Erlotinib prevents pulmonary metastasis in curatively resected breast carcinoma using a mouse model

YOUNG-JIN CHOI[1*], SEOK-JIN NAM[1*], MYUNG JIN SON[2*], DAE-KYEUM KIM[1], JEONG-HAN KIM[1], JUNG-HYUN YANG[1], MI-HYUN KIM[2], HYUN SEOK SONG[2], DO-HYUN NAM[2] and SA IK BANG[3]

Departments of [1]Surgery, [2]Neurosurgery and [3]Plastic and Reconstructive Surgery, Samsung Medical Center and Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea

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Abstract. Metastatic breast cancer is still defined as an incurable disease, with the lungs being the most common metastatic sites in breast cancer patients. Epidermal growth factor receptor (EGFR), a member of receptor tyrosine kinase family, is known to be involved in survival, migration, angiogenesis and metastasis of cancer. The spontaneous pulmonary metastasis mouse model was applied to evaluate the effects of the EGFR tyrosine kinase inhibitor, erlotinib, on the prevention of pulmonary metastasis in curatively resected breast carcinoma. The expression of EGF and EGFR was significantly strong in pulmonary metastatic nodules compared to those in primary breast carcinoma tissue. A treatment of erlotinib (oral gavage, 50 mg/kg/day, every day for 6 weeks) given to mastectomized mice inhibited the incidence of pulmonary metastasis. The number of metastatic pulmonary nodules was significantly reduced in the erlotinib-treated group compared with the control. Therefore, erlotinib may play a role in preventing pulmonary metastasis, which shows the strong expression of EGF and EGFR after curative resection of primary breast cancer.

Introduction

Breast carcinoma is the most common female cancer, and the incidence has been increasing annually. The estimated 5-year survival rate is approximately 85%, but differs significantly depending on stage and prognostic factors (1). The advancement of multidisciplinary treatment such as chemotherapy and hormonal therapy has significantly impacted the survival of these patients. However, patients with breast cancer and clinically detectable metastases beyond the regional lymph nodes are generally believed to have reached a final and fatal step in tumor progression (2). Therefore, the aim of treating breast carcinoma is to minimize and delay distant metastasis, which is the direct cause of death. The lungs and bones are frequent target sites of breast cancer metastasis (3).

An improved understanding of the cell signaling pathways involved in cancer growth allowed molecular-targeted therapy using new generations of anti-cancer compounds. EGFR is a member of the type 1 receptor tyrosine kinase family involved in survival, cell migration, angiogenesis and metastasis of cancer and expressed in several solid tumors, including breast carcinoma (4). The expression of EGFR in breast carcinoma has been reported to be in the range of 14-91%, and EGFR modulates growth properties of transformed cells. Targeting EGFR pathways in cancer treatment showed an inhibition of proliferation and angiogenesis, the induction of apoptosis, and an increase of tumor sensitivity to cytotoxic therapy (5). Moreover, other studies have reported the effect of EGFR tyrosine kinase inhibitors in breast carcinoma (6-8). However, the effects of the EGFR inhibitor in pulmonary metastasis after the curative resection of primary breast carcinoma were not evaluated.

Erlotinib (tarceva®) is a small molecule targeting EGFR and it represents a class of compounds that prevents EGFR autophosphorylation. It interrupts downstream signaling of EGFR, resulting in the inhibition of tumor cell proliferation (9). In this study, we evaluated whether EGF and EGFR expression was different in primary breast cancer tissue from metastatic pulmonary nodules with immunohistochemistry and whether erlotinib inhibits the incidence of pulmonary metastasis and reduces the number of metastatic nodules after curative resection of primary breast carcinoma in a mouse model.

