Expression of epidermal growth factor (EGF)/transforming growth factor-α by human lung cancer cells determines their response to EGF receptor tyrosine kinase inhibition in the lungs of mice

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Abstract

Epidermal growth factor receptor (EGFR) has been extensively targeted in the treatment of non–small cell lung cancer, producing responses in a small number of patients. To study the role of ligand expression in mediating response to EGFR antagonism, we injected NCI-H441 [EGFR and EGF/transforming growth factor-α (TGF-α) positive] or PC14-PE6 (EGFR positive and EGF/TGF-α negative) human lung adenocarcinoma cells into the lungs of nude mice. We randomized the mice to receive treatment with the EGFR tyrosine kinase inhibitors gefitinib or AEE788 or vehicle. Treatment of mice bearing NCI-H441 but not PC14-PE6 lung tumors resulted in a significant reduction in primary tumor growth, pleural effusion, and lymph node metastasis. Immunohistochemical analyses revealed that NCI-H441 and PC14-PE6 cells expressed EGFR but that the expression of EGF/TGF-α was high in NCI-H441 cells and very low in PC14-PE6 cells. Consequently, EGFR was activated in both tumor and tumor-associated endothelial cells in the NCI-H441 tumors but not in the PC14-PE6 tumors. Antagonism of EGFR signaling by treatment of mice with AEE788 decreased proliferation and increased apoptosis of both tumor cells and tumor-associated endothelial cells in NCI-H441 tumors but not in PC14-PE6 tumors. However, after transfection of PC14-PE6 cells with TGF-α, lung tumors derived from the transfected cells expressed and activated EGFR in both tumor and tumor-associated endothelial cells and tumors responded to treatment with AEE788. Collectively, these results strongly suggest that the response of human lung cancers growing orthotopically in mice to the inhibition of EGFR signaling is determined by ligand (EGF/TGF-α) expression by tumor cells. Our findings provide an additional explanation for the susceptibility of lung cancers to treatment with EGFR tyrosine kinase inhibitors. [Mol Cancer Ther 2007;6(10):2652–63]

Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States and throughout the world (1, 2). At the time of diagnosis of lung cancer, >50% of patients have advanced metastatic disease and the only therapeutic option for these patients is systemic chemotherapy. Unfortunately, the majority of these patients eventually die of their disease (3, 4). Clearly, new strategies for the therapy of lung cancer are urgently needed. One such strategy has been to target epidermal growth factor receptor (EGFR) signaling. EGFR, a member of the Erb superfamily of tyrosine kinases, plays a crucial role in cell proliferation, survival, angiogenesis, and metastasis (5). The EGFR signaling pathway is believed to contribute to tumor progression by triggering downstream signaling molecules, such as Akt and mitogen-activated protein kinase (MAPK; refs. 6–8). Studies have detected overexpression of EGFR in ≥80% of non–small cell lung cancer (NSCLC) cases and coexpression of EGFR and its ligand(s) in 38% of NSCLC specimens (9–12). The close association between EGFR and EGF/transforming growth factor-α (TGF-α) coexpression in tumor cells and tumor progression, chemotherapy resistance, and poor survival in patients with lung cancer has advanced EGFR as a logical target in therapy for lung cancer (13).

Investigators have extensively studied small-molecule EGFR tyrosine kinase inhibitors in clinical trials for the treatment of relapsed NSCLC (14–16). However, only a small percentage of patients with lung cancer respond to EGFR antagonists given as single agents. For this reason, and because preclinical and clinical data suggest that EGFR expression in tumor cells is not a reliable marker in determining the response of NSCLC to EGFR antagonism (17, 18), identifying predictive biomarkers of EGFR pathway inhibitor activity is important. Several groups

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have reported the association of specific mutations in the tyrosine kinase domain of EGFR (19–22) or a high EGFR gene copy number (23, 24) with sensitivity to tyrosine kinase inhibitors. Because these mutations also occur, albeit at a low frequency, in patients who do not have a response to EGFR inhibitors, the sensitivity of NSCLC to this therapy may be caused by additional mechanisms (25, 26).

Angiogenesis plays an important role in tumor growth and metastasis (27), and microvessel density in tumors is an independent prognostic marker in patients with NSCLC (28). Growth factors secreted by tumor and tumor-associated cells, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and EGF/TGF-α, play key roles in angiogenesis. EGF/TGF-α contributes to tumor angiogenesis (5) and the binding of EGF/TGF-α to EGFR stimulates the expression of VEGF in tumor cells (8, 29). The inhibition of EGFR signaling can indirectly inhibit angiogenesis by decreased production of VEGF by tumor cells and can in some cases directly target angiogenesis by inducing apoptosis of endothelial cells that express and activate EGFR (30–32).

We hypothesized that expression of EGF/TGF-α in tumor cells is important for the activation of EGFR in tumor cells in an autocrine manner and for its expression and activation in tumor-associated endothelial cells in a paracrine manner. Thus, blockade of EGFR signaling could then have potent antivascular and antitumor effects if its ligand is expressed. To test this hypothesis, we studied two human lung adenocarcinoma cell lines, whose growth patterns after injection in the lung have been well characterized in our laboratory (33, 34), which both express EGFR but differ in their production of EGF/TGF-α. Specifically, we evaluated the association between expression of EGF/TGF-α in human NSCLC cells that express EGFR growing orthotopically in nude mice and the response to the therapeutic antagonism of EGFR signaling with the small molecules gefitinib or AEE788.

Materials and Methods

Cell Culture

NCI-H441 human lung adenocarcinoma cells (American Type Culture Collection) were cultured in RPMI 1640 with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution, and penicillin-streptomycin (Flow Laboratories). PC14-PE6 lung adenocarcinoma cells (35), a variant of the PC14 human lung adenocarcinoma cell line, were provided by Nagahiro Saijo (National Cancer Research Institute, Tokyo, Japan) and maintained in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution, and penicillin-streptomycin. A mouse lung endothelial cell (MLEC) line was established (36) from transgenic *H-2K^b-ts*A58 mice (CBA/ca × C57BL/10 hybrid; Charles River Laboratories). MLECs were maintained in DMEM with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, vitamin solution, and penicillin-streptomycin. Cells were incubated in a mixture of 5% CO_2 and 95% air at 37°C in an incubator free of *Mycoplasma* and pathogenic murine viruses (assayed by Microbiological Associates).

Orthotopic Lung Tumor Models

Male athymic nude mice, 6 to 8 weeks old (obtained from the Animal Production Area of the National Cancer Institute at Frederick Cancer Research and Development Center, Frederick, MD), were anesthetized using sodium pentobarbital (50 mg/kg body weight) and placed in the right lateral decubitus position. Logarithmically growing NCI-H441 or PC14-PE6 single-cell suspensions containing 2.5×10^5 or 5×10^5 cells with ≥90% viability in 50 μL PBS containing 50 μg of growth factor–reduced Matrigel (BD Biosciences) were injected into the left lateral thorax of the mice at the lateral dorsal axillary line. After the tumor cell injection, the mice were turned to the left lateral decubitus position and observed for 45 to 60 min until they recovered fully. Mice were housed and maintained under specific pathogen-free conditions in accordance with the guidelines of the American Association for Laboratory Animal Care and experiments met all of the current regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the NIH.

Treatment of Orthotopic Human Lung Tumors

Gefitinib (AstraZeneca Pharmaceuticals) was dissolved in vehicle containing 1% Tween 80. AEE788 (Novartis Pharmaceuticals) was dissolved in vehicle containing 90% polyethylene glycol 300 and 10% 1-methyl-2-pyrrolidinone. Five days after the implantation of the NCI-H441 or PC14-PE6 tumor cells into their lungs, mice (8–10 per group) were randomized to treatment with thrice weekly oral administration of 50 mg/kg AEE788 (37) or vehicle control. In a similar but separate study, mice implanted with PC14-PE6 or NCI-H441 lung tumors were randomized to treatment (8–10 per group) with once daily oral administration of 50 mg/kg of gefitinib or vehicle control. All of the mice were killed and autopsied when control animals became moribund and primary lung tumor weight (total tumor-bearing lung weight minus the normal lung weight of 0.17 g), incidence and volume of pleural effusion, and the presence of metastasis were evaluated.

