

Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Does Not Improve Paclitaxel Effect in an Orthotopic Mouse Model of Lung Cancer

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ABSTRACT

Purpose: The purpose is to evaluate whether inhibition of epidermal growth factor receptor (EGFR) activation by PKI166, an EGFR-tyrosine kinase inhibitor, affects growth of human lung cancer implanted orthotopically into the lungs of nude mice.

Experimental Design: Lungs of mice were injected with NCI-H358 human bronchioloalveolar cancer cells. In three experiments, groups of mice ($n = 10$ per group) were randomized 7 days after tumor implantation to receive one of the following treatments: i.p. paclitaxel 100 or 200 μg (4 or 8 mg/kg) once per week, oral PKI166 100 or 200 mg/kg three times per week, paclitaxel plus PKI166, or i.p. saline and oral PKI166-vehicle (control) for 5 weeks. Mice were killed 6.5 to 8 weeks after tumor implantation. The experiments were repeated with PC14PE6 human lung adenocarcinoma cells to assess effect on survival.

Results: Immunohistochemical analyses revealed the expression and phosphorylation of EGFR in the growing tumors. Treatment with PKI166 alone or in combination with paclitaxel diminished activation of EGFR on tumor cells, yet maximal therapeutic effect was observed in mice treated with paclitaxel alone. Activated mitogen-activated protein kinase and basic fibroblast growth factor expression

were similar in all treatment groups. Survival in mice treated with the combination of paclitaxel and PKI166 was shorter than in those treated with paclitaxel alone.

Conclusions: Our results suggest that concurrent administration of EGFR-tyrosine kinase inhibitor and chemotherapy is equivalent and may indeed be inferior to chemotherapy alone, even if EGFR is functional and its phosphorylation effectively inhibited. Our data show that the interaction of EGFR-TKIs and chemotherapy is complex and suggest that other growth factors may activate the downstream signaling events.

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is the most common form of lung cancer and the leading cause of cancer-related death in the world (1). Systemic chemotherapy is the main treatment for the majority of patients with NSCLC because most are diagnosed with advanced inoperable disease. However, recent studies have shown that modern chemotherapeutics may have reached the ceiling of their clinical efficacy, and the 5-year survival rate for NSCLC has plateaued at 14% (2). These findings show that a new approach to the therapy of lung cancer is mandatory.

Targeting the epidermal growth factor receptor (EGFR) is one appealing strategy for the treatment of lung cancer because the EGFR plays a pivotal role in tumor cell proliferation, survival, adhesion, migration, differentiation, and angiogenesis (3). Indeed, increased expression of EGFR, one of the earliest and most frequently detected abnormalities in the bronchial epithelium of heavy smokers (4), is pronounced in the majority of NSCLC cases (5, 6). However, the relationship between EGFR expression and the prognosis of NSCLC patients is still not well established, and retrospective studies of EGFR expression in stage I disease have yielded conflicting results (7). Several approaches to block EGFR-mediated signaling pathways are under evaluation, including using monoclonal antibodies (MAbs) against the ligand or receptor, ligand-toxin conjugates, antisense oligonucleotides, and receptor tyrosine kinase inhibitors (8). A recent phase II trial of monotherapy with the anti-EGFR-tyrosine kinase inhibitor gefitinib (Iressa, ZD1839, AstraZeneca Pharmaceuticals, Wilmington, De) showed tumor response rate of 10%, a stable disease of 30%, and a symptom response rate of 40% (9, 10) for lung cancer patients. Paez *et al.* (11) and Lynch *et al.* (12) recently studied EGFR mutation status in patients treated with gefitinib and found that somatic mutations in tyrosine kinase site characterize responders to therapy.

The clinical effects of several EGFR-tyrosine kinase inhibitors were studied intensively both *in vitro* and *in vivo*, either alone or in combination with diverse chemotherapeutics. Ciardiello *et al.* (13) and Sirotnak *et al.* (14) studied the effect

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of gefitinib in combination with different chemotherapeutics. PKI166 (Novartis, Switzerland) was studied by Bruns *et al.* (15) in an orthotopic model of human pancreatic cancer in nude mice and by Kim *et al.* (16) and Weber *et al.* (17) in models of human prostate cancer and renal cell cancer growing in the bones of nude mice. In these models, therapy with PKI166 was associated with decreased tumor growth and metastasis, and the anti-tumor effect was enhanced by chemotherapy. We recently developed an orthotopic lung cancer model (18). In this system, human lung cancer cells were injected into the left lung of nude mice. Tumors formed from a single focus of disease and progressed to a widespread and fatal thoracic process, characterized by diffuse dissemination of lung cancer in both lungs and metastasis to intra- and extrathoracic lymph nodes.

