Induction of lung adenocarcinoma in transgenic mice expressing activated *EGFR* driven by the SP-C promoter

Kadoaki Ohashi,[1] Kammei Rai,[1] Yoshiro Fujiwara,[1] Masahiro Osawa,[1] Seiki Hirano,[2,4] Katsuyoshi Takata,[3] Eisaku Kondo,[3] Tadashi Yoshino,[3] Minoru Takata,[2,5] Mitsune Tanimoto[1] and Katsuyuki Kiura[1,6]

[1]Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, 700-8558; [2]Department of Immunology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192; [3]Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

(Received February 1, 2008/Revised April 30, 2008/Accepted May 7, 2008/Online publication June 28, 2008)

To investigate the role of an activating *epidermal growth factor receptor* (*EGFR*) mutation in lung cancer, we generated transgenic mice expressing the delE748-A752 mutant version of mouse *EGFR* driven by the SP-C promoter, which is equivalent to the delE746-A750 mutation found in lung cancer patients. Strikingly, the mice invariably developed multifocal lung adenocarcinomas of varying sizes at between 5 and 6 weeks of age, and they died from tumor progression approximately 2 months later if left untreated. Daily oral administration of the EGFR tyrosine kinase inhibitor (TKI) gefitinib (5 mg/kg/day) reduced the total and phosphorylation levels of EGFR to those in wild-type mouse lung tissue; in addition, it abrogated tumor growth within 1 week and prolonged survival to >30 weeks. Interestingly, phosphorylated ErbB2, ErbB3, and thyroid transcriptional factor-1 increased in the transgenic mice compared with those in wild-type mice. They might play some roles in tumors progression in the transgenic mice. This model will be useful for studying the mechanisms of carcinogenesis, chemoprevention, and acquired resistance to EGFR TKIs in lung cancer patients carrying activating *EGFR* mutations. (*Cancer Sci* 2008; 99: 1747–1753)

Epidermal growth factor receptor (EGFR) signaling regulates processes that are essential for the initiation and progression of cancer, including cell motility, cell adhesion, tumor invasion, cell survival, angiogenesis, and cellular proliferation.[1] Because EGFR is expressed in ~50% of non-small cell lung cancers (NSCLCs), it is an excellent candidate for a therapeutic target in the treatment of these types of cancer.[2] The reversible EGFR tyrosine kinase inhibitor (TKI) gefitinib was initially selected as a therapeutic agent for NSCLC because of its ability to inhibit wild-type EGFR tyrosine kinase (TK); its expected effects included disease control and/or long-term disease stability.[1]

Unexpectedly, however, dramatic responses to gefitinib were observed in a small but substantial subset of NSCLC patients characterized by female gender, adenocarcinoma histology, and Asian ethnicity.[3–5] Somatic *EGFR* mutations were subsequently discovered in those patients who responded to gefitinib or erlotinib.[6–10] Deletion mutations in exon 19 and the substitution of leucine by arginine at codon 858 (L858R) account for ~90% of all *EGFR* mutations.[4,11,12] These mutations appear to drastically sensitize cancer cells to the growth-suppressive effects of EGFR inhibitors.[13,14] Such activating *EGFR* mutations are presumed to play important roles in cell growth, survival, and carcinogenesis in lung cancer. *EGFR* mutations have also been detected in the histologically normal lung tissue surrounding adenocarcinomas,[15] and in atypical adenomatous hyperplasias (AAH) of the lung.[16] Further, activated *EGFR* mutants display the capacity to transform NIH3T3 cells and to promote cytokine-independent growth in Ba/F3 cells.[17,18] The basis for the activation of EGFR by these mutations has been delineated in recent structural studies.[19] Transgenic mice with a mutated version of the *EGFR* gene driven by a tetracycline-inducible promoter developed lung adenocarcinomas following the administration of doxycycline, although they disappeared upon cessation of the inducing agent.[20,21] Taken together, these observations suggest that activating *EGFR* mutations promote carcinogenesis, as well as maintenance of the induced tumor; however, the mechanistic details of these processes are unclear.

