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Chemopreventive Effects of Gefitinib on Nonsmoking-Related Lung Tumorigenesis in Activating Epidermal Growth Factor Receptor Transgenic Mice

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Abstract

Twenty-five percent of all lung cancer cases are not attributable to smoking. Epidermal growth factor receptor (EGFR) mutations, which are involved in ~50% of nonsmoker lung cancer, are positively correlated with responsiveness to gefitinib, and inversely correlated with smoking history. Activating *EGFR* mutations play a critical role in the carcinogenesis of nonsmoking-related lung cancer. To investigate the chemopreventive effects of gefitinib on nonsmoking-related lung cancer, we generated transgenic mice expressing EGFR L858R in type II pneumocytes constitutively using the surfactant protein-C promoter. The transgenic mice invariably developed atypical adenomatous hyperplasia at age 4 weeks and multifocal adenocarcinoma of varying sizes at age 7 weeks. Notably, the expression levels of phosphorylated and total ErbB2, ErbB3, and thyroid transcription factor-1 were elevated in the transgenic mice compared with wild-type controls at age 3 weeks. Administration of gefitinib to 3-week-old transgenic mice for 1 week before carcinogenesis reduced the amount of phosphorylated EGFR in the lungs of the mice to the baseline level. Gefitinib (5 mg/kg/d; n = 5, 5, and 15) or vehicle (n = 5, 5, and 15) was administered to transgenic mice from age 3 to 8, 13, and 18 weeks, respectively. The numbers of lung tumors in the control and gefitinib-treated groups were 1.75, 5.8, 10.2, and 0 ($P < 0.05$), respectively. No fatal toxic events occurred in either group, and gefitinib inhibited tumorigenesis completely in this mouse model. These results suggest the utility of molecular targeted chemoprevention against nonsmoking-related lung cancer. [Cancer Res 2009;69(17):7088–95]

Introduction

Age-adjusted mortality rates for lung cancer have begun to decline in the United States and Japan due to intensive antismoking campaigns (1). However, an estimated 15% of men and 53% of women with lung cancer worldwide are lifelong nonsmokers (2). In Japan, ~30% of the male and 70% of the female patients with lung cancer were found to have no history of smoking (3). In addition, ethnically Asian people are relatively more resistant to the carcinogenic effects of tobacco smoke compared with African Americans or Caucasians (4). Note that lung cancer in lifelong nonsmokers is the seventh most common cause of cancer-related death worldwide, ahead of cervical, pancreatic, and prostate cancers (5). Consequently, nonsmoking-related lung cancer will become an increasingly important issue in the future.

Recent advances in lung cancer research have raised the possibility of a molecular-based method for classifying tumors [e.g., epidermal growth factor receptor (*EGFR*) and *KRAS* mutation-related lung cancers; ref. 6] that could replace the older WHO/International Association Society of Lung Cancer system, which is based on tumor morphology. Tumor biology and pathogenesis are reportedly different between smoking- and nonsmoking-related lung cancers (7).

To date, most chemopreventive studies of lung cancer have targeted factors that were related to smoking. In addition, no clinical trials or preclinical studies have investigated specific molecules associated with the pathogenesis of lung cancer (8, 9). Preclinical studies generally rely on mice sensitive to the carcinogens in tobacco smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[*a*] pyrene, as a smoking-related lung cancer model, and the majority of tumors in these mice contain point mutations in *K-ras* (10–12). We and other groups have achieved major, and sometimes complete, success in preventing smoking-related tumorigenesis using chemopreventive agents such as epigallocatechin gallate and cyclooxygenase-2 inhibitors (13–15). Similar results have been obtained using gefitinib, an EGFR tyrosine kinase inhibitor (TKI; refs. 16, 17); however, the inhibitory effect was weaker than expected because EGFR is not closely associated with tumorigenic signaling in smoking-related lung cancer (16, 17). In contrast, no previous chemopreventive studies have reported on nonsmoking-related lung cancer.

In patients with lung cancer, the presence of *EGFR* mutations is strongly associated with a history of never smoking cigarettes (18–20), Asian ethnicity, and female gender (20, 21). In addition, transfection with mutated *EGFR* can transform NIH3T3 or HBECS cells to show EGF-independent growth (22, 23), but wild-type *EGFR* shows no such effect (24). Consistent with these observations, we and two other groups revealed the essential role of *EGFR* mutations in lung carcinogenesis using transgenic mice. Specifically, activating *EGFR* mutations in the mice induced lung adenocarcinoma, which up-regulated EGFR signaling (25–27); however, wild-type *EGFR* induced only minor pathologic changes in the lungs of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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mice (25). Thus, activating *EGFR* mutations appear to play a critical role in the carcinogenesis of nonsmoking-related lung cancer. Approximately 90% of the activating *EGFR* mutations identified in these studies involved deletions in exon 19 and the substitution of arginine for leucine at codon 858 (L858R; refs. 6, 20). These mutations were also positively correlated with an improved clinical response to treatment with gefitinib or erlotinib, a reversible EGFR TKI (28, 29). The binding strength of gefitinib to the ATP-binding pocket in EGFR is ~20-fold higher for mutated EGFR L858R than for wild-type EGFR; in contrast, the binding of ATP to the ATP-binding pocket in EGFR is 5-fold lower for mutated EGFR than for wild-type EGFR. Consequently, gefitinib inhibits the activity of the kinase more strongly and efficiently in mutated EGFR than in wild-type EGFR (30). Therefore, activating *EGFR* mutations may be excellent molecular targets for chemoprevention of nonsmoking-related lung cancer.