Materials and methods

Cell line. MDA-MB-435 LvBr cells were derived from pleural exudates of a pulmonary metastasis breast carcinoma

Correspondence to: Dr Sa Ik Bang, Department of Plastic and Reconstructive Surgery, Samsung Medical Center and Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea
E-mail: sibang55@smc.samsung.co.kr

Dr Do-Hyun Nam, Department of Neurosurgery, Samsung Medical Center and Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea
E-mail: nsnam@smc.samsung.co.kr

*Contributed equally

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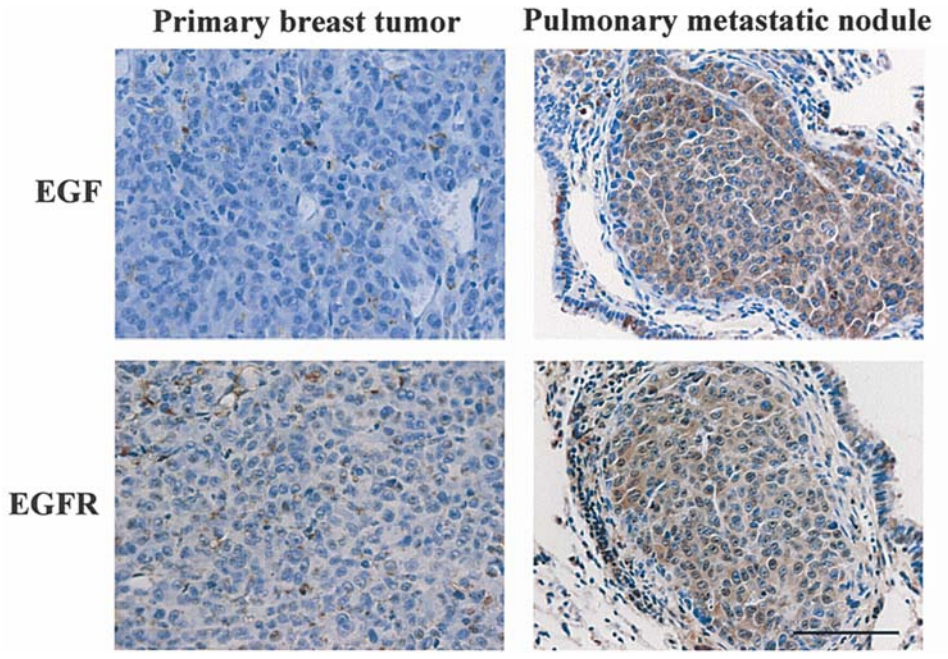

Figure 1. Expression of EGF and EGFR in a primary and pulmonary metastatic tumor. MDA-MB-435 LvBr cells were inoculated into the mammary fat pad of mice. Six weeks after inoculation, a radical mastectomy was performed. A further 6 weeks after primary tumor resection, the mice were necropsied and pulmonary metastatic tumors were excised. Paraffin sections of primary and metastatic tumors were stained with EGF and EGFR antibodies, respectively. Scale bar, 100 μm.

patient and are hormone-independent (kindly provided by Dr J.E. Price, MD Anderson Cancer Center). This cell line was known to develop spontaneous pulmonary metastasis after mastectomy in 90% of cases (10). The cells were cultured in Eagle's minimal essential medium (Cambrex) supplemented with 10% fetal bovine serum and antibiotics (Life Technologies) at 37˚C and 5% CO_2.

Mouse model. Balb/c-nu female mice at 8 weeks of age were housed and maintained under specific pathogen-free conditions in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International and in accordance with current regulations and standards of the Laboratory Animal Research Center under the Samsung Biomedical Research Institute. To produce orthotopic breast tumors, a 1.5-cm incision was made on the chest slightly right of the sternum, and the skin was separated from the chest by gentle blunt dissection. MDA-MB-435 LvBr cells ($1x10^6$ cells/100 μl of HBSS) were injected into the mammary fat pad of each animal and the incision was closed with wound clips. When the tumor size was 1.25-1.50 cm, tumor resection (mastectomy) was performed. The tumor mass was gently removed with a small margin of surrounding skin, and metastatic axillary lymph nodes were also excised.

