proteins. Gefitinib would be an effective antitumor agent in chondrosarcoma and therapeutic interventions that are designed to block EGFR signaling may provide a new approach to the treatment of this difficult to treat malignancy.

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