Here, we report the establishment of a nonsmoking-related lung cancer mouse model for use in chemopreventive studies. In this model, mice expressing EGFR L858R died due to lung tumors at age ~30 weeks if left untreated with gefitinib. Using this model, we analyzed the molecular targeted chemopreventive effect of gefitinib on tumorigenesis in nonsmoking-related lung cancer.

Materials and Methods

Production of transgenic mice. To generate mice constitutively expressing mutant human EGFR in the lung, we used the *surfactant protein-C* promoter, which is active only in type II alveolar epithelial cells (31). The plasmid containing the *surfactant protein-C* promoter and SV40 small T intron and polyadenylation sequence was described previously (31). Full-length cDNA encoding human *EGFR* tagged at the COOH-terminal end with FLAG was inserted into the plasmid between the *surfactant protein-C* promoter and SV40 small T intron. The entire sequence of the construct was subsequently confirmed by sequencing. Next, a point mutation, L858R, was introduced using a QuikChange XL kit (Stratagene) in accordance with the manufacturer's instructions. The *EGFR* expression cassette was then excised from the parental vector using *Hind* III and *Not* I and injected into fertilized C57BL/6Cr eggs (Japan SLC).

Animal husbandry and gefitinib treatment. All animals were kept under pathogen-free conditions and provided with food and water *ad libitum* as specified in the guidelines of the Department of Animal Resources of Okayama University Advanced Science Research Center. Gefitinib kindly provided by AstraZeneca UK was given once a day, 5 days/wk, by gavage as a 5 mg/kg suspension. The suspension was prepared in 1% polysorbate 80 by homogenization and ball-milled with glass beads for 24 h. All procedures were done in accordance with institutional guidelines for the care and use of experimental animals.

PCR genotyping. Genomic DNA was isolated from the tails of the mice using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocol. Transgenic lines were identified by PCR using primers specific for human *EGFR* (human EGFR-F1 5′-GGCACGGTGTAAGGGACTC-3′ and human EGFR-R1 5′-CAAACGGTCACCCCGTAGCTCCAGA-3′). The amplified products were resolved by electrophoresis on 1.6% agarose gels.

Histology and immunohistochemistry. The animals were killed by cervical dislocation, and the lungs were then excised. The left lung was flash-frozen in liquid nitrogen for molecular analysis, and the right lung was inflated with 10% paraformaldehyde in PBS. The right lung was stained with each antibody as described previously (27). Briefly, sections were incubated with the primary antibody (1:50 dilution) at 4°C for 12 h followed by 30 min incubation with EnVision+ System–labeled polymer-horseradish peroxidase anti-rabbit (DAKO). Sections were stained with 3,3′-diaminobenzidine chromogen and counterstained with Mayer's hematoxylin. Rabbit polyclonal anti-EGFR antibodies (Santa Cruz Biotechnology) and anti-phosphorylated EGFR Y1068, phosphorylated Akt, total Akt, phosphorylated mitogen-activated protein kinase (MAPK), and total MAPK (Cell Signaling Technology) were used as the primary antibodies. Proliferating cell nuclear antigen (PCNA) was detected using a PCNA Staining kit (Zymed Laboratories).

Real-time PCR and quantitative PCR analysis. RNA samples were prepared using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol, and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Primers human EGFR-F1 and human EGFR-R1 were then used to specifically amplify the mutant human *EGFR*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as a control using primers GAPDH-F (5′-CGTAGACAAAATGGTGAAGG-3′) and GAPDH-R (5′-GTTGTCATGGATGACCTTGG-3′). Quantitative PCR was done by monitoring in real-time the increase in fluorescence of SYBR Green dye (Qiagen) with an ABI 5700 sequence detection system (Applied Biosystems). Amplification of the total *EGFR* transcript level and copy number including both the endogenous mouse *EGFR* and the mutant human *EGFR* were determined by quantitative real-time PCR (RT-PCR) using the following primers having a highly similar sequence to amplify the exon 15 region from both human and mouse *EGFR* loci as described by Ji and colleagues (26). *GAPDH* was coamplified in the same reaction mixture as an endogenous reference gene using GAPDH. The average level of *EGFR* expression and the copy number were determined from the differences in the threshold amplification cycles between *EGFR* and *GAPDH*.

Immunoblotting and immunoprecipitation. Proteins were extracted and transferred as described previously (27). Briefly after quantification by a protein assay (Bio-Rad), ~50 μg aliquots of each extract were separated by SDS-PAGE using precast 5% to 15% gels (Bio-Rad) and transferred onto nitrocellulose membranes. For immunoprecipitation, the cells were lysed in 0.1% Triton [0.1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate containing protease inhibitors], subsequently sonicated, and incubated with an appropriate antibody overnight at 4°C. The immunoprecipitates were collected using anti-FLAG affinity gel (Sigma). Specific proteins were detected by enhanced chemiluminescence (GE Healthcare) using antibodies against the following: phosphorylated EGFR Y1068, total EGFR, phosphorylated Akt, total Akt, phosphorylated MAPK, total MAPK, pMet, phosphorylated insulin-like growth factor-I receptor (IGF-IR), and total IGF-IR (1:1,000 dilution; Cell Signaling Technology); phosphorylated ErbB2 and phosphorylated ErbB3 (1:1,000 dilution; Cell Signaling Technology); ErbB2 and ErbB3 (1:1,000 dilution; Upstate Biotechnology); total Met and thyroid transcription factor-1 (TTF-1; 1:200 dilution; Santa Cruz Biotechnology); actin (1:5,000 dilution; Chemicon); and FLAG (1:1,000 dilution; Sigma). The secondary antibodies were anti-rabbit IgG (horseradish peroxidase–linked, species-specific whole antibody) and anti-mouse IgG (horseradish peroxidase–linked, species-specific whole antibody; GE Healthcare), both of which were used at a 1:5,000 dilution.