To investigate the mechanisms of carcinogenesis, chemoprevention, and acquired resistance to gefitinib in cases of lung cancer involving the activation of the *EGFR* mutation, we generated transgenic mice expressing the delE748-A752 mutant version of mouse *EGFR* (*EGFR*$^{de1748\text{-}752}$), which corresponds to delE746-A750 in human *EGFR* (*EGFR*$^{delE\ N746\text{-}A750}$). Constitutive and lung-specific expression of the transgene was achieved using the pulmonary surfactant protein C (SP-C) promoter, which is active only in type II pneumocytes.[22] Here, we describe the generation and initial characterization of a transgenic mouse model for NSCLC based on an activating *EGFR* mutation.

Materials and Methods

Production of transgenic mice. To generate mice that constitutively express mutant mouse *EGFR* in the lung, we used the SP-C promoter, which is active in type II pneumocytes.[22] The plasmid containing the SP-C promoter and the SV40 small T intron and polyadenylation sequence has been described previously.[22] Full-length mouse *EGFR* cDNA was amplified from liver cDNA (kindly provided by Dr Hiroshi Osawa, Division of Neurology, Kawasaki Medical School, Kurashiki, Japan) using primers that contained a *Sal*I site. After digestion with *Sal*I, the amplified product was inserted into the *Sal*I site of the plasmid between the SP-C promoter (kindly provided by Dr Jeffrey A. Whitsett, the Division of Neonatology Perinatal and Pulmonary Biology at Cincinnati Children's Hospital Medical Center, USA) and SV40 small T intron. The entire sequence of the construct was subsequently confirmed by sequencing. A 15-bp in-frame deletion (nucleotides 2242–2256) was then introduced using a QuikChange XL kit (Stratagene, La Jolla, CA, USA) in accordance with the manufacturer's instructions. The EGFR expression cassette was excised from the parental vector using *Nde*I and *Not*I, and injected into fertilized C57BL/6Cr eggs (Biotechnical Center of Japan SLC, Shizuoka, Japan).

[4]Current address: CR-UK Oxford Cancer Center, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK; [5]Radiation Biology Center, Kyoto University, Kyoto, Japan
[6]To whom correspondence should be addressed. E-mail: kkiura@md.okayama-u.ac.jp

doi: 10.1111/j.1349-7006.2008.00875.x

Animal husbandry. All animals were kept under pathogen-free conditions with abundant food and water, as specified in the guidelines of the Department of Animal Resources, Okayama University Advanced Science Research Center. Gefitinib was given once a day, 5 days per week, 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.

Polymerase chain reaction (PCR) genotyping. Genomic DNA was isolated from the tails of the mice using a QIAamp DNA mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Transgenic lines were identified by PCR using primers specific for mouse *EGFR* cDNA: EGFRF1 (5′-CCTATTCATGCGAAGACGTC-3′) and EGFRR1 (5′-AAGTCACGGTGCACCAAACG-3′). The amplified products were resolved using 1.6% agarose gels.

Histology, immunohistochemistry, and immunofluorescence. The animals were sacrificed by cervical dislocation. 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 phosphate-buffered saline (PBS). The lungs were subsequently fixed in 10% paraformaldehyde overnight at room temperature, embedded in paraffin, and sectioned every 5 μm (West Japan Pathology, Okayama, Japan). Antigen retrieval was performed using Pascal (Dako, Glostrup, Denmark), according to the manufacturer's protocol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 10 min. After incubation with blocking solution for 60 min, the sections were incubated with primary antibody (1:50 dilution) at 4°C for 12 h, followed by 30 min of incubation with EnVision + System Labeled polymer HRP antirabbit (Dako, Glostrup, Denmark). Staining was achieved with diaminobenzidine chromogen, and counter-staining with Mayer's hematoxylin. The primary antibody used was rabbit polyclonal anti-EGFR antibody and TTF-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proliferating cell nuclear antigen (PCNA) was stained using a PCNA staining kit (Zymed Laboratories, Carlsbad, CA, USA). For the immunofluorescence to detect the apoptotic cells on the lung tissues in the transgenic mice with or without gefitinib treatment for 2 days, the deparaffinized sections were incubated with rabbit biotin-conjugated antiactive Caspase-3 polyclonal antibody (BD Biosciences, Tokyo, Japan), were sequentially reacted with Cy3-conjugated Streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA, USA) after several time washings. Nuclei were counterstained with bisBenzamid (Hoechst33258; Sigma-Aldrich, St. Louis, MO, USA).