Reverse Transcription-PCR Analysis

Total RNA was isolated using the Trizol reagent kit (Life Technologies) following the manufacturer's protocol. cDNA generated by reverse transcription of the extracted RNA was amplified using PCR. Following an initial incubation at 94°C, each cycle consisted of incubation for 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. After the final cycle, the samples were incubated for a further 7 min at 72°C and then kept at 4°C before analysis using agarose gel electrophoresis. The primer sequences were as follows: actin forward, 5′-GCACTCTTCCAGCCTTCCTTCCTG-3′; actin reverse, 5′-GGAGTACTTGCGCTCAGGAGGAGC-3′;

EGF forward, 5′-AGCCAGCTCTGATCTAATCTGG-3′; EGF reverse, 5′-TTTTGCAAATATGTTCACAGCC-3′; TGF-α forward, 5′-GACGGAGTTCTTGACAGAGT-3′; TGF-α reverse, 5′-CTGGCTGTCCTTATCATCAC-3′.

EGFR Nucleotide Sequence Analysis

DNA was extracted from NCI-H441 or PC14-PE6 cells using a PicoPure DNA extraction kit (Arcturus). Exons 18 to 21 of EGFR were amplified, and PCR fragments were sequenced and analyzed (20). All of the sequences were confirmed using two independent PCR amplifications. The primer sequences were as follows: exon 19 forward, 5′-CAGATCACTGGGCAGCATGTGGCACC-3′; exon 19 reverse, 5′-GGCAGCTGCTCTGCTCTAGACCCTGCT-3′; exon 20 forward, 5′-TCCTTCTGGCCACCATGCG-3′; exon 20 reverse, 5′-CCTCACATGCGGTCTGCGCT-3′; exon 21 forward, 5′-GACGTGGAGAGGCTCAGAGC-3′; and exon 21 reverse, 5′-CACATGCAGGGGAGGATGCT-3′.

EGFR Gene Copy Number Analysis

Glass slides with NCI-H441, PC14-PE6, PC14-PE6/TGF-α, and PC14-PE6/Neo in monolayers were fixed with 10% formalin and washed with 2× SSC buffer (pH 7.0). The gene copy number per cell was determined using fluorescence *in situ* hybridization with the Vysis LSI EGFR Spectrum Orange/CEP7 Spectrum Green probe (Abbott Laboratories) according to the published protocol. After incubation in 2× SSC at 75°C for 15 to 25 min, slides were digested with proteinase K [0.25 mg/mL in 2× SSC (pH 7.0)] at 37°C for 15 to 25 min, rinsed in 2× SSC at room temperature for 5 min, and dehydrated using ethanol at increasing concentrations (70%, 85%, and 100%). The EGFR/CEP7 probe set was used following the manufacturer's instructions and the hybridization area was covered with a glass coverslip and sealed with rubber cement. The slides were incubated at 80°C for 8 to 10 min for codenaturation of chromosomal and probe DNA and then placed in a humidified chamber at 37°C for 20 h to allow hybridization to occur. Posthybridization washes were done in 1.5 mol/L urea and 0.1× SSC (pH 7.0–7.5) at 45°C for 30 min and in 2× SSC for 2 min at room temperature. After the samples were dehydrated in ethanol as described above, 4′,6′-diamidino-2-phenylindole (0.15 mg/mL in Vectashield mounting medium; Vector Laboratories) was applied for chromatin counterstaining. The slides were examined under a florescence microscope that was equipped with a set of appropriate filters. Signals were scored in at least 200 nonoverlapping intact nuclei. Specimens were considered to be amplified for EGFR when >10% of the cells exhibited either an EGFR to CEP7 ratio of >2 or innumerable tight clusters of signals of the locus probe.

Transfection of the *TGF-α* Gene into PC14-PE6 Cells

Plasmids containing a 925-bp cDNA *Eco*RI fragment coding the full precursor peptide were transfected into PC14-PE6 cells as described previously (38). PC14-PE6 cells plated at a density of 4×10^5 per well in six-well plates were transfected and incubated for 16 h with the *TGF-α* gene using the FuGENE 6 reagent (Roche Molecular Systems) according to the manufacturer's instructions and then washed and fed with fresh DMEM. Selection of cells for resistance to neomycin was initiated 48 h after transfection by adding 800 μg/mL G418 (Life Technologies) to DMEM, which was replaced every 3 days. Three weeks later, single G418-resistant colonies were transferred to individual wells of 48-well plates. The expression of TGF-α in individual clones was determined using an ELISA.

TGF-α Measurement *In vitro*

Conditioned media was collected from TGF-α–transfected cells grown in medium supplemented with 5% fetal bovine serum for 24 h. The collected media were assayed for the presence of TGF-α using a commercial ELISA kit (R&D System, Inc.) following the manufacturer's protocol.

Western Blotting

Cell lysis and Western blotting were described in detail previously (37). Briefly, total cellular proteins were resolved using 7.5% SDS-PAGE and probed with antibodies against human EGFR (Upstate); MAPK p42/44 (Zymed); human phosphorylated EGFR (Tyr^{845}), phosphorylated MAPK p42/44, Akt, and phosphorylated Akt (Cell Signaling Technology); and β-actin (Sigma Chemical Co.). The protein bands were scanned using a densitometer, and the relative intensities were quantified using the ImageQuant software program (Molecular Dynamics).

Histologic and Immunohistochemical Analysis

Tumor tissue specimens were fixed with formalin, embedded in paraffin or frozen in OCT compound, and then sectioned and stained with H&E. Immunostaining for EGF, TGF-α, EGFR (Zymed), phosphorylated EGFR (Biosource International), VEGF receptor (VEGFR), phosphorylated VEGFR, phosphorylated Akt, phosphorylated MAPK (Cell Signaling Technology), Ki67 (DAKO), and/or CD31/PECAM-1 (PharMingen) was done using methods and antibodies described previously (37, 39). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was done using a commercial apoptosis detection kit (Promega) with modifications (34). Microvessel density was quantified in 10 random 0.159-mm^2 fields at a magnification of ×100 for each tumor as described previously (34). Both Ki67- and TUNEL-positive endothelial cells were counted in 10 high-power fields at a magnification of ×200, and the percentages of positive cells were calculated.

Statistical Analysis

The data were analyzed using the χ^2 test (for incidence only), Wilcoxon rank sum test, and two-tailed Student's t test ($P < 0.05$ is defined as the P value for significance).

Results

EGF/TGF-α and EGFR Expression by NCI-H441 and PC14-PE6 Human Lung Adenocarcinoma Cells *In vitro*

We first examined the expression of EGFR and its ligands in cultured NCI-H441 and PC14-PE6 lung adenocarcinoma cells. Cultured NCI-H441 cells had high levels of EGF/TGF-α expression and consequently had high expression and activation of EGFR. PC14-PE6 cells expressed EGFR but had low or undetectable levels of EGF/TGF-α and virtually no EGFR activation. There was no discernible difference in the level of total EGFR expression between the two cell lines (Fig. 1A).

To investigate the interaction between NSCLC cells and lung endothelial cells (MLECs), we examined the expression and activation of EGFR in MLECs cultured with NCI-H441 or PC14-PE6 lung adenocarcinoma cells using a Transwell coculture system (Costar). EGFR was activated in MLECs cocultured with NCI-H441 cells (which had high expression of EGF/TGF-α) but not in PC14-PE6 cells (which had low expression of EGF/TGF-α; Fig. 1B).