Experimental design. Fifty 3-week-old transgenic mice were divided into two groups treated with either gefitinib ($n = 25$) or vehicle ($n = 25$) for 5, 10, and 15 weeks. At age 8, 13, and 18 weeks, 5, 5, and 15 mice, respectively, were killed in each group. The diameter of superficial lung tumors was measured with an Absolute Coolant Proof Caliper (Mitutoyo) using a megaloscope and the tumor number of the left lung per mouse (long axis exceeding 1 mm) was counted.

Statistical analysis. Tumor incidence and proportions of EGFR- and PCNA-positive cells were analyzed by the Mann-Whitney test. Changes in body weight were analyzed by paired t test. Statistical significance was defined as $P < 0.05$. All statistical analyses were carried out using SPSS version 10.0 (SPSS).

Results

Production of transgenic mice. We mutated a single nucleotide in human *EGFR* known to be substituted in patients with lung cancer (EGFR L858R) to generate a mutant *EGFR-FLAG* construct consisting of the *surfactant protein-C* promoter, open reading frame of the mutated *EGFR-FLAG*, SV40 small T intron, and SV40 polyadenylation signal (Fig. 1*A*). Following injection of

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the construct into fertilized eggs, 27 pups were born and examined for integration of the transgene by PCR using tail genomic DNA. Of these, eight mice were positive and two (3-6 and 4-3) later developed lung adenocarcinoma. Subsequently, lines 3-6 and 4-3 produced offspring, each of which developed lung tumors at age ≥7 weeks. However, transgenic line 3-6 mice were lost because of mating error. No lung tumors were detected in any of the other mice until age 30 weeks. We attempted to produce transgenic mice expressing human EGFR delE746-A750 at the same time but failed to generate a founder.

Analysis of copy number and expression of the transgene. RT-PCR using tail genomic DNA from heterozygous transgenic mice showed an ~1.5- to 3-fold greater expression of the transgene compared with endogenous *Egfr* in mice of transgenic lines 3-6 and 4-3 (Fig. 1*B*). Immunoblotting using multiple tissues from 7-week-old transgenic (line 4-3) and control mice using anti-FLAG antibodies indicated that mutant *EGFR* expression was restricted to the lung tissue of the transgenic mice (Fig. 1C, *a*). In addition, the presence of the point mutation in the lung tissue of the transgenic mice was confirmed by direct sequencing of the RT-PCR products (Fig. 1*C*, *b*).

We then evaluated EGFR expression in the lungs of 3-week-old transgenic mice showing no overt signs of lung tumors by Western blotting. The level of EGFR expression in the lungs of the transgenic mice was significantly higher than that in the lungs of the control mice (Fig. 1*D*, *a*). We also evaluated the levels of phosphorylated and total ErbB2, ErbB3, Met, IGF-IR, Akt, MAPK, and TTF-1 in the same manner. The transgenic mice expressed more phosphorylated ErbB2, ErbB3, Akt, MAPK, and TTF-1 in their lungs than did the control mice, but Met and IGF-IR was not activated in the transgenic mice compared with controls (Fig. 1*D*, *a*). In addition, we consistently detected numerous alveolar epithelial cells expressing high levels of EGFR in the lungs of the 3-week-old transgenic mice by immunohistochemistry (Supplementary Fig. S1*A*). The expression levels of *EGFR* mRNA were examined by SYBR Green RT-PCR. The transgenic lines 3-6 and 4-3 mice with multiple lung tumors expressed 10 to 30 times more *EGFR* in their lungs than the normal nontransgenic controls (Fig. 1*D*, *b*). These observations suggest an increase in the number of cells expressing mutated *EGFR* during tumor development.

Histology of the lungs of the transgenic mice. To determine the oncogenic effect of the activating *EGFR* mutation in the lungs,