The purpose of the present study was to evaluate whether inhibition of EGFR activation by PKI166 affects tumor growth and metastasis of human lung cancer implanted orthotopically into the lungs of nude mice. We studied human bronchioloalveolar cancer (NCI-H358) and lung adenocarcinoma (PC14PE6), which both express the EGFR and the activated EGFR. The results of these studies show that therapy with PKI166 either alone or in combination with paclitaxel inhibited EGFR activation for lung cancer cells growing in the lung. However, the therapeutic efficacy of the regimen of concurrent PKI166 and paclitaxel was inferior to that of paclitaxel alone. These results are similar to those of recent human clinical trials with gefitinib (19, 20), which demonstrated that gefitinib showed no added benefit compared with standard chemotherapy alone in patients with advanced lung cancer. Our data strongly suggest that other growth factors may bypass the EGFR blockade and activate the downstream signaling events.

MATERIALS AND METHODS

Cell Lines and Tissue Culture Conditions. Bronchioloalveolar lung carcinoma cells (NCI-H358) were obtained from the American Type Culture Collection (Manassas, VA). A variant (PC14PE6) of the lung adenocarcinoma cell line PC14 that was selected to produce pleural effusion when injected into mice had been described previously (21, 22). Human bladder cancer (253J-BV), a cell line that highly expresses EGFR and activated EGFR (23), was used as a positive control for the *in vitro* studies. Adherent monolayer cultures of NCI-H358 and 253J-BV cells and floating and adherent monolayer cultures of PC14PE6 cells were incubated at 37°C in 5% CO₂-95% air. NCI-H358 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin, and 253J-BV cells were maintained in DMEM supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution (Life technologies, Inc., Grand Island, NY), and a penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). The tumor cell cultures were free of *Mycoplasma* and the following pathogenic murine viruses: retrovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by Microbiological Associates, Bethesda, MD).

Reagents. PKI166, an EGFR-tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). For *in vivo* administration, PKI166 was dissolved in DMSO/0.5% Tween 80 and then diluted 1:20 in HBSS (15, 16); paclitaxel (Taxol) was purchased from Bristol-Myers Squibb (Princeton, NJ) and dissolved in water for i.p. injections; Matrigel Matrix Growth Factor Reduced was purchased from Becton Dickinson & Co. (San Jose, CA; ref. 18); all antibodies were purchased as listed: (a) rabbit anti-basic fibroblast growth factor-2 (bFGF), rabbit anti-EGF, and rabbit anti-EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); (b) rabbit anti-phospho-EGFR (activated EGFR; Tyr⁸⁴⁵), rabbit polyclonal phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr²⁰²/Tyr²⁰⁴) were purchased from Cell Signaling Technology, Inc. (Beverly, MA); and (c) the enhanced chemiluminescence detection system was purchased from Amersham, Inc. (Arlington Heights, IL).

Animals and Animal Care. Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 6 to 10 weeks old.

Western Blot Analysis of EGFR, EGFR Phosphorylation of Lung Cancer Cell Lines, and Response to Treatment with PKI166. Serum-starved NCI-H358 cells were studied for the expression of EGFR and activated EGFR, with or without the stimulation of 40 ng/mL recombinant human EGF for 15 minutes. Serum-starved NCI-H358 cells were then treated with PKI166 (0, 0.5, 1, 2.5, and 5 μmol/L) for 60 minutes and then incubated with or without 40 ng/mL recombinant human EGF for 15 minutes, washed, scraped into PBS containing 5 mmol/L EDTA and 1 mmol/L sodium orthovanadate, and centrifuged. The pellet was resuspended in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L leupeptin, and 0.15 unit/mL aprotinin], sonicated, and centrifuged to recover insoluble protein. Immunoprecipitation was done with monoclonal antibody anti-EGFR (clone EGF-RI) as described previously (15-17). Immunoprecipitates were analyzed on 7.5% SDS-PAGE and transferred onto 0.45-μm nitrocellulose membranes. The filters were blocked with 3% BSA in TDS [20 mmol/L Tris-HCl (pH 7.5) and 150 mmol/L NaCl], probed with either polyclonal sheep antihuman EGFR (1:1000) or monoclonal anti-phospho-tyrosine (monoclonal antibody 4G10; 1:2000) in TTBS (0.1% Tween 20 in TBS), and incubated with horseradish peroxidase-conjugated donkey antisheep IgG (1:2000; Sigma, St. Louis, MO) or sheep antimouse IgG (1:2000), respectively, in TTBS. Protein bands were visualized by the enhanced chemiluminescence detection system.