Drug administration. For 6 weeks after primary tumor resection, erlotinib (OSI-774, tarceva; kindly provided by OSI Pharmaceuticals) or paclitaxel (Taxol, Bristol-Myers Squibb Co.) was administered orally at 50 mg/kg/day or intraperitoneally at 8 mg/kg (twice a week), respectively. The mice were then necropsied, and the incidence of pulmonary metastasis of each group was evaluated by Bouin's solution staining.

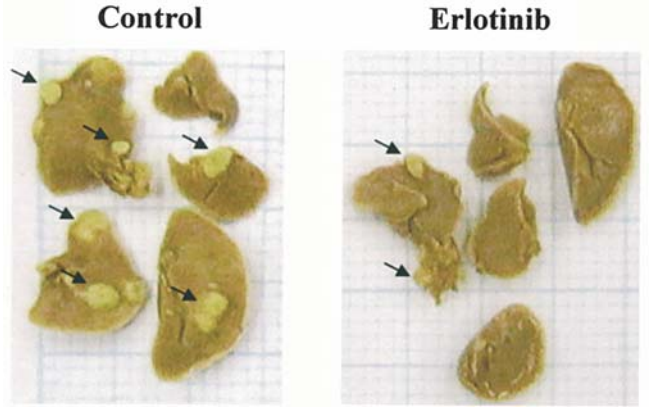

Figure 2. The metastatic pulmonary nodules in control and erlotinib-treated groups. Erlotinib was administered orally at 50 mg/kg/day for 6 weeks after primary breast tumor resection. The mice were then necropsied, and the excised lungs were stained for metastatic pulmonary nodules (arrows) with Bouin's solution.

Histology and immunohistochemical analysis. The number of metastatic nodules was examined under a dissecting microscope. The lungs from each mouse were fixed in 10% neutral-buffered formalin, embedded in paraffin, and thin sectioned (4 μm). Tissue sections were stained with H&E and examined under light microscopy for the detection of microscopic metastasis. EGF and EGFR were immunohistochemically stained with monoclonal antibody (Santa Cruz Biotechnology, Inc.) according to the manufacturer's manual.

Statistical analysis. Data are expressed as the inhibitory rate and mean ± SE. Statistical analyses were performed using SPSS 12.0 software and one-way ANOVA.

Table I. Effect of erlotinib on pulmonary metastasis after curative resection of primary breast cancer.

Treatment group[a]	No. of mice	No. of mice with pulmonary metastasis (%)
Control	11	11 (100%)
Erlotinib-treated	12	9 (75%)

[a]Tumor cells were orthotopically implanted into the mammary fat pad of mice, and a radical mastectomy was performed 6 weeks after inoculation. Erlotinib was administered orally at 50 mg/kg/day every day for 6 weeks after primary tumor resection. The mice were then necropsied, and the incidence of pulmonary metastasis of each group was evaluated.

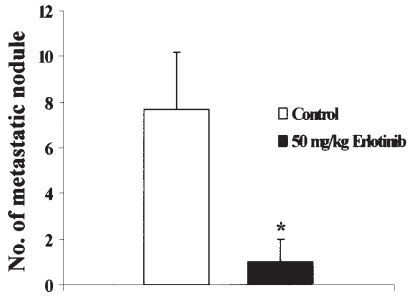

Figure 3. Comparison of the number of metastatic pulmonary nodules in the control and erlotinib-treated groups. The number of metastatic nodules stained with Bouin's solution was examined under a dissecting microscope. The numbers are shown as the mean ± SD. *p<0.05.

Results

Expression of EGF and EGFR in the primary breast and pulmonary metastatic tumor. The MDA-MB-435 LvBr cell line is known to have spontaneous metastatic potential. We also found that pulmonary metastasis was detected in all mice 6 weeks after primary breast tumor resection. To evaluate whether the expression of EGF and EGFR were different between the primary and metastatic tumor, we performed immunohistochemistry on the primary breast cancer specimens and metastatic pulmonary nodules (Fig. 1). The metastatic pulmonary nodules expressed EGF and EGFR stronger than primary breast cancer.