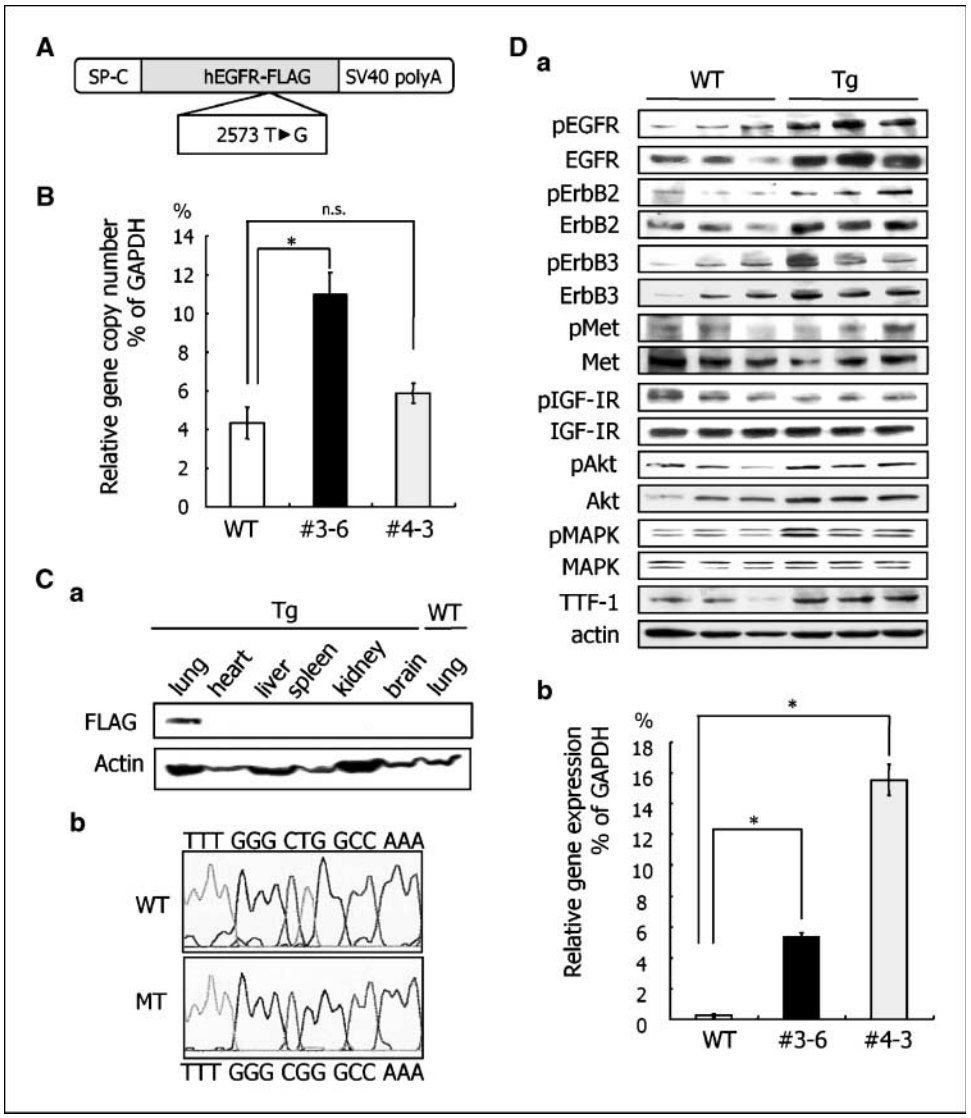

Figure 1. Generation of transgenic mice carrying mutated *EGFR* (*EGFR*^{L858R}) and analyses of the transgene copy number and expression. *A,* construction of the transgenic human *EGFR*^{L858R} mice. *B, EGFR* copy number in the lungs of wild-type (*WT*) and transgenic mice. *Bars,* SE. *, $P < 0.05$; n.s., not significant. *C, a,* lung tissue-specific expression of the transgene; *b,* direct sequencing of the RT-PCR products from the lungs of wild-type (*top*) and mutant (*MT*; *bottom*) *EGFR* mice. *D, a,* Western blotting for phosphorylated and total EGFR, ErbB2, ErbB3, Akt, MAPK, Met, IGF-IR, and TTF-1 expression in the lungs of 3-week-old tumor-free transgenic (*Tg*) and control mice; *b, EGFR* mRNA expression in the lungs of the transgenic mice with lung cancer and the control mice by SYBR Green RT-PCR. *Bars,* SE. *, $P < 0.05$.

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transgenic mice from line 4-3 and control mice were killed at various time points for pathologic examination. Histologic changes consistent with human bronchioloalveolar carcinoma were observed at age 4 to 5 weeks (Supplementary Fig. S1*D*, *c* and *d*). At age 7 weeks, we observed adenocarcinoma with solid features similar to human adenocarcinoma (Supplementary Fig. S1*D*, *e* and *f*), and the tumors continued to progress (Supplementary Fig. S1*D*, *g* and *h*). The tumors showed strong immunoreactivity with anti-total and phosphorylated EGFR, Akt, and MAPK antibodies (Supplementary Fig. S1*A* and *C*). Ultimately, the mice died at age ~30 weeks if left untreated with gefitinib. Macroscopically, we found multiple nodules on the lung surface (Supplementary Fig. S1*D*, *i*), although metastatic lesions were not detected in any other organ. In marked contrast, no lung tumors developed in the control mice, and all of the controls survived for >1 year.

To test for increased cellular proliferation caused by the transgene, we used PCNA staining in successive specimens taken from the lungs of the transgenic mice. No significant differences were observed in the number of PCNA-positive cells between wild-type and transgenic mice before lung tumor development (age 3 weeks; Supplementary Fig. S1*B*, *a* and *b*; data not shown). However, beginning at 4 weeks, the number of PCNA-positive cells, most of which seemed to be tumor cells, markedly increased in the transgenic mice (Supplementary Fig. S1*B*, *c* and *d*). Moreover, the tumors observed at 15 weeks were highly positive for PCNA (Supplementary Fig. S1*B*, *e* and *f*).

Crosstalk of ectopic human EGFR with endogenous signaling protein in mice. Immunoprecipitation by the FLAG protein tagged in human EGFR at the COOH-terminal end using lung tissue from 7-week-old transgenic with or without gefitinib treatment indicated that ectopic human EGFR colocalized with endogenous ErbB2, ErbB3, and p85, a subunit of phosphoinositide 3-kinase (Supplementary Fig. S2). These results suggested that crosstalk between ectopic human EGFR and endogenous signaling proteins may occur in the transgenic mice.