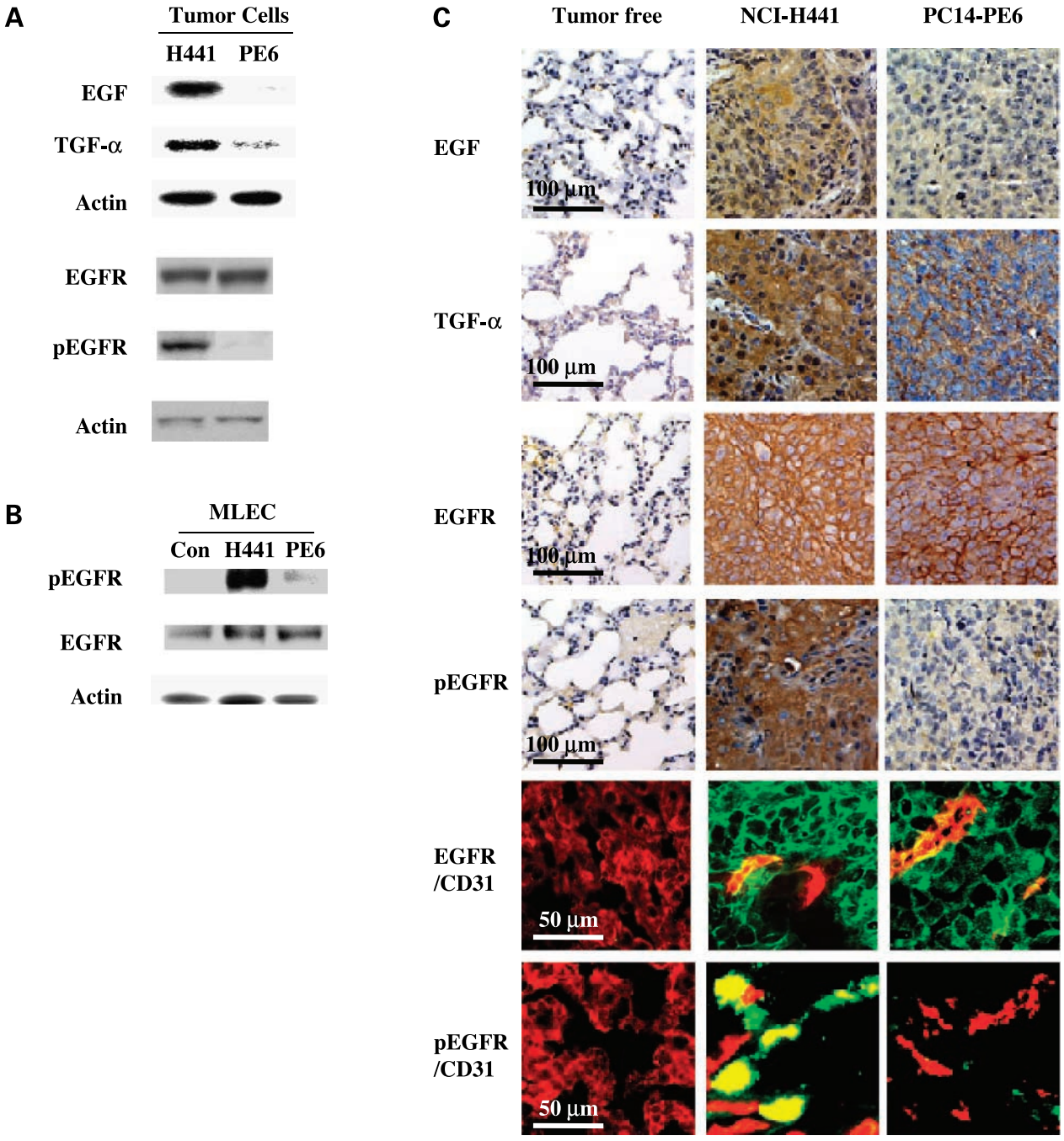

Figure 1. Expression and activation of EGFR in tumor cells, MLECs, and orthotopic NCI-H441 and PC14-PE6 lung tumors. **A,** expression of EGFR and its ligands in NCI-H441 (*H441*) or PC14-PE6 (*PE6*) cells *in vitro*. Expression of EGF and TGF-α was analyzed using reverse transcription PCR. EGFR and phosphorylated EGFR (*pEGFR*) expression levels were determined by Western blotting. **B,** Western blot analysis of EGFR and phosphorylated EGFR levels in MLECs cocultured with NCI-H441 or PC14-PE6 lung cancer cells compared with control (*Con*). **C,** representative immunohistochemical images of the expression (*EGFR*) and phosphorylation (*pEGFR*) of EGFR and its ligands (*EGF* and *TGF-α*) for tumor tissue sections for NCI-H441 or PC14-PE6 lung tumors and adjacent tumor-free lung tissue. For the dual immunofluorescence studies (*bottom two rows*), *red fluorescence* was used to identify endothelial cells (CD31) and *green fluorescence* was used to identify EGFR or phosphorylated EGFR. *Yellow fluorescence* indicates endothelial cell expression (*EGFR/CD31*) or activation (*pEGFR/CD31*) of EGFR.

Table 1. Effects of systemic administration of AEE788 on NCI-H441 or PC14-PE6 human lung adenocarcinomas growing orthotopically in the lungs of nude mice

Group	Lung tumor			Pleural effusion (incidence)	Metastasis to lymph nodes (incidence)
	Body weight (g), median (range)	Incidence	Weight (g), median (range)		
NCI-H441					
Vehicle	27.8 (21.1-33.6)	20/20	0.4 (0.1-0.9)	18/20	20/20
AEE788	27.5 (23.3-32.7)	13/20*	0.03 (0-0.1)†	0/20†	1/20†
PC14-PE6					
Vehicle	27.8 (23.3-34.5)	16/16	0.2 (0.02-0.5)	14/16	16/16
AEE788	27.3 (24.1-34.5)	16/16	0.2 (0.01-0.7)	8/16	16/16

NOTE: NCI-H441 or PC14-PE6 cells (2.5×10^5) were injected into the left lobe of the lung. Treatment with AEE788 (50 mg/kg orally thrice weekly) or vehicle control was initiated 5 d after tumor injection. All mice were killed and tumors were harvested 24 (NCI-H441) or 26 (PC14-PE6) days after tumor injection when mice in the control group became moribund. Lung tumor weight was determined by subtracting the normal lung weight (0.17 g) from the weight of the tumor-containing lung.

*$P < 0.05$, compared with vehicle control according to the χ^2 test (for incidence) and Student's two-tailed t test.

†$P < 0.001$, compared with vehicle control according to the χ^2 test (for incidence) and Student's two-tailed t test.

EGFR and EGF/TGF-α Expression by NCI-H441 and PC14-PE6 Human Lung Adenocarcinomas Growing in the Lungs of Mice

We next characterized the patterns of growth and expression of EGFR and EGF/TGF-α after orthotopic injection of the human adenocarcinoma cell lines NCI-H441 and PC14-PE6 into the lungs of mice. Both cell lines established microscopic lung tumors within 5 days of tumor cell injection. Tumors progressed with similar incidence, durations of host survival, and incidence of