Preparation of Cell Suspension and Orthotopic Implantation of Tumor Cells. Cells were injected with Growth Factor Reduced Matrigel, which anchors the tumor cells, thereby preventing their diffusion into the thorax. For all experiments, a

stock solution of 500 μg of Matrigel in 1 mL of PBS was used. Suspensions were prepared of equal volumes of cells in PBS and Matrigel stock. Matrigel was thawed on ice, and all cell line suspensions, syringes, and needles were kept on ice before the injections. Suspensions of tumor cells in Matrigel were prepared as described previously (18). For intrathoracic injection, mice were anesthetized with sodium pentobarbital (50 mg/kg body weight) and placed in the right lateral decubitus position. Cell inoculum was injected percutaneously into the left lung with 1-ml tuberculin syringes (Becton Dickinson & Co.) with 30-gauge hypodermic needles.

Therapy for Established Human Lung Cancer Tumors Growing in the Lung of Athymic Nude Mice. In the initial set of experiments, the presence of microscopic tumor lesions was determined for each of the cell lines. Three mice, who received injections of NCI-H358 bronchioloalveolar cells (1×10^6 cells in 75 μL in Matrigel), and three mice, who received injections of PC14PE6 adenocarcinoma cells (0.5×10^6 cells in 75 μL in Matrigel), were killed, and histologic examination confirmed the lesions to be actively growing lung cancer 9 days (in case of PC14PE6 tumor cells) or 14 days (in case of NCI-H358 tumor cells) after tumor implantation. Mice were then injected orthotopically into the lung with NCI-H358 tumors (1×10^6 cells in 75 μL in Matrigel). Seven days after implantation, the mice were randomized into the following treatment groups ($n = 10$): once-per-week i.p. administration of paclitaxel (100 or 200 μg ; 4 or 8 mg/kg), three times-per-week oral administration of PKI166 (100 or 200 mg/kg), and a combination of paclitaxel and PKI166 (same dosages and frequencies of each of the drugs given alone). Control mice received the oral vehicle solution for PKI166 (DMSO/0.5% Tween 80 diluted 1:20 in HBSS) and i.p. HBSS (15–17). Three separate experiments were carried out with NCI-H358 tumors for a total of 5 weeks of therapy. The experiments were terminated when the control mice became moribund, and all mice were then simultaneously killed, autopsied, and their tumor tissues were harvested.

Using similar methodologies, the therapy experiment was repeated with PC14PE6 human lung adenocarcinoma cells with overall survival as an end point. Mice were implanted with PC14PE6 cells into the lung and were randomized to the four treatment groups ($n = 10$) on day 7. The mice were killed and necropsied when they became moribund. Survival was evaluated by the Kaplan-Meier method (24).

Necropsy and Tissue Preparation. Mice were killed with a lethal dose of sodium pentobarbital (100 mg/kg body weight). After a laparotomy, the thoracic cavity was inspected through the diaphragm for evidence of pleural effusion. Pleural effusions were collected, and the thoracic organs were then removed en block, including all lymph nodes and tumors. After dissection and removal of the heart, the lung and tumor mass were washed in cold PBS and weighed. Other visceral organs were removed and inspected for the presence of metastases. For immunohistochemical and H&E-staining procedures, one part of the tumor was fixed in formalin and embedded in paraffin.

Immunohistochemical Determination of EGFR, Activated EGFR, bFGF, and Activated MAPK. Paraffin-embedded tissues were used to identify EGFR, activated EGFR, bFGF, and phosphorylated MAPK. Immunohistochemical pro-

cedures for EGFR, activated EGFR, and bFGF were performed as described previously (15–17, 25). Activated MAPK was determined as described by Albanell *et al.* (26). Briefly, tissue sections (4 to 6 μm) were mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) and dried overnight. The sections were deparaffinized in xylene, treated with a graded series of alcohol [100, 95, and 80% etomideate/double distilled H_2O (v/v)], and then rehydrated in PBS (pH 7.5). For antigen retrieval, sections used for the activated EGFR and activated MAPK analyses were prepared in 10 mmol/L ethylene diamine tetraacetic acid buffer (pH 8) for 10 minutes in a microwave oven at 600 W. To determine EGFR, slides were prepared by pepsin digestion for 10 minutes. Before being stained for bFGF, the tissues were treated with pepsin for 20 minutes at 37°C and washed with PBS. The tissues were incubated with primary antibodies overnight at 4°C at the following dilutions: EGFR 1/200, activated EGFR 1/1000, activated MAPK 1/80, and bFGF 1/100. Control samples exposed to a secondary antibody alone showed no staining.