Chemopreventive effect of gefitinib on activating *EGFR* mutation-induced lung adenocarcinoma. We first investigated the inhibitory effects of gefitinib on EGFR phosphorylation in lung tissue taken from the transgenic mice before tumor formation. Gefitinib or vehicle alone was administered to transgenic mice at age 3 weeks by gavage as a 5 mg/kg suspension for 5 days. Staining with H&E versus that with PCNA showed no significant difference; however, staining for EGFR indicated that the level of pneumocyte expression of EGFR was slightly higher in the control group than in the gefitinib-treated group (Fig. 2*A*). In addition, Western blotting confirmed that the levels of total and phosphorylated EGFR were greater in the lungs of the transgenic mice than in those of the wild-type mice and that gefitinib reduced the phosphorylation of EGFR before carcinogenesis (Fig. 2*B*).

We next investigated the effects of gefitinib on tumor number, results of histologic analysis of the lung, and body weight in 3-week-old transgenic mice treated with gefitinib or vehicle alone for 5 weeks (n = 5), 10 weeks (n = 5), and 15 weeks (n = 15), respectively. The numbers ± SE of superficial left lung tumors with a long axis exceeding 1 mm in the control and gefitinib-treated groups were 1.75 ± 1.5 and 0 (P < 0.05), 5.8 ± 2.17 and 0 (P < 0.005), and 10.2 ± 2.44 and 0 (P < 0.001), respectively (Mann-Whitney test; Fig. 3*A*). Gefitinib prevented lung tumorigenesis completely. When treated with gefitinib, lung tumors were not detected either macroscopically (Fig. 3*A*) or histologically (Fig. 3*B*) at three points.

The body weight of each animal was determined every week, but no significant difference was detected between the two groups

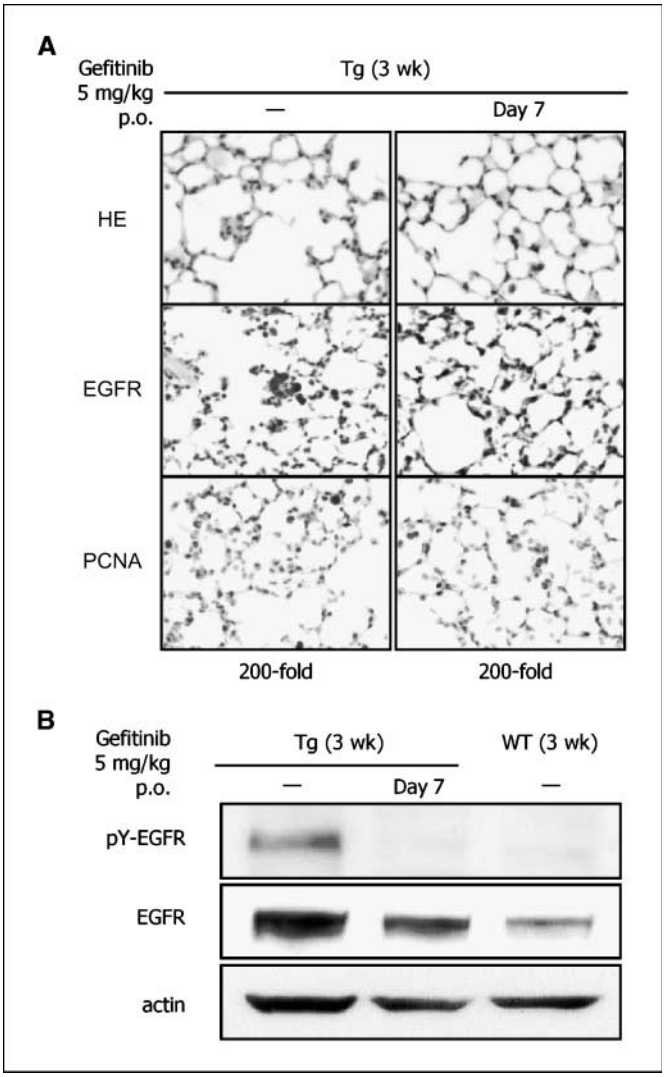

Figure 2. Inhibitory effect of gefitinib on EGFR phosphorylation in the transgenic mice before tumor formation. *A,* lungs of 3-week-old gefitinib- and vehicle-treated transgenic mice were examined by H&E, EGFR, and PCNA staining. *B,* levels of total and phosphorylated EGFR in the lungs of the transgenic mice treated with or without gefitinib and wild-type controls by Western blotting.

(P = 0.971, paired t test; Fig. 3*C*). We found no skin rash, diarrhea, or interstitial lung disease in either group.

We next used EGFR and PCNA immunostaining to analyze the lungs of the animals in the two groups. Multiple tumors with strongly EGFR- and PCNA-positive cells were detected in the control group, whereas largely normal tissues with weakly EGFR- and PCNA-positive cells were seen in the gefitinib group (Fig. 4*A*). That gefitinib reduced the level of EGFR phosphorylation in the gefitinib group was confirmed by Western blotting (Fig. 4*C*). EGFR- and PCNA-positive and EGFR- and PCNA-negative cells were counted 10 times (1,000-fold view fields) in five transgenic mice and the ratio of EGFR- and PCNA-positive cells (number of EGFR- and PCNA-positive cells / number of cells counted × 100) was calculated. The ratios ± SE of EGFR- and PCNA-positive cells in the gefitinib group versus the control group were 48.6 ± 1.5% versus 4.7 ± 0.72% (P < 0.001, Mann-Whitney test) and 72.3 ± 1.77% versus 30.7 ± 1.81% (P < 0.001, Mann-Whitney test), respectively. EGFR- and PCNA-positive cell numbers decreased

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significantly in the gefitinib group compared with the control group (Fig. 4*B*).