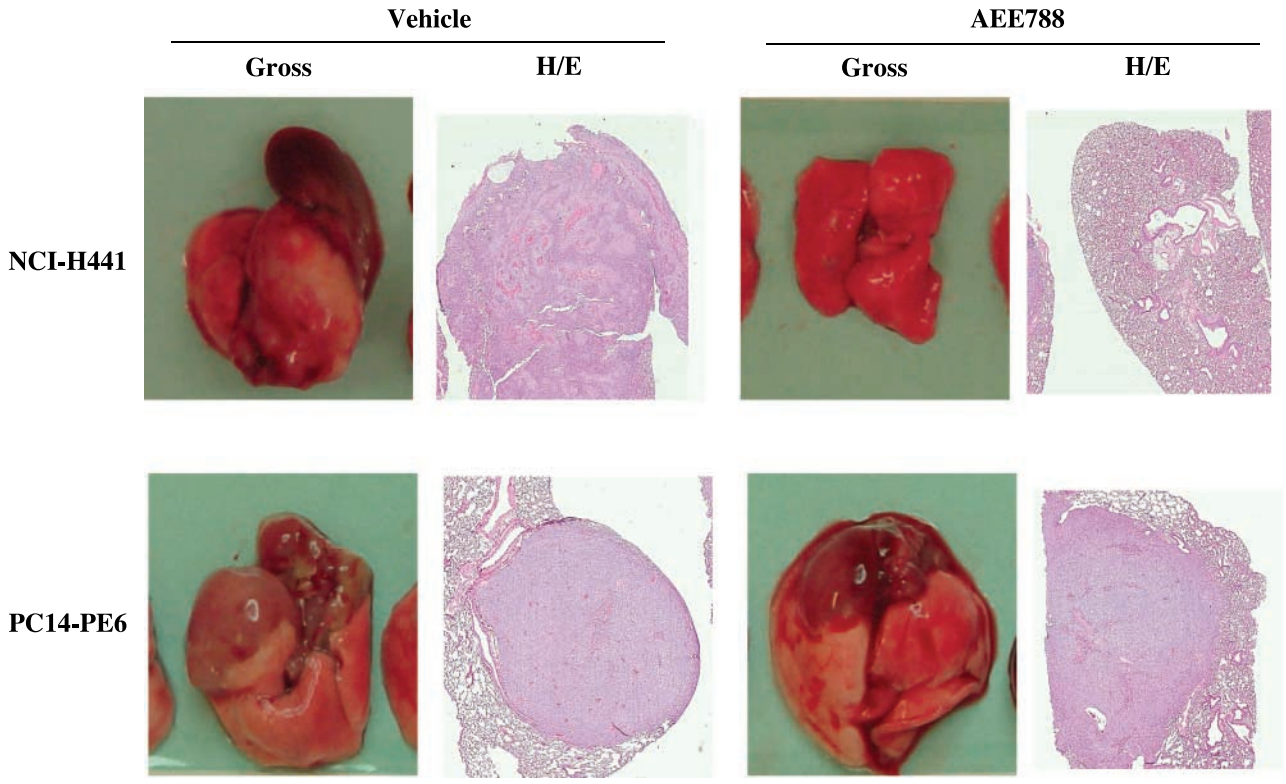

Figure 2. Effect of systemic administration of AEE788 on NCI-H441 or PC14-PE6 human lung adenocarcinomas growing orthotopically in the lungs of nude mice. NCI-H441 or PC14-PE6 cells (2.5×10^5) were injected into the left lobe of the lung. Treatment with AEE788 (50 mg/kg orally thrice weekly) or vehicle control was initiated 5 d after tumor injection. All mice were killed and autopsied 24 (NCI-H441) or 26 (PC14-PE6) days after tumor injection when mice in the control group became moribund and the lungs were removed. Representative gross lung specimens and H&E-stained histologic lung sections.

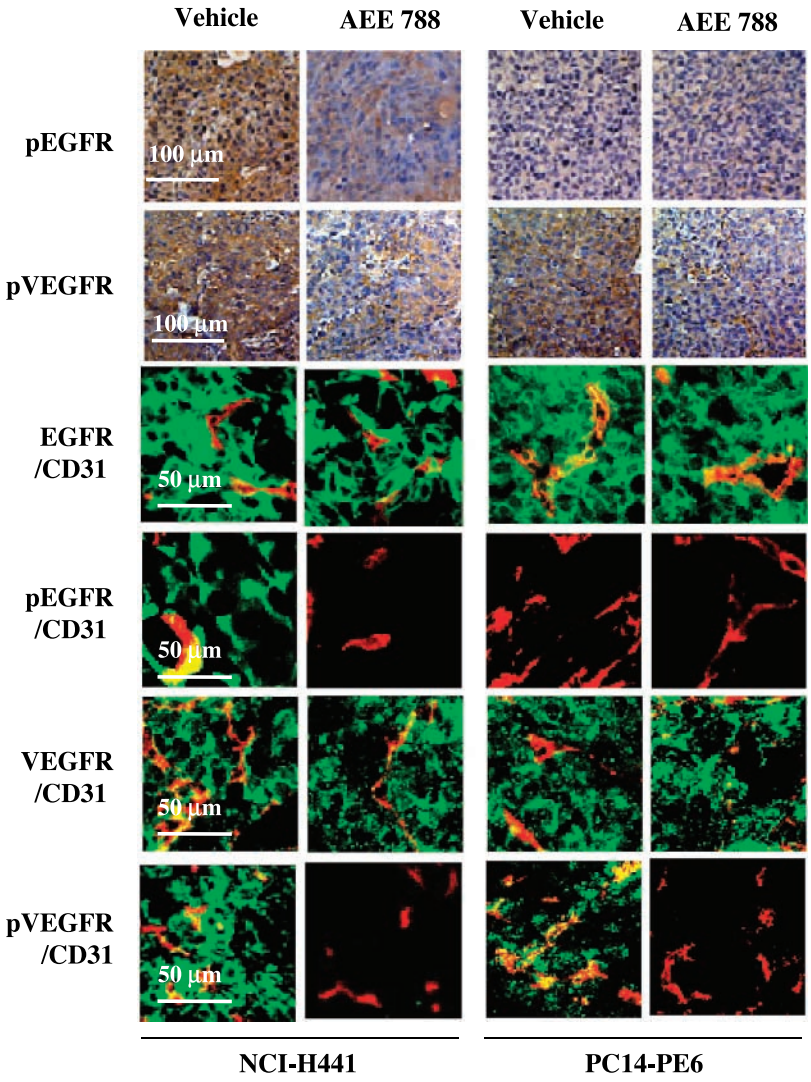

Figure 3. Activation of EGFR and VEGFR in lung tumor cells and expression and activation of EGFR and VEGFR in lung tumor-associated endothelial cells for NCI-H441 or PC14-PE6 lung tumors after treatment with AEE788 or vehicle control. Representative immunohistochemical images for tumor tissue sections for NCI-H441 or PC14-PE6 lung tumors. For the dual immunofluorescence studies, *red fluorescence* was used to identify endothelial cells (CD31) and *green fluorescence* was used to identify EGFR or VEGFR or phosphorylated EGFR or VEGFR (*pVEGFR*). *Yellow fluorescence* indicates endothelial cell expression (*EGFR/CD31* or *VEGFR/CD31*) or activation (*pEGFR/CD31* or *pVEGFR/CD31*) of EGFR or VEGFR, respectively.

metastasis to lymph nodes for both cell lines. Lung tumors derived from mice injected with PC14-PE6 or NCI-H441 lung tumors were subjected to immunohistochemical analyses for EGFR and its ligand EGF/TGF-α (Fig. 1C). EGFR was expressed by both NCI-H441 and PC14-PE6 lung tumors. EGF/TGF-α was markedly overexpressed in NCI-H441 lung tumors, whereas little or no expression of EGF/TGF-α was observed for PC14-PE6 lung tumors. For the NCI-H441 tumors, EGFR was expressed and activated by tumor cells and tumor-associated endothelial cells. In contrast, EGFR was expressed but not activated by tumor cells in PC14-PE6 lung adenocarcinomas and EGFR expression was not observed for tumor-associated endothelial cells for the PC14-PE6 lung tumors (Fig. 1C). These findings show that EGFR expression by tumor-associated endothelial cells is dependent on the presence of its ligand EGF/TGF-α that is secreted by tumor cells in the local microenvironment and the ligand is necessary for receptor activation by tumor and tumor-associated endothelial cells.

Differential Response of Orthotopic NCI-H441 and PC14-PE6 Human Lung Cancers to Inhibition of EGFR Phosphorylation by AEE788

To study the effects of EGFR antagonism on EGFR-positive lung cancers with varying expression of the EGFR ligand, we treated mice with AEE788 5 days after orthotopic implantation of NCI-H441 (EGFR positive and EGF/TGF-α negative) or PC14-PE6 (EGFR positive and EGF/TGF-α negative) human lung adenocarcinomas. AEE788 is a small molecule that primarily targets EGFR phosphorylation but also has additional reported activity against VEGFR phosphorylation (37). However, when administered on a thrice-weekly schedule, the drug selectively targets EGFR phosphorylation. Orally administered AEE788 significantly decreased lung tumor burden, incidence of tumors, pleural effusion, and metastasis to lymph nodes of NCI-H441 tumors compared with treatment with the vehicle control (Table 1; Fig. 2). In contrast, administration of AEE788 did not significantly affect lung tumor growth and progression for PC14-PE6 lung tumors compared with the control group

Table 2. Effects of systemic administration of gefitinib on NCI-H441 or PC14-PE6 human lung adenocarcinomas growing orthotopically in the lungs of nude mice

Group	Lung tumor			Pleural effusion (incidence)	Metastasis to lymph nodes (incidence)
	Body weight (g), median (range)	Incidence	Weight (g), median (range)		
NCI-H441					
Vehicle	28.1 (26.4-31.5)	10/10	0.37 (0.11-0.72)	9/10	10/10
Gefitinib	29.7 (27.5-36.8)	10/10	0.10 (0.04-0.24)*	2/10†	8/10
PC14-PE6					
Vehicle	30.5 (25.9-31.9)	8/8	0.22 (0.15-0.52)	5/8	7/8
Gefitinib	28.2 (27.0-31.3)	8/8	0.27 (0.16-0.71)	7/8	7/8

NOTE: NCI-H441 or PC14-PE6 cells (2.5×10^5) were injected into the left lobe of the lung. Treatment with gefitinib (50 mg/kg orally once daily) or vehicle control was initiated 5 days after tumor injection. All mice were killed and tumors were harvested 24 (NCI-H441) or 30 (PC14-PE6) days after tumor injection when mice in the control group became moribund. Lung tumor weight was determined by subtracting the normal lung weight (0.17 g) from the weight of the tumor-containing lung.