Quantification of Immunohistochemical Studies. To quantify the intensity of the immunohistochemical reaction, the absorbance of 100 bFGF- and activated MAPK-positive cells in 10 random 0.039- mm^2 fields at $\times 200$ of treated tumor tissues was measured with Optimas Image Analysis software (15–17, 25). The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the immunohistochemical reaction. bFGF and activated MAPK cytoplasmic immunoreactivity were evaluated by computer-assisted image analysis and expressed as the ratio of tumor cell expression to normal pancreatic gland expression multiplied by 100 (27, 28).

RESULTS

EGFR Is Expressed and Activated in Human Lung Cancer Cells, and the Activation of EGFR Is Blocked by PKI166.

In the first set of experiments, we determined that the NCI-H358 tumor cells express the EGFR and the activated EGFR. A Western blot analysis showed that NCI-H358 tumor cells express the EGFR protein constitutively. The activated EGFR protein was found after stimulation with the ligand EGF. Tumor cells incubated for 15 minutes in medium free of serum but containing EGF exhibited the activated EGFR (M_r 170,000 band), as detected by antisera on Western blot analyses of anti-EGFR immunoprecipitated cell lysates. We next determined that *in vitro* treatment of the cell lines with PKI166 inhibited EGF-stimulated tyrosine phosphorylation of the EGFR. Pretreatment of cells with PKI166 for 1 hour followed by a 15-minute treatment with EGF inhibited the phosphorylation in a dose-dependent manner (0 to 5 $\mu\text{mol/L}$). The identity of the M_r 170,000 band was confirmed by Western blot analysis with anti-EGFR antisera (Fig. 1). Similar results were noted with PC14PE6 cells (data not shown).

Inhibition of NSCLC Tumor Growth and Metastasis.

Athymic nude mice were injected into their left lungs with NCI-H358 cells in three separate experiments. The mice were killed 6.5 to 8 weeks after tumor implantation, when the control mice became moribund. In all three experiments, oral PKI166 decreased the median lung and tumor weight of the treated mice as compared with that of the control mice but was less effective

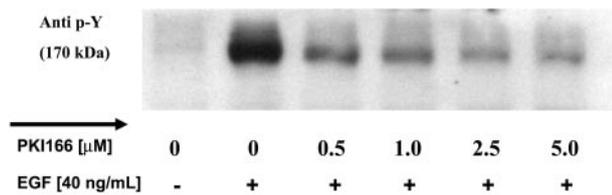


Fig. 1 Inhibition of EGF-induced autophosphorylation of EGFR: NCI-H358 NSCLC cells growing *in vitro* in serum-free medium were stimulated for 15 minutes with EGF (40 ng/mL) in the presence or absence of PKI166 (0 to 5 μ mol/L). The cells were washed and lysed, and insoluble proteins in the lysate were immunoprecipitated with an anti-EGFR monoclonal antibody, separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and sequentially probed with antisera to phosphotyrosine (Anti-pY) and EGFR (see Materials and Methods). The immunoreactive proteins were detected by incubating the blot with the corresponding peroxidase-conjugated IgG and visualized with the enhanced chemiluminescence system. Densitometric quantitation of the ratio between the M_r 170,000 phospho-tyrosine-specific and the M_r 170,000 EGFR-specific bands was compared in each case with the untreated cells, whose ratio was defined as 1.0.

than weekly paclitaxel alone. The antitumor effects observed in the mice treated with the combination of PKI166 and paclitaxel were equivalent or inferior to those observed in the mice treated with paclitaxel alone (Table 1). Treatment with PKI166 alone or in combination with paclitaxel was well tolerated, as evidenced by maintenance of body weight (data not shown).

For the survival studies, we used the same therapeutic model with human lung adenocarcinoma cells (PC14PE6) injected into the left lungs of 10 mice in each group. The mice were killed and necropsied when they became moribund. As in the therapeutic studies in NCI-H358 cells, results of the studies in PC14PE6 cells showed that the survival of mice treated with a combination of PKI166 and paclitaxel were equivalent to or inferior to the survival of mice treated with paclitaxel alone. The survival data are presented in Fig. 2. A detailed necropsy revealed that all of the mice had thoracic tumors.

Histology and Immunohistochemical Analysis. The NCI-H358 tumor specimens were processed for routine histology and immunohistochemical analyses. Immunohistochemistry with specific anti-EGFR, activated EGFR, bFGF, and activated MAPK showed differences in the level of expression for the different treatment groups. The lesions did not show differences

in the expression of EGFR; yet, the expression of activated EGFR was diminished in the groups treated with PKI166 alone or in combination with paclitaxel (Fig. 3). The expression of bFGF and activated MAPK was determined. Activated MAPK expression was decreased in PKI166-treated tumors, whereas bFGF level was elevated in PKI166 or paclitaxel-treated tumors and even higher in tumors treated with concurrent PKI166 and paclitaxel (Fig. 4).