Finally, we investigated whether the effect of gefitinib was temporary in transgenic mice treated with gefitinib for 15 weeks after a 1-week follow-up period ($n = 3$). Tumors were detected in the lungs of the mice, similar to the control transgenic mice. Notably, at 1 week after stopping gefitinib treatment, we detected the reexpansion of the tumor cells (data not shown) because gefitinib is a reversible EGFR TKI; however, other growth signals may be associated with regrowth. Recently, Met and/or IGF-IR activation were reported as the reasons for acquired resistance to gefitinib (32, 33). In these transgenic mice treated with 5 mg/kg gefitinib for 15 weeks (Fig. 4*D*) and then discontinued gefitinib for 1 week, Met, IGF-IR, and ErbB3 status were not activated (data not shown).

Discussion

In the present study, we found that gefitinib completely inhibited tumorigenesis in EGFR L858R transgenic mice. This nonsmoking-related lung cancer mouse model is the first chemopreventive paradigm that does not involve induction of tumorigenesis by specific carcinogens found in tobacco smoke. In addition, our results showed that a molecular targeted agent can inhibit specific oncogene-induced carcinogenesis completely.

Human EGFR was overexpressed, but ectopic human EGFR could show crosstalk with endogenous ErbB2, ErbB3, and p85 and also activate Akt or MAPK in the lungs of the transgenic mice. Constitutive human EGFR activation could induce lung adenocarcinoma in the mice because EGFR inhibition resulted in complete tumor prevention. Sato and colleagues reported that the *EGFR* mutation alone was not sufficient for acquisition of fully malignant phenotype (23). Our transgenic mice showed no distant metastasis in other organs (e.g., brain and liver) when mice died due to lung tumors at ~30 weeks. These observations suggest that activating *EGFR* mutations play a critical role in tumorigenesis, although further genetic alterations were needed to obtain the fully malignant phenotype (34). The majority of patients with lung cancer having activating *EGFR* mutations also show high copy numbers of *EGFR*