*$P < 0.01$, compared with vehicle control according to the χ^2 test (for incidence) and Student's two-tailed t test.

†$P < 0.05$, compared with vehicle control according to the χ^2 test (for incidence) and Student's two-tailed t test.

treated with vehicle alone (Table 1; Fig. 2). There was minimal toxicity and no difference in body weight between the control and treatment groups.

Lung tumor tissues from the mice bearing NCI-H441 or PC14-PE6 lung adenocarcinomas were obtained after treatment with AEE788 or vehicle control and subjected to immunohistochemical analyses for EGFR and VEGFR expression and activation (Fig. 3). EGFR was expressed in each of the tumor tissues studied but was only activated in the NCI-H441 tumor treated with vehicle control. The activation of EGFR in NCI-H441 lung tumors was blocked in mice treated with AEE788. Dual immunofluorescence studies for CD31 (endothelial cells) and total or phosphorylated EGFR and VEGFR were completed to characterize endothelial cell expression and activation of EGFR and VEGFR. EGFR was expressed by the tumor cells and tumor-associated endothelial cells for both NCI-H441 and PC14-PE6 tumors. Consequently, activation of EGFR in tumor-associated endothelial cells only occurred in the NCI-H441 tumors treated with vehicle control. The

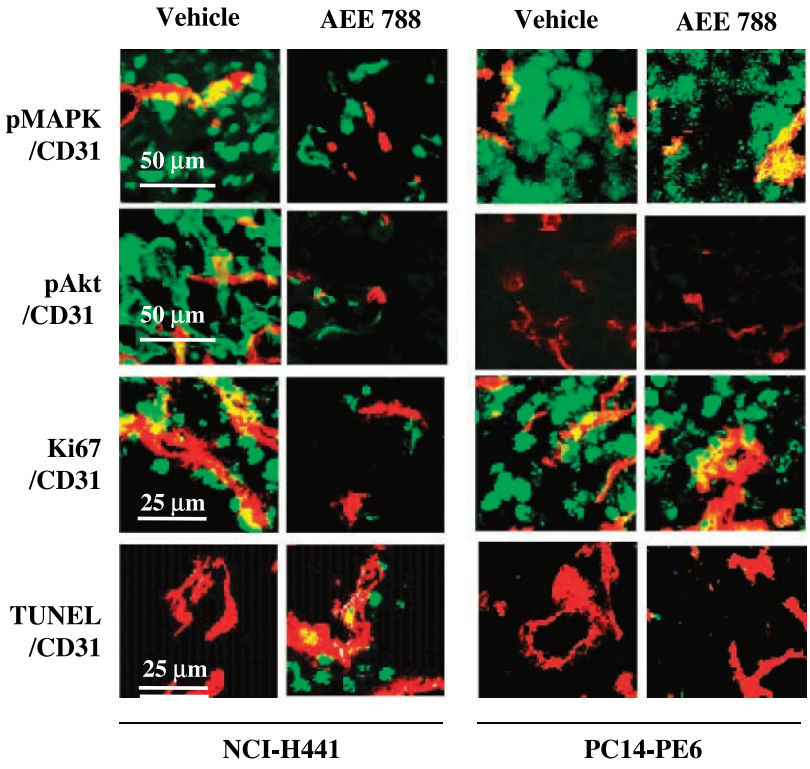

Figure 4. Activation of MAPK [phosphorylated MAPK (*pMAPK*)] and Akt [phosphorylated Akt (*pAkt*)] and proliferation (Ki67) and apoptosis (TUNEL) in lung tumor cells and lung-tumor associated endothelial cells (CD31) for NCI-H441 or PC14-PE6 lung tumors after treatment with AEE788 or vehicle control. Representative dual immunofluorescence immunohistochemical images for tumor tissue sections for NCI-H441 or PC14-PE6 lung tumors. *Red fluorescence* was used to identify endothelial cells (CD31) and *green fluorescence* was used to identify phosphorylated MAPK (*first row*), phosphorylated Akt (*second row*), proliferating cells (*third row*), or apoptotic cells (*last row*). *Yellow fluorescence* indicates endothelial cell activation of MAPK (*pMAPK/CD31*) or Akt (*pAkt/CD31*) or endothelial cell proliferation (*Ki67/CD31*) or apoptosis (*TUNEL/CD31*).

activation of EGFR in the vasculature of NCI-H441 lung tumors was blocked in mice treated with AEE788. Collectively, these data show that NCI-H441 lung adenocarcinomas are sensitive to treatment with AEE788, whereas PC14-PE6 lung adenocarcinomas are not and that this sensitivity correlates with EGFR activation status and ligand expression.

Differential Response of Orthotopic NCI-H441 and PC14-PE6 Human Lung Cancers to Inhibition of EGFR Phosphorylation by Gefitinib

To further study the effects of EGFR antagonism on EGFR-positive lung cancers with varying expression of the EGFR ligand, we evaluated the therapeutic effects of gefitinib (13), a more highly selective EGFR tyrosine kinase inhibitor, on the growth and spread of NCI-H441 and PC14-PE6 human lung cancer cells within the lung and thorax of mice after orthotopic injection. Gefitinib therapy was highly effective against NCI-H441 (EGFR positive and EGF/TGF-α positive) lung adenocarcinomas but was ineffective against PC14-PE6 (EGFR positive and EGF/TGF-α negative) lung adenocarcinomas (Table 2). Orally administered gefitinib significantly inhibited lung tumor growth and progression and prevented pleural effusion formation by NCI-H441 tumors when compared with treatment with vehicle but had no effect on the growth and progression of PC14-PE6 tumors. For the NCI-H441 tumors, gefitinib treatment was more effective in preventing lung tumor progression within the lung than on the progression of lymph node metastases within the mediastinum. When taken together with the findings for AEE788, these data further show that NCI-H441 lung adenocarcinomas are sensitive to EGFR antagonism, whereas PC14-PE6 lung adenocarcinomas are not and that this sensitivity correlates with EGFR activation status and ligand expression.

Analysis of EGFR Mutations in NCI-H441 and PC14-PE6 Cells and Orthotopic Lung Tumors

Specific mutations affecting the tyrosine kinase domains of EGFR are associated with sensitivity to small-molecule antagonists of EGFR (21, 22). To determine whether the differential response of NCI-H441 and PC14-PE6 cells to AEE788 is associated with EGFR mutations, we amplified and sequenced exons 18 to 22 of EGFR, which encode the tyrosine kinase domain, from NCI-H441 or PC14-PE6 genomic DNA. No EGFR mutations were found for either cell line (data not shown). These data indicate that the sensitivity of NCI-H441 lung tumors to the EGFR kinase inhibitors AEE788 and gefitinib is not associated with the previously identified EGFR mutations.

Analysis of EGFR Gene Copy Numbers in NCI-H441 and PC14-PE6 Cells and Orthotopic Lung Tumors

EGFR gene copy number can in some cases affect response to EGFR tyrosine kinase inhibition (23, 24). We performed fluorescence *in situ* hybridization analyses to determine the EGFR gene copy number in the NCI-H441, PC14-PE6, PC14-PE6/Neo, and PC14-PE6/TGF-α cell lines. All cell lines have trisomy (three copies in >10% of the cells, four or more copies in <10% of the cells) for chromosome 7. Based on EGFR gene copy number, the NCI-H441 cells, but not PC14-PE6 cells, have an amplification of the *EGFR* gene and exhibited innumerable tight clusters of signals in their nuclei (data not shown).