DISCUSSION

The effects of EGFR-tyrosine kinase inhibitors in combination with chemotherapy on subcutaneous ectopic tumors have been extensively studied. Ciardiello *et al.* (13), with gefitinib on human cancer lines that co-expressed EGFR and its ligand transforming growth factor α , observed an increased antitumor effect and potentiation of cytotoxic drugs both *in vitro* and *in vivo* for s.c. xenografts. Sirotiak *et al.* (14) also showed a potentiation of cytotoxic treatment with coadministration of gefitinib for s.c. xenografts, but the effects did not correlate with the level of EGFR expression by the target tumor. However, the level of the activated EGFR, which is the presumptive actual target for gefitinib, was not tested in these studies. Furthermore, the relevance of s.c. tumor growth in animal models to that in human studies has to be carefully ascertained because experimental preclinical results frequently cannot be translated to the clinic. One reason for this is that lower doses of chemotherapy agents often used in animal models to benefit from a synergy with biological therapies (29). Another limitation of animal models is related to tumor implantation. The use of ectopic tumors does not adequately take into account the interaction between the specific organ environment and tumor cells (*i.e.*, lung cancer cells interacting with the lung microenvironment; refs. 30–32) and may therefore alter the tumor response to therapy. Indeed, we have recently shown that paclitaxel is more effective in treating s.c. NSCLC tumors than in orthotopic ones, suggesting that the orthotopic model may be more suitable for the study of lung cancer (18).

In the present study, we examined the clinical and biological effects of targeted therapy against the EGFR alone and in combination with chemotherapy in orthotopic lung cancer models. Our data showed that concurrent therapy with an EGFR-tyrosine kinase inhibitor and paclitaxel in two human lung

Table 1 PKI166 and paclitaxel therapy of NCI-H358 (human NSCLC) tumors

	Control			PKI166			Paclitaxel			PKI166 + paclitaxel		
	Incidence*	Median	Percentage†	Incidence*	Median	Percentage†	Incidence*	Median	Percentage†	Incidence*	Median	Percentage†
Study 1	9/9	0.319	100%	9/10	0.318	99%	10/10	0.271	85%	10/10	0.287	90%
Study 2	9/9	0.345	100%	9/9	0.217	63%	9/9	0.215	62%	9/10	0.226	65%
Study 3‡	9/9	0.340	100%	10/10	0.297	87%	8/9	0.248	73%	9/9	0.245	72%

NOTE. Weight (grams) designates lung and tumor weight.

* Number of positive mice/number of treated mice.

† Percentage of control mice weight.

‡ NCI-H358 human NSCLC cells (1×10^6) were injected into the left lung of nude mice. Seven days later, groups of mice were treated with once-a-week i.p. paclitaxel [studies 1 and 2: 200 μ g (8 mg/kg); study 3: 100 μ g (4 mg/kg)], three times-a-week oral administration of PKI166 [studies 1 and 2: 200 mg/kg; study 3: 100 mg/kg], and combination of paclitaxel and PKI166. Control mice received oral vehicle solution for PKI166 and i.p. injections saline for a total of five weekly courses of therapy. All mice were killed on 6.5 to 8 weeks after tumor injection when control mice became moribund.

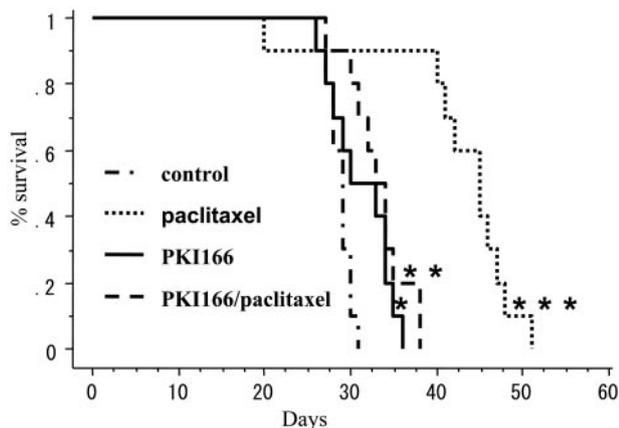


Fig. 2 Therapeutic effects of EGFR blockade and paclitaxel: percentage of cumulative survival. PC14PE6 adenocarcinoma cells were implanted in the lungs of nude mice. The mice were randomized into the above four treatment groups ($n = 10$) and were killed when moribund. Survival analysis was computed by the Kaplan-Meier method. * $P = 0.1$; ** $P = 0.004$; *** $P = 0.001$. Compared to the control, PKI166 alone improved survival by 2.5 days, combination PKI166 and paclitaxel by 4.5 days, and paclitaxel alone by 13.8 days.