The effect of erlotinib on pulmonary metastasis. The effect of erlotinib on the incidence of pulmonary metastasis is summarized in Table I. Lung metastasis occurred in 9 of 12 (75%) mice with erlotinib treatment and in all mice in the control group (11/11, 100%). Moreover, the erlotinib-treated group showed a potent reduction of metastatic pulmonary nodules on the surface of the lungs (Fig. 2). The number of metastatic pulmonary nodules was significantly reduced in the erlotinib-treated group (1.00±1.00) compared with the control group (7.67±2.52) (p=0.032) (Fig. 3). These results demonstrate that erlotinib decreases the number of metastatic pulmonary nodules.

We also examined whether a pilot trial of combination therapy with erlotinib and a cytotoxic drug (paclitaxel) could synergistically decrease the incidence of pulmonary metastasis. The combination therapy with erlotinib and paclitaxel significantly reduced the incidence by 50% (data not shown). These results suggest that a combination treatment with erlotinib and a cytotoxic drug may be more effective than either treatment alone in the prevention of pulmonary metastasis in curatively resected breast carcinoma.

Discussion

Erlotinib (OSI-774, tarceva) is an orally active EGFR tyrosine kinase inhibitor with inhibitory effects on the growth of diverse tumors, and it has received approval as a monotherapy for the treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) after failure of at least one prior chemotherapy regimen (11). The role of erlotinib was also studied in diverse malignancies including hepatocellular carcinoma (HCC), ovary cancer and glioma, but its effect on metastatic breast cancer remains unclear (12-14).

In this study, the orthotopically implanted MDA-MB-435 LvBr cell line developed lung metastasis in all cases 6 weeks after primary tumor resection. The expression of EGF and EGFR were stronger in metastatic nodules than primary tumors. This is the rationale for using erlotinib to inhibit pulmonary metastasis. The incidence of metastasis was decreased to 75% in the erlotinib-treated group compared to 100% in the control group, showing no statistical significance (p=0.269) (Table I). However, the number of metastatic nodules showed a statistically significant decrease in the erlotinib-treated group compared to the control group (p=0.032) (Fig. 3). Upregulation of EGFR expression was frequently observed in breast carcinoma, which correlated with a lack of response to endocrine therapy in recurrent breast carcinoma and was associated with poor prognosis (15). Therefore, the blockade of activation and function of EGFR might be a potential therapeutic strategy for metastatic breast cancer.

However, the study on the effect of erlotinib on metastatic breast cancer by Tan *et al* did not show any clinical response in patients with metastatic breast cancer after treatment with erlotinib (8). They reported that the metastatic tumors were EGFR-negative except one, and the inhibitory effects of erlotinib are evident in normal tissue and an EGFR-positive tumor. It is unclear whether EGFR expression in a tumor is essential for it to respond to EGFR inhibitor. The study of gefitinib, an EGFR tyrosine kinase inhibitor, showed that breast carcinoma cell lines with either low or high levels of EGFR are inhibited by gefitinib treatment (8,16). Therefore, the changes in gene expression after erlotinib treatment and unknown pathways in metastatic breast cancer, except EGFR signaling, should be further studied.

We also found that the combination therapy of erlotinib and paclitaxel, a chemotherapeutic agent, inhibited pulmonary metastasis of breast carcinoma. However, other studies on NSCLC about the simultaneous use of a cytotoxic agent and EGFR inhibitor showed no added clinical benefit (8,17). The appropriate timing and sequence of the EGFR inhibitor and cytotoxic agents are considered important factor(s).

In summary, we demonstrated that pulmonary metastasis after the curative resection of primary breast carcinoma was

inhibited by erlotinib, which is dependent on EGFR expression. Moreover, we suggest that the inhibitory effect of erlotinib is further enhanced by combination treatment with the cytotoxic agent, paclitaxel.

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