EGFR Blockade Impairs EGFR Signaling Pathways and Affects Proliferation and Apoptosis of Tumor and Tumor-Associated Endothelial Cells in NCI-H441 Lung Tumors but Not in PC14-PE6 Lung Tumors

To better assess the differential response of NCI-H441 and PC14-PE6 lung tumors to EGFR blockade, we used fluorescence double-labeling techniques to evaluate the effects of treatment with AEE788 on EGFR signaling pathways (Akt and MAPK phosphorylation) and on cell proliferation (Ki67) and apoptosis (TUNEL) for tumor and tumor-associated endothelial cells. MAPK and Akt activation were inhibited in both tumor and tumor-associated endothelial cells in the NCI-H441 tumors but not in the PC14-PE6 tumors (Fig. 4). Treatment with AEE788 suppressed the proliferation of tumor and tumor-associated endothelial cells for NCI-H441 tumors but not in PC14-PE6 tumors (Table 3; Fig. 4). The percentage of cell nuclei staining positively for Ki67, a marker for cell proliferation, in the NCI-H441 tumors was 48.8% ± 4.5% in the control group and only 9.4% ± 4.4% in the AEE788 treatment

Table 3. Effect of systemic administration of AEE788 on normal lung and on tumor and tumor-associated endothelial cell proliferation and apoptosis for NCI-H441 and PC14-PE6 tumors

Group	Adjacent normal lung		Tumor cells		Tumor-associated endothelial cells		
	% Ki67	% TUNEL	% Ki67	% TUNEL	CD31	% Ki67	% TUNEL
NCI-H441							
Vehicle	0.1 ± 0.3	0.1 ± 0.3	48.8 ± 4.5	0.6 ± 0.6	45 ± 4	8.2 ± 2.3	0.4 ± 1.0
AEE788	0.1 ± 0.2	0.1 ± 0.3	9.4 ± 4.4*	17.7 ± 2.0*	9 ± 2*	1.8 ± 2.7*	21.1 ± 6.8*
PC14-PE6							
Vehicle	0.3 ± 0.4	0.1 ± 0.3	50.0 ± 12.2	0.6 ± 0.3	36 ± 6	6.5 ± 1.9	0.5 ± 1.1
AEE788	0.1 ± 0.3	0.1 ± 0.3	46.4 ± 5.5	6.0 ± 0.9	34 ± 6	5.8 ± 1.3	0.6 ± 1.2

NOTE: % Ki67 is the ratio of the number of proliferating cells to total number of cells in 10 random ×200 fields. % TUNEL is the ratio of the number of apoptotic cells to total number of cells in 10 random ×200 fields. Data are presented as mean ± SE.
*$P < 0.01$, compared with vehicle control according to Student's two-tailed t test.

groups. In contrast, there was no significant difference in the tumor cell proliferation rate in the PC14-PE6 tumors (50% ± 12.2% in the control group and 46.4% ± 5.5% in the AEE788 treatment groups).

Treatment with AEE788 significantly increased the rate of apoptosis in both tumor and tumor-associated endothelial cells in NCI-H441 tumors but not in PC14-PE6 tumors as shown by the TUNEL assay and CD31 staining (Table 3; Fig. 4). There was no evidence of cellular proliferation or apoptosis of the endothelial cells in the adjacent normal lung tissues in any of the groups (Table 3). Additionally, treatment with AEE788 markedly reduced tumor vascularization, as determined by microvessel density, in NCI-H441 tumors but not in PC14-PE6 tumors (Table 3). Taken together, these results provide additional support for our hypothesis that activated EGFR in lung tumor and tumor-associated endothelial cells is sensitive to treatment with an EGFR tyrosine kinase inhibitor only when the ligand is expressed.

Activation of EGFR in PC14-PE6 Tumor Cells Transfected with TGF-α

To more precisely define the role of EGF/TGF-α in determining the response of lung tumors and the lung tumor vasculature to EGFR antagonism, we transfected the *TGF-α* gene into PC14-PE6 cells, which have low levels of TGF-α expression. Cells transfected with the empty cDNA vector served as controls. As shown in Fig. 5, TGF-α–transfected PC14-PE6 cells (PC14-PE6/TGF-α) secreted 350 pg of TGF-α per 10^6 cells every 48 h, whereas the control cells (PC14-PE6/Neo) secreted only 5 pg of TGF-α per 10^6 cells every 48 h (Fig. 5A). Analysis of activated EGFR protein levels by Western Blotting showed an increase in the PC14-PE16/TGF-α cells (data not shown).

We next injected the PC14-PE6/TGF-α or PC14-PE6/Neo cells into the lungs of nude mice. The resulting PC14-PE6/TGF-α lung tumors expressed higher levels of TGF-α than did the PC14-PE6/Neo lung tumors (Fig. 5B). The TGF-α levels produced by PC14-PE6/TGF-α tumors were substantially similar to the TGF-α levels observed for NCI-H441 lung tumors. EGFR was expressed by both the PC14-PE6/TGF-α and the PC14-PE6/Neo tumor cells but was only activated in the PC14-PE6/TGF-α tumors. No endothelial activation of EGFR was observed for the PC14-PE6/Neo tumors, whereas EGFR was both expressed and activated in the tumor-associated endothelial cells of the PC14-PE6/TGF-α lung tumors (Fig. 5B). These data confirm that the expression of the ligand for EGFR results in expression and activation of the receptor for the tumor and vascular components of lung tumors.

TGF-α Transfection of PC14-PE6 Lung Cancer Cells Makes Them Susceptible to EGFR Antagonism in an Orthotopic Lung Tumor Model

We next assessed the effects of TGF-α transfection of PC14-PE6 lung adenocarcinoma cells on their response to inhibition of EGFR activation by treatment with AEE788 after implantation into the lungs on mice. The left lungs of mice were injected with PC14-PE6/TGF-α or PC14-PE6/Neo lung cancer cells and, after 5 days of tumor growth,

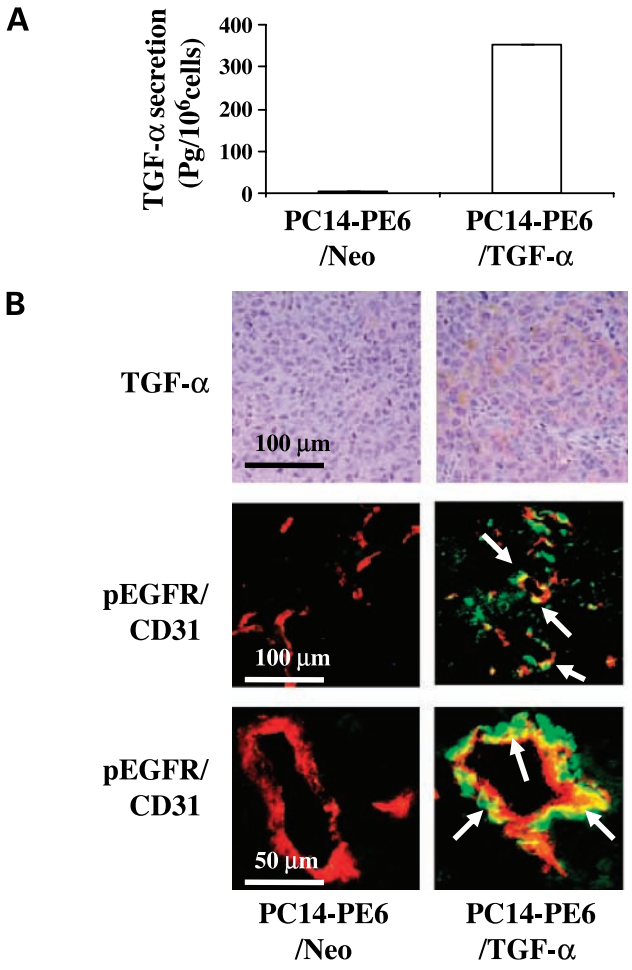

Figure 5. Expression of TGF-α by PC14-PE6 lung cancer cells transfected with TGF-α (PC14-PE6/TGF-α) or an empty vector (PC14-PE6/Neo) and activation of EGFR in lung tumor and tumor-associated endothelial cells. **A**, ELISA of *in vitro* TGF-α secretion by PC14-PE6 lung cancer cells transfected with TGF-α (PC14-PE6/TGFα) or an empty vector (PC14-PE6/Neo). **B**, representative immunohistochemical sections of primary lung tumors from PC14-PE6 lung cancer cells transfected with TGF-α (PC14-PE6/TGFα) or an empty vector (PC14-PE6/Neo). The sections were stained to detect TGF-α (*first row*; brown staining) expression or to detect endothelial cells (CD31) and/or activation of EGFR (phosphorylated EGFR). For the dual immunofluorescence studies (*second and last rows*), *red fluorescence* was used to identify endothelial cells (CD31) and *green fluorescence* was used to identify phosphorylated EGFR. *Yellow fluorescence* (*arrows*) indicates endothelial cell activation (*pEGFR/CD31*) of EGFR.