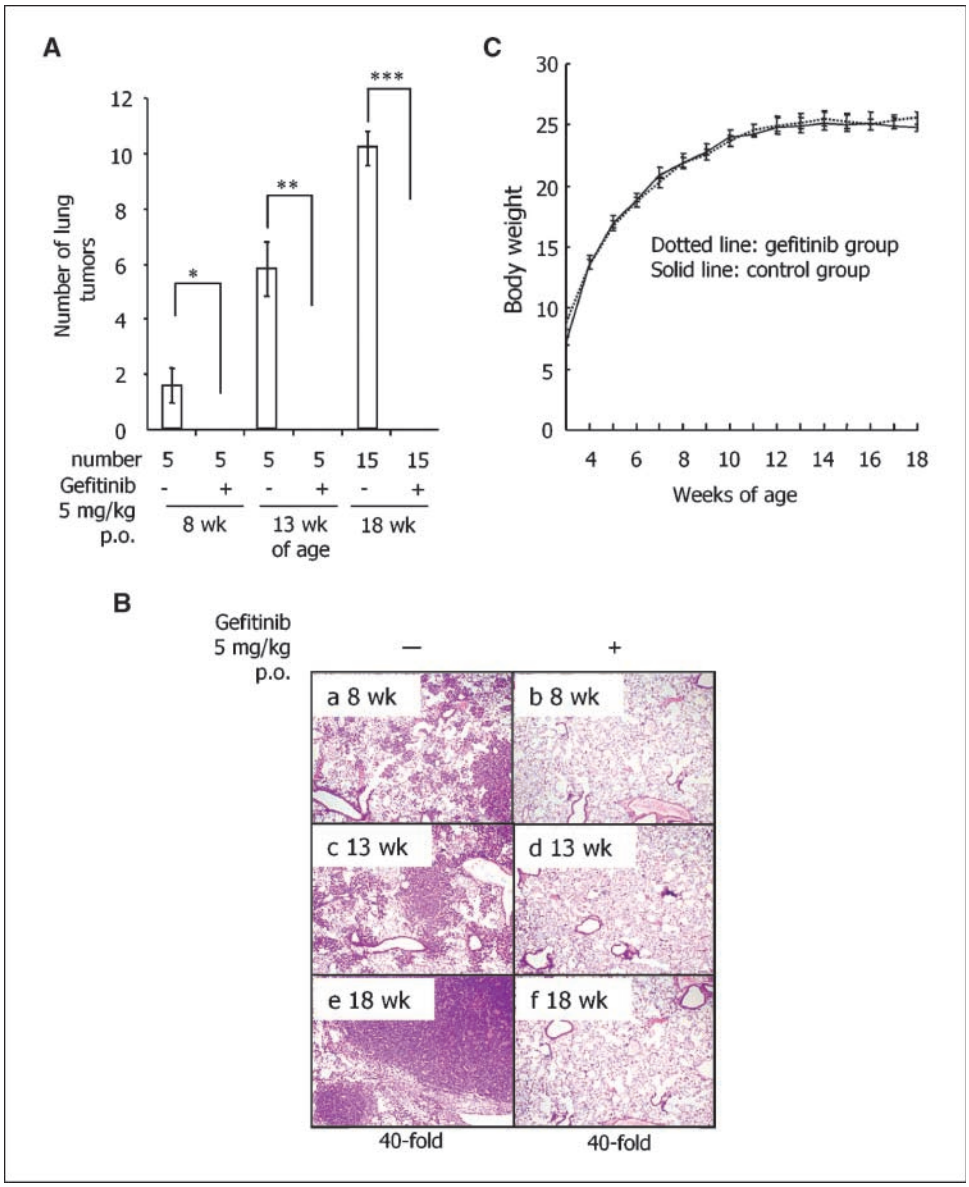

Figure 3. Chemopreventive effects of gefitinib on tumor number and body weight in the transgenic mice. *A,* 3-week-old transgenic mice were treated with gefitinib ($n = 5$, 5, and 15) or vehicle alone ($n = 5$, 5, and 15) for 5, 10, and 15 wk, respectively. The numbers ± SE of superficial left lung tumors with a long axis exceeding 1 mm in the control and gefitinib-treated groups were 1.75 ± 1.5 and 0 (*, $P < 0.05$), 5.8 ± 2.17 and 0 (**, $P < 0.005$), and 10.2 ± 2.44 and 0 (***, $P < 0.001$), respectively (Mann-Whitney test). *Bars,* SE. *B,* gefitinib prevented lung tumorigenesis completely. *C,* no significant difference in body weight was observed between the two groups ($P = 0.971$, paired *t* test). *Bars,* SE.

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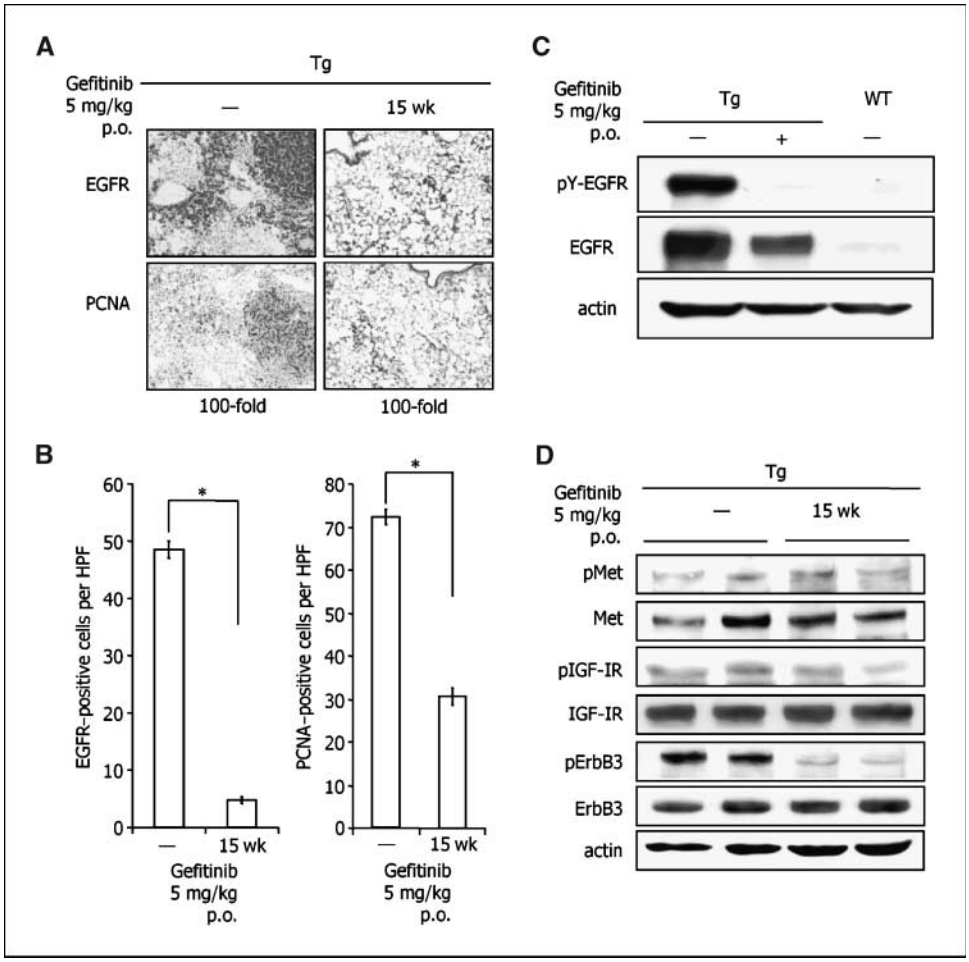

Figure 4. Inhibitory effect of gefitinib on EGFR phosphorylation in the transgenic mice after tumor formation. *A,* immunostaining of EGFR and PCNA in the lungs of transgenic mice treated with or without gefitinib. *B,* percent EGFR- and PCNA-positive cells in the lungs of transgenic mice treated with or without gefitinib. *Bars,* SE. *, $P < 0.001$. *C,* levels of total and phosphorylated EGFR in the lungs of the transgenic mice treated with or without gefitinib and wild-type controls by Western blotting. *D,* expression levels of phosphorylated Met, IGF-IR, and ErbB3 in the lungs of the transgenic mice treated with or without gefitinib for 15 wk.