cancers growing orthotopically in mice is equivalent to or inferior to therapy with paclitaxel alone, although therapy with PKI166 inhibited EGFR phosphorylation. Our studies were among the first to show the discrepancy between biological activity and clinical results in an orthotopic model of human lung cancer in nude mice. Both lung cancer models studied expressed the EGFR, the presumed target for therapy.

Our data were consistent with those from the clinical trials of EGFR-tyrosine kinase inhibitors and chemotherapy for the treatment of lung cancer. Recent phase III clinical trials of gefitinib or erlotinib in combination with chemotherapy in patients with advanced NSCLC showed that concurrent gefitinib or erlotinib and chemotherapy did not improve overall survival or other efficacy as compared with chemotherapy alone (19, 20, 33, 34). The lack of improved efficacy in the combination groups was observed with different combinations of chemotherapy (carboplatin/paclitaxel or gemcitabine/cisplatin) and with two dosages of gefitinib (250 or 500 mg/day). Similar clinical results have been observed for erlotinib (35).

Two recent studies suggested that response to monotherapy with gefitinib was associated with EGFR mutations. In these important reports, the authors suggested that patients whose tumors express EGFR mutations were likely to respond to gefitinib, whereas patients whose tumors did not express the mutations did not show response (11, 12). Our cell lines did not express EGFR mutations (data not shown). We propose that other mechanisms may be involved in response or resistance to EGFR-tyrosine kinase inhibitors. In our orthotopic lung model, the expression of bFGF by lung tumors was increased in all of the treatment groups with a trend of higher level in tumors treated with the combination of PKI166 and paclitaxel. Furthermore, the expression and activation of MAPK were lower in tumors treated with PKI166, as shown in prior studies with gefitinib (26). MAPK

can be activated by bFGF (36), and our data suggested that the effects of EGFR blockade could possibly be offset by downstream signaling events mediated by bFGF and other growth factors, thereby promoting tumor cell survival. Combination therapy of bFGF signaling inhibitors, *e.g.*, IFNs (37) and anti-EGFR agents, may be warranted. Furthermore, when taken together with the clinical trials, our data suggested that the sequencing of conventional therapy and biological therapy with EGFR-tyrosine kinase inhibitors has to be considered and that concurrent therapy may be detrimental. In this regard, Magne *et al.* (38) recently found differential effect of gefitinib in different combinations with chemotherapy and radiotherapy.

An additional intriguing explanation for the discrepancy between the biological effect and the clinical efficacy of PKI166 was provided by recent studies in animal models that showed a significant response to the combination of PKI166 therapy with