mice from each group were randomized to treatment with AEE788 or vehicle control. As for the wild-type cells, there was no effect of AE788 treatment on tumor growth, pleural effusion formation, or lymphatic metastasis for the PC14-PE6/Neo group (Table 4). In contrast, antagonism of EGFR signaling by AEE788 treatment blocked tumor growth, pleural effusion, and metastasis for the PC14-PE6/TGF-α tumors. These data show that the expression of the EGFR ligand TGF-α causes lung cancer cells to become susceptible to EGFR antagonism in the lung microenvironment.

Table 4. Effect of systemic administration of AEE788 on lung tumors derived from TGF-α–transfected or vector-transfected PC14-PE6 cells growing orthotopically in the lungs of nude mice

Group	Lung tumor			Pleural effusion (incidence)	Metastasis to lymph node (incidence)
	Body weight (g), median (range)	Incidence	Weight (g), median (range)		
PC14-PE6/Neo					
Vehicle	28.5 (22.6-33.2)	9/9	0.11 (0.01-0.43)	5/9	2/9
AEE788	28.3 (19.9-32.3)	9/9	0.11 (0.01-0.51)	3/9	1/9
PC14-PE6/TGF-α					
Vehicle	28.3 (24.0-31.5)	9/9	0.31 (0.06-0.85)	6/9	2/9
AEE788	27.8 (24.1-31.1)	9/9	0.16 (0.01-0.25)*	1/9*	1/9

NOTE: PC14-PE6/Neo or PC14-PE6/TGF-α cells (2.5×10^5) were injected into the left lobe of the lung. Treatment with AEE788 (50 mg/kg orally thrice weekly) or vehicle control was initiated 5 d after tumor injection. All mice were killed and tumors were harvested 30 days after tumor injection when mice in the control group became moribund. Lung tumor weight was determined by subtracting the normal lung weight (0.17 g) from the weight of the tumor-containing lung.
*$P < 0.05$, compared with vehicle control tumors according to the χ^2 test (for incidence) and Student's two-tailed t test.

Discussion

The effect of autocrine growth mechanisms of EGFR in driving malignant progression has been extensively studied (5). Activation of EGFR depends on the binding of its ligands (EGF and TGF-α) and subsequent dimerization with itself or with other Erb family members (including HER2, HER3, and HER4; ref. 40). Expression of EGF/TGF-α by tumor cells is associated with a more aggressive phenotype (41–43) and the treatment of tumors with antisense TGF-α or anti-TGF-α monoclonal antibodies can inhibit tumor growth *in vitro* and *in vivo* (44–46). A growing number of reports show that inhibition of EGFR signaling in tumor-associated endothelial cells significantly blocks tumor growth and metastasis, showing the importance of EGFR signaling to tumor vascularization and progression (30, 47).

In the current study, we found that the response of orthotopically growing human lung adenocarcinomas that express wild-type EGFR to treatment with EGFR tyrosine kinase inhibitors is determined by ligand (EGF/TGF-α) expression by tumor cells and the resultant activation of EGFR in both tumor and tumor-associated endothelial cells. EGFR in tumor-associated endothelial cells was only activated in tumors secreting EGF or TGF-α, such as those formed by NCI-H441 cells or TGF-α–transfected PC14-PE6 cells. Consequently, only these tumors responded to treatment with AEE788 or gefitinib. *In vitro*, EGFR in lung endothelial cells (MLECs) was activated by coculture with NCI-H441 tumor cells, which have high levels of EGF/TGF-α expression, but not by coculture with PC14-PE6 cells, which have low levels of EGF/TGF-α expression. These findings suggest that secretion of EGF or TGF-α by tumor cells in the lung microenvironment activates EGFR in tumor cells in an autocrine manner and in tumor-associated endothelial cells in a paracrine manner and thereby plays a crucial role in the response of tumors to EGFR antagonism. Our studies further reveal that antagonism of EGFR *in vitro* and *in vivo* significantly suppresses the activation of Akt and MAPK and that this suppression is associated with the inhibition of proliferation, invasion, and metastasis and the induction of apoptosis for tumor and tumor-associated endothelial cells in lung tumors that express EGF/TGF-α. Taken together, our studies provide compelling evidence to support the important role of paracrine activation of EGFR in tumor-associated endothelial cells in lung which then other tumors in mediating response to EGFR kinase inhibitors.

Recent studies suggest that EGFR gene copy number can be associated with response to EGFR agents in a subset of cases (23, 24). In our studies, NCI-H441 cells were EGFR gene amplified, whereas PC14-PE6 cells were not. Gene amplification did not correlate with enhanced sensitivity to EGFR inhibition *in vitro* (data not shown). Furthermore, transfection of the gene for TGF-α into PC14-PE6 lung cancer cells did not result in EGFR gene amplification but did make the cells susceptible to EGFR antagonism, showing that EGFR amplification was not necessary for the enhanced sensitivity. Thus, our results suggest that the activation of EGFR in the orthotopic lung tumors and tumor-associated endothelial cells is dependent on expression of EGF/TGF-α which then provided a sensitive target for EGFR kinase inhibition.

Previous investigations identified EGFR mutations, HER2 expression, and Akt activation as predictive markers for response to treatment with EGFR antagonists (20, 22, 48). Our data suggest that EGFR ligand expression (EGF or TGF-α) by tumor cells is also an important predictor of response to treatment with EGFR antagonists. Recent studies show that head and neck (49), colon (32), pancreatic (50), brain (51), prostate (52), and ovarian (53) tumors that express wild-type EGFR and EGF/TGF-α with activated EGFR in tumor-associated endothelial cells respond to treatment with AEE788 and that expression of TGF-α constitutively drives activation of EGFR in human pancreatic cancer cells and regulates their sensitivity to gefitinib (54). Thus, screening for both the expression of EGFR and its ligands in tumor cells and tumor-associated endothelial cells could become crucial when designing therapeutic strategies for lung cancers.

Endothelial cells are usually quiescent in normal tissues but undergo accelerated growth in clinical human lung cancer tumor specimens (55). This finding was supported and extended by our observation that the actively dividing tumor-associated endothelial cells of lung tumors expressed and activated EGFR and underwent apoptosis in response to treatment with AEE788, whereas the nondividing endothelial cells in tumor-adjacent lung tissues were not affected by AEE788 treatment. These differences between dividing tumor-associated endothelial cells and quiescent endothelial cells in adjacent normal tissue provide an opportunity for selective therapeutic intervention with EGFR inhibitors.

In summary, the secretion of EGF and TGF-α by NSCLC cells is necessary to activate EGFR in tumor and tumor-associated endothelial cells and EGFR signaling in tumor-associated endothelial cells and may well predict the response of human lung cancers to treatment with AEE788, gefitinib, and other EGFR kinase inhibitors. Our data confirm the importance of EGFR signaling for growth, vascularization, and metastasis of human lung cancer and suggest that the inhibition of EGFR signaling pathways in both tumor cells and tumor-associated endothelial cells can have therapeutic benefits. Future studies will seek to expand these findings to additional models of primary and metastatic lung cancer and will provide the basis for clinical studies in lung cancer patients.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43 – 66.