(19). Thus, *EGFR* mutations and overexpression seem to be important for tumor development in nonsmoking-related lung cancer. Consequently, mutated EGFR is an excellent target for chemoprevention in nonsmoking-related lung cancer.

Previously, we showed that a high dose (50 mg/kg) of gefitinib partially inhibited 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone–induced tumorigenesis in A/J mice (16). Similarly, a very high dose (100 mg/kg) of gefitinib significantly reduced benzo[*a*]pyrene–induced tumorigenesis in A/J mice (17). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[*a*]pyrene are potent carcinogens found in tobacco smoke that induce oncogenic *K-ras* mutations in A/J mice (12–14). In those classic smoking-related lung cancer mouse models, however, gefitinib could not inhibit Ras-related signaling as completely as suggested in recent *ex vivo* studies (35, 36). Thus, if the chemopreventive agents do not match the pathogenic mechanism, the result will be incomplete. In contrast, we showed that a clinical dose (5 mg/kg) of gefitinib completely inhibited tumorigenesis using our nonsmoking-related lung cancer mouse model because we targeted a specific molecule with a critical role in carcinogenesis.

If a population with a high risk of non–small cell lung cancer induced by a mutated *EGFR* is selected, EGFR TKI can be used clinically as a chemopreventive agent. Future advances in epidemiologic research will provide the predictors necessary for selecting such a population with higher precision instead of relying on data regarding smoking history, sex, and ethnicity. In addition, the risk of developing a second primary lung cancer in patients who survived after the resection of primary early-stage of non–small cell lung cancer is ~1% to 2% per patient per year (37). If the first primary lung cancer has activating *EGFR* mutations, the second primary lung cancer is likely to have *EGFR* mutations as well because the patient's lungs would have been exposed to the same carcinogens and be of the same genetic background as the primary lung cancer. In a similar fashion, chemoprevention trials using molecular targeted agents associated with the tumorigenesis of primary lung cancer might be feasible not only in mutated *EGFR*-related lung cancer but also in other pathogenesis-related lung cancers. However, in applying EGFR TKI to a chemoprevention trial, considering the potential for severe adverse events such as interstitial lung disease is necessary (38, 39). Accordingly, the development of a new EGFR TKI without adverse events of interstitial lung diseases or the discovery of predictive factors of intestinal lung disease is expected.

One of the most effective ways to prevent *EGFR* mutation-induced non–small cell lung cancer is to remove the cause of the *EGFR* mutation. X-ray–induced lung tumors carrying *EGFR* mutations were recently reported in rats (40) and are presumed to be one of the causes of activating *EGFR* mutations in humans. However, the mutated *EGFR* in the rats did not correspond to major activating *EGFR* mutations in humans. Moreover, the oncogenesis of mutated *EGFR* in rats has not yet been reported.

These findings raised the questions of what causes activating *EGFR* mutations. One study indicated a decreasing trend in the

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incidence of *EGFR* mutations with increasing exposure to smoke (6). Moreover, *EGFR* mutations are rare in other types of human cancer. Nevertheless, the majority of human epithelial cancers are marked by the functional activation of *EGFR*, which suggests that the aspiration of an environmental carcinogen other than tobacco smoke is the cause of *EGFR* mutations. In addition, recent epidemiologic studies have shown that a longer total fertile life, resulting from increased exposure to estrogen, increased the frequency of mutations in *EGFR* (41), whereas the consumption of soy, which contains soybean isoflavones with antiestrogenic effects, decreased the frequency (42). Thus, hormones appear to play an important role in *EGFR* mutagenesis. Moreover, a dysfunctional single nucleotide polymorphism in many DNA repair enzymes was reported to be more frequent in Asians than in Whites (43). Taken together, exposure to environmental carcinogens other than tobacco smoke, hormonal imbalances, and defects in DNA repair may explain why some *EGFR* mutations are detected more frequently among nonsmokers, females, and Asians. If dysfunctional single nucleotide polymorphisms in DNA repair enzymes are important causes of *EGFR* mutations, chemoprevention by genetic stratification may be possible.

We investigated other factors related to the activation of EGFR signaling in our transgenic mice. The status of ErbB2 and ErbB3 has been reported to predict gefitinib sensitivity (44, 45). Phosphorylated and total ErbB2 and ErbB3 were also up-regulated in our transgenic mice compared with the wild-type controls. A recent study suggested that increased EGFR signaling induced the up-regulation of *ErbB3* in a mouse model (46). ErbB2 and ErbB3 may have been up-regulated by altered *EGFR* signaling in our transgenic mice. In addition, *EGFR* mutations were specifically detected in terminal respiratory unit–type adenocarcinoma, which constitutes a major subset of adenocarcinoma based on its distinctive morphology and expression of TTF-1 and surfactant protein (47). Note that TTF-1 expression was significantly higher in our mouse model compared with the wild-type controls. TTF-1 was recently shown to be crucial for the survival of a subset of lung adenocarcinomas (48). ErbB2, ErbB3, and TTF-1 may have been involved in carcinogenesis and the progression of lung tumors in our transgenic mice. Therefore, they could also serve as excellent chemopreventive targets.

In conclusion, gefitinib completely inhibited tumorigenesis in a nonsmoking-related lung cancer mouse model, which was induced by an activating *EGFR* mutation. Our results suggest that molecular targeted chemoprevention is a viable approach to the treatment of lung cancer.

Disclosure of Potential Conflicts of Interest

K. Kiura: honoraria from speakers bureau, AstraZeneca. The other authors disclosed no potential conflicts of interest.

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