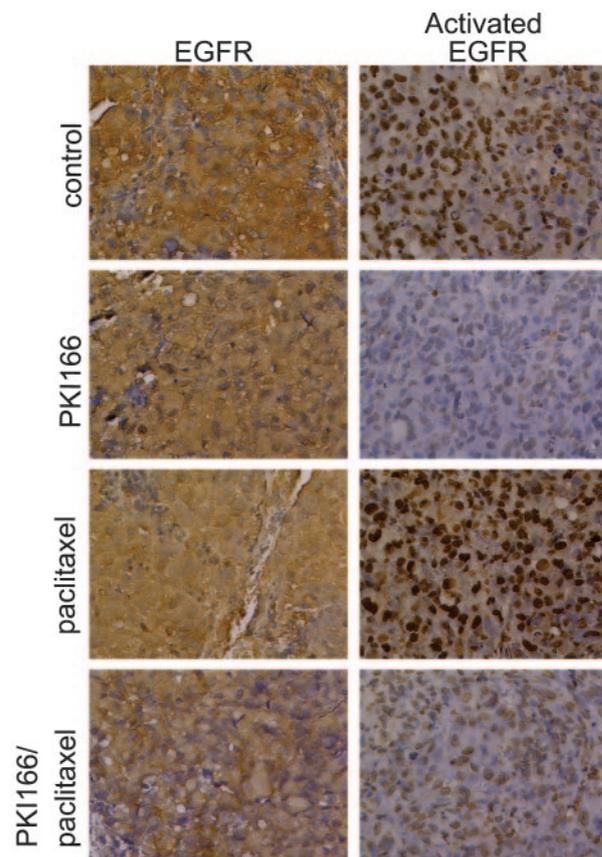


Fig. 3 Representative panels of immunohistochemical determination of EGFR and activated EGFR in lung cancer tumors ($\times 200$ magnification): human NCI-H358 tumors from the lung of nude mice were harvested and processed for histology and immunohistochemical analyses 6.5 to 8 weeks after treatment with once-per-week i.p. paclitaxel (100 to 200 μg ; 4 to 8 mg/kg), three times-per-week oral PKI166 (100 to 200 mg/kg), combination of paclitaxel and PKI166, or control. Tissue sections were stained for expression of tyrosine-phosphorylated, activated EGFR, and total EGFR as described previously (15–17). Tumors from all treatment groups stained positive for EGFR. Only tumors from control mice or mice treated with paclitaxel alone stained positive for activated EGFR.

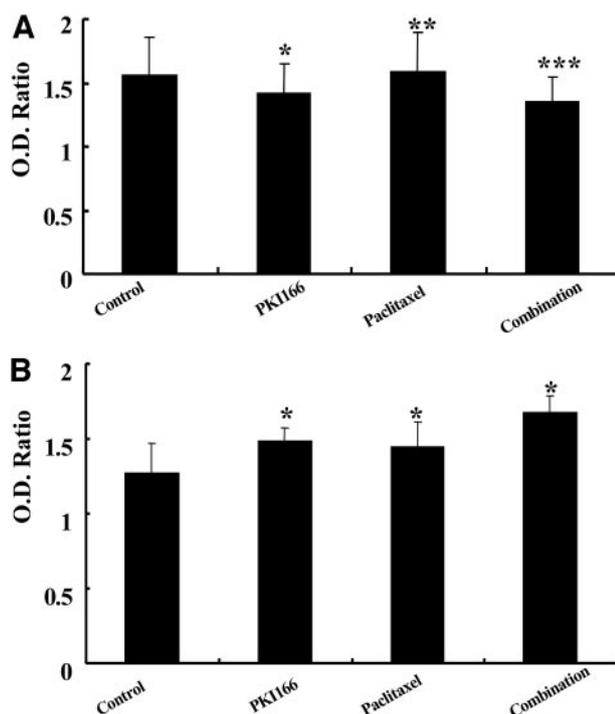


Fig. 4 For quantification of the immunohistochemistry reaction intensity, the absorbance of 100 phosphorylated MAPK-positive cells (A) or bFGF-positive cells (B) from each of the differently treated tumor tissues were measured in 10 random fields at $\times 200$ magnification with the Optimas Image Analysis software (Bioscan). The cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and was expressed as a ratio of tumor cell expression to normal muscle cell expression multiplied by 100. Student's *t* test was compared the results between control and each of the three different treatment groups. A, **P* = 0.003; ***P* = 0.5; ****P* < 0.00001. B, **P* < 0.00001.

chemotherapy (15–17) in an orthotopic model of pancreatic cancer and an orthotopic model of prostate carcinoma metastasis to the bone. Interestingly, in these studies, both the tumor cells and the tumor-associated blood vessels expressed the target receptor, and the tumor cells expressed the ligand transforming growth factor α (25). In contrast, in our studies of lung cancer, the NCI-H358 and the PC14PE6 tumors did not express transforming growth factor α , and the tumor endothelial cells did not express EGFR (data not shown).

In summary, our data suggest that in lung cancer, EGFR signaling may have an important role, yet it is insufficient to control tumor growth. The combination of several-targeted molecular therapies with conventional chemotherapy may be the basis of future therapy for lung cancer. Future directions for the study of EGFR inhibition in lung cancer should focus on the following topics: identifying the actual target for EGFR-tyrosine kinase inhibitors; identifying the best tumor biological profile that predicts response to therapy with EGFR inhibition, including the role of transforming growth factor α and the expression of EGFR by tumor endothelial cells; and the sequencing of chemotherapy and targeted therapy.