2. Weir HK, Thun MJ, Hankey BF, et al. Annual report to the nation on the status of cancer, 1975-2000, featuring the uses of surveillance data for cancer prevention and control. J Natl Cancer Inst 2003;95:1276 – 99.

3. Mountain CF, Lukeman JM, Hammar SP, et al. Lung cancer classification: the relationship of disease extent and cell type to survival in a clinical trials population. J Surg Oncol 1987;35:147 – 56.

4. 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;346:92 – 8.

5. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000;19:6550 – 65.

6. Ciardiello F, Bianco R, Damiano V, et al. Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. Clin Cancer Res 2000;6: 3739 – 47.

7. Marmor MD, Skaria KB, Yarden Y. Signal transduction and oncogenesis by ErbB/HER receptors. Int J Radiat Oncol Biol Phys 2004;58:903 – 13.

8. Ravindranath N, Wion D, Brachet P, Djakiew D. Epidermal growth factor modulates the expression of vascular endothelial growth factor in the human prostate. J Androl 2001;22:432 – 43.

9. Veale D, Kerr N, Gibson GJ, Harris AL. Characterization of epidermal growth factor receptor in primary human non-small cell lung cancer. Cancer Res 1989;49:1313 – 7.

10. Liu C, Tsao MS. *In vitro* and *in vivo* expressions of transforming growth factor-α and tyrosine kinase receptors in human non-small-cell lung carcinomas. Am J Pathol 1993;142:1155 – 62.

11. Rusch V, Klimstra D, Venkatraman E, Pisters PW, Langenfeld J, Dmitrovsky E. Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor α is frequent in resectable non-small cell lung cancer but does not predict tumor progression. Clin Cancer Res 1997;3:515 – 22.

12. 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;10:136 – 43.

13. Herbst RS, Fukuoka M, Baselga J. Gefitinib—a novel targeted approach to treating cancer. Nat Rev Cancer 2004;4:956 – 65.

14. Baselga J, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. J Clin Oncol 2005; 23:2445 – 59.

15. 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. JAMA 2003; 290:2149 – 58.

16. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353: 123 – 32.

17. Cappuzzo F, Gregorc V, Rossi E, et al. Gefitinib in pretreated non-small-cell lung cancer (NSCLC): analysis of efficacy and correlation with HER2 and epidermal growth factor receptor expression in locally advanced or metastatic NSCLC. J Clin Oncol 2003;21:2658 – 63.

18. Perez-Soler R, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. J Clin Oncol 2004;22:3238 – 47.

19. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. J Clin Oncol 2005;23: 2493 – 501.

20. 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;350:2129 – 39.

21. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304: 1497 – 500.

22. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. N Engl J Med 2005;353: 133 – 44.

23. Cappuzzo F, Varella-Garcia M, Shigematsu H, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. J Clin Oncol 2005;23:5007 – 18.

24. Daniele L, Macri L, Schena M, et al. Predicting gefitinib responsiveness in lung cancer by fluorescence *in situ* hybridization/chromogenic *in situ* hybridization analysis of EGFR and HER2 in biopsy and cytology specimens. Mol Cancer Ther 2007;6:1223 – 9.

25. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from ''never smokers'' and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306 – 11.

26. Marchetti A, Martella C, Felicioni L, et al. EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 2005;23:857 – 65.

27. Folkman J. What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 1990;82:4 – 6.

28. Ushijima C, Tsukamoto S, Yamazaki K, Yoshino I, Sugio K, Sugimachi K. High vascularity in the peripheral region of non-small cell lung cancer tissue is associated with tumor progression. Lung Cancer 2001;34:233 – 41.

29. Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. Mol Biol Cell 1993;4:121 – 33.

30. 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; 161:929 – 38.

31. Bruns CJ, Harbison MT, Davis DW, et al. Epidermal growth factor

receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. Clin Cancer Res 2000;6:1936 – 48.

32. Yokoi K, Thaker PH, Yazici S, et al. Dual inhibition of epidermal growth factor receptor and vascular endothelial growth factor receptor phosphorylation by AEE788 reduces growth and metastasis of human colon carcinoma in an orthotopic nude mouse model. Cancer Res 2005; 65:3716 – 25.

33. 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;9:5532 – 9.

34. Wu W, Onn A, Isobe T, et al. Targeted therapy of orthotopic human lung cancer by combined vascular endothelial growth factor and epidermal growth factor receptor signaling blockade. Mol Cancer Ther 2007;6:471 – 83.

35. 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.

36. Langley RR, Ramirez KM, Tsan RZ, Van Arsdall M, Nilsson MB, Fidler IJ. Tissue-specific microvascular endothelial cell lines from H-2K(b)-tsA58 mice for studies of angiogenesis and metastasis. Cancer Res 2003;63: 2971 – 6.

37. Traxler P, Allegrini PR, Brandt R, et al. AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. Cancer Res 2004;64:4931 – 41.

38. Clarke R, Brunner N, Katz D, et al. The effects of a constitutive expression of transforming growth factor-α on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. Mol Endocrinol 1989;3:372 – 80.

39. Langley RR, Fan D, Tsan RZ, et al. Activation of the platelet-derived growth factor-receptor enhances survival of murine bone endothelial cells. Cancer Res 2004;64:3727 – 30.

40. Heldin CH. Dimerization of cell surface receptors in signal transduction. Cell 1995;80:213 – 23.

41. De Jong KP, Stellema R, Karrenbeld A, et al. Clinical relevance of transforming growth factor α, epidermal growth factor receptor, p53, and Ki67 in colorectal liver metastases and corresponding primary tumors. Hepatology 1998;28:971 – 9.

42. Hamburger AW, Fernandes A, Murakami M, Gerwin BI. The role of transforming growth factor α production and ErbB-2 overexpression in induction of tumorigenicity of lung epithelial cells. Br J Cancer 1998;77: 1066 – 71.

43. Uhlman DL, Nguyen P, Manivel JC, et al. Epidermal growth factor receptor and transforming growth factor α expression in papillary and nonpapillary renal cell carcinoma: correlation with metastatic behavior and prognosis. Clin Cancer Res 1995;1:913 – 20.

44. Endo S, Zeng Q, Burke NA, et al. TGF-α antisense gene therapy inhibits head and neck squamous cell carcinoma growth *in vivo*. Gene Ther 2000;7:1906 – 14.

45. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. Proc Natl Acad Sci U S A 2000;97:4227 – 32.

46. Laird AD, Brown PI, Fausto N. Inhibition of tumor growth in liver epithelial cells transfected with a transforming growth factor α antisense gene. Cancer Res 1994;54:4224 – 32.

47. Baker CH, Pino MS, Fidler IJ. Phosphorylated epidermal growth factor receptor on tumor-associated endothelial cells in human renal cell carcinoma is a primary target for therapy by tyrosine kinase inhibitors. Neoplasia 2006;8:470 – 6.

48. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2004;96:1133 – 41.

49. Yigitbasi OG, Younes MN, Doan D, et al. Tumor cell and endothelial cell therapy of oral cancer by dual tyrosine kinase receptor blockade. Cancer Res 2004;64:7977 – 84.

50. Yokoi K, Kim SJ, Thaker P, et al. Induction of apoptosis in tumor-associated endothelial cells and therapy of orthotopic human pancreatic carcinoma in nude mice. Neoplasia 2005;7:696 – 704.

51. Goudar RK, Shi Q, Hjelmeland MD, et al. Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition. Mol Cancer Ther 2005;4:101 – 12.

52. Yazici S, Kim SJ, Busby JE, et al. Dual inhibition of the epidermal growth factor and vascular endothelial growth factor phosphorylation for antivascular therapy of human prostate cancer in the prostate of nude mice. Prostate 2005;65:203 – 15.

53. Thaker PH, Yazici S, Nilsson MB, et al. Antivascular therapy for orthotopic human ovarian carcinoma through blockade of the vascular endothelial growth factor and epidermal growth factor receptors. Clin Cancer Res 2005;11:4923 – 33.

54. Pino MS, Shrader M, Baker CH, et al. Transforming growth factor α expression drives constitutive epidermal growth factor receptor pathway activation and sensitivity to gefitinib (Iressa) in human pancreatic cancer cell lines. Cancer Res 2006;66:3802 – 12.

55. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res 2000; 60:1388 – 93.