REFERENCES

- Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA - Cancer J Clin* 2003;53(1):5–26.
- Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346(2):92–8.
- Huang SM, Harari PM. Epidermal growth factor receptor inhibition in cancer therapy: biology, rationale and preliminary clinical results. *Investig New Drugs* 1999;17:259–69.
- Franklin WA, Veve R, Hirsch FR, Helfrich BA, Bunn PA Jr. Epidermal growth factor receptor family in lung cancer and premalignancy. *Semin Oncol* 2002;29(1 Suppl 4):3–14.
- Hendler FJ, Ozanne BW. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J Clin Investig* 1984;74(2):647–51.
- Onn A, Correa AM, Gilcrease M, et al. Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. *Clin Cancer Res* 2004;10(1 Pt 1):136–43.
- Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001;37(Suppl 4):S9–15.
- Mendelsohn J. Targeting the epidermal growth factor receptor for cancer therapy. *J Clin Oncol* 2002;20(18 Suppl):1S–13S.
- Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *J Am Med Assoc* 2003;290(16):2149–58.
- Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003;21(12):2237–46.
- Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304(5676):1497–500.
- Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350(21):2129–39.
- Ciardiello F, Caputo R, Bianco R, et al. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001;7(5):1459–65.
- Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000;6(12):4885–92.
- Bruns CJ, Solorzano CC, Harbison MT, et al. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 2000;60(11):2926–35.
- Kim SJ, Uehara H, Karashima T, Shepherd DL, Killion JJ, Fidler IJ. Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. *Clin Cancer Res* 2003;9(3):1200–10.
- Weber KL, Doucet M, Price JE, Baker C, Kim SJ, Fidler IJ. Blockade of epidermal growth factor receptor signaling leads to inhibition of renal cell carcinoma growth in the bone of nude mice. *Cancer Res* 2003;63(11):2940–7.
- Onn A, Isobe T, Itasaka S, et al. Development of an orthotopic model to study the biology and therapy of primary human lung cancer in nude mice. *Clin Cancer Res* 2003;9(15):5532–9.
- Herbst RS, Giaccone G, Schiller JH, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 2. *J Clin Oncol* 2004;22(5):785–94.

20. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. *J Clin Oncol* 2004;22(5):777–84.
21. Yano S, Nokihara H, Hanibuchi M, et al. Model of malignant pleural effusion of human lung adenocarcinoma in SCID mice. *Oncol Res* 1997;9:573–9.
22. Yano S, Herbst RS, Shinohara H, et al. Treatment for malignant pleural effusion of human lung adenocarcinoma by inhibition of vascular endothelial growth factor receptor tyrosine kinase phosphorylation. *Clin Cancer Res* 2000;6(3):957–65.
23. Dinney CP, Fishbeck R, Singh RK, et al. Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. *J Urol* 1995;154(4):1532–8.
24. Kaplan EL, Meier P. Nonparametric estimation from incomplete observation. *J Am Stat Assoc* 1958;53:457–81.
25. Baker CH, Kedar D, McCarty MF, et al. Blockade of epidermal growth factor receptor signaling on tumor cells and tumor-associated endothelial cells for therapy of human carcinomas. *Am J Pathol* 2002;161(3):929–38.
26. Albanell J, Rojo F, Averbuch S, et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. *J Clin Oncol* 2002;20(1):110–24.
27. Yoneda J, Kuniyasu H, Crispens MA, Price JE, Bucana CD, Fidler IJ. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst (Bethesda)* 1998;90(6):447–54.
28. Radinsky R, Risin, Fan, et al. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin Cancer Res* 1995;1(1):19–31.
29. Kerbel RS. What is the optimal rodent model for anti-tumor drug testing? *Cancer Metastasis Rev* 1998–99;17(3):301–4.
30. Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM, Fidler IJ. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res* 1988;48(23):6863–71.
31. Katakai A, Scheid P, Piet M, et al. Tumor infiltrating lymphocytes and macrophages have a potential dual role in lung cancer by supporting both host-defense and tumor progression. *J Lab Clin Med* 2002;140(5):320–8.
32. Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. *J Clin Invest* 2004;113(6):913–23.
33. Gatzemeier U, Pluzanska A, Szczesna E, et al. Results of a phase III trial of erlotinib (OSI-774) combined with cisplatin and gemcitabine chemotherapy in advanced non-small cell lung cancer. *Proc Am Soc Clin Oncol* 2004;23:617.
34. Herbst RS, Prager D, Hermann R, et al. Tribute: a phase III trial of erlotinib HCl (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non-small cell lung cancer. *Proc Am Soc Clin Oncol* 2004;23:617.
35. Akita RW, Sliwkowski MX. Preclinical studies with Erlotinib (Tarceva). *Semin Oncol* 2003;30(3 Suppl 7):15–24.
36. Motamed K, Blake DJ, Angello JC, et al. Fibroblast growth factor receptor-1 mediates the inhibition of endothelial cell proliferation and the promotion of skeletal myoblast differentiation by SPARC: a role for protein kinase A. *J Cell Biochem* 2003;90(2):408–23.
37. Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ. Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci USA* 1995;92(10):4562–6.
38. Magne N, Fischel JL, Tiffon C, et al. Molecular mechanisms underlying the interaction between ZD1839 ('Iressa') and cisplatin/5-fluorouracil. *Br J Cancer* 2003;89(3):585–92.

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