Reverse transcription (RT)–PCR analysis. RNA samples were prepared for RT-PCR using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). To specifically amplify the mutant version of mouse *EGFR*, the primer EGFR F8del (5′-CCTATTCATGCGAAGACGT-3′) was designed to span the deleted portion of the *EGFR* cDNA and used with EGFR R5 (5′-AAAGTTGGAGAGTCTGTAGGGCT-3′). *GAPDH* was amplified as a control using the primers GAPDH F (5′-CGTAGACAAAATGGTGAAGG-3′) and GAPDH R (5′-GTTGTCATGGATGACCTTGG-3′). Duplex TaqMan real-time PCR was used to analyze the relative *EGFR* expression level, as well as the *EGFR* copy number, in the transgenic and wild-type mice using an ABI PRISM 5700 Sequence Detection System (Japan Applied Biosystems, Tokyo, Japan). The *EGFR* primers were 5′-CTGCCAAAAGTTCCAAGATGAGG-3′ and 5′-GGGGCACTTCTTCACACAGG-3′. The probe (5′-AGACACCTGCCCACCACTCATGCT-3′) was labeled with the reporter dye 6-carboxyfluorescein (FAM). *GAPDH* was coamplified in the same reaction mixture as the endogenous reference gene using TaqMan GAPDH Control Reagents (Sigma-Aldrich, Tokyo, Japan). The average *EGFR* expression and the copy number were determined from the differences in the threshold amplification cycles between *EGFR* and *GAPDH*.

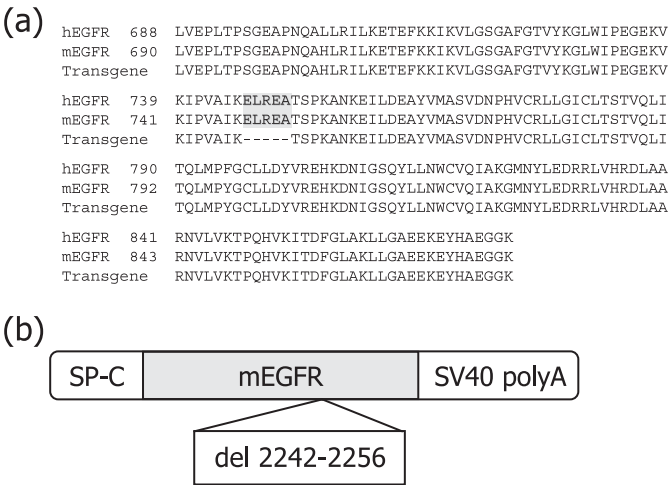

Fig. 1. Generation of transgenic mice carrying mutated *EGFR* (*EGFR*$^{de1748\text{-}752}$). (a) Comparison of the kinase domains in humans, mice, and our mutated version of mouse *EGFR*. Two non-identical residues between humans and mice and the deleted portion are darkened. (b) Diagram of the construct used to produce transgenic mice. *EGFR*$^{de1748\text{-}752}$ was cloned between the SP-C promoter and SV40 small T intron. SP-C, SP-C promoter.

Immunoblotting. Protein extracts were prepared from crushed tissue samples that were incubated in lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol phosphate, 10 mM NaF, and 1 mM Na-orthovanadate) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) and centrifuged at 14 500 r.p.m. for 20 min at 4°C. After quantification by Bio-Rad protein assay, ~50 μg of protein per sample was separated by SDS-PAGE using 5–15% precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Specific proteins were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) using the following antibodies: phospho-EGFR Y1068, total EGFR, phospho-ErbB2, and phospho-ErbB3 (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA); ErbB2, ErbB3 (1:1000 dilution; Upstate biotechnology, Waltham, MA, USA); TTF-1 (1:200 dilution; Santa Cruz Biotechnology); and antiactin (1:5000 dilution; Chemicon, Temecula, CA, USA). The secondary antibodies were antirabbit IgG and antimouse IgG (horseradish peroxidase–linked, species-specific whole antibodies; GE Healthcare), each of which was used at a 1:5000 dilution.

Statistical analysis. Survival time was defined as the period from birth to death or the most recent follow-up evaluation. Survival curves were calculated using the Kaplan–Meier method. The log-rank test was used to detect differences in survival. Statistical significance was defined as $P < 0.05$. All statistical analyses were carried out using SPSS version 10.0 (SPSS, Chicago, IL, USA).

Results

Production of transgenic mice. The kinase domain of *EGFR* is highly conserved between humans and mice (Fig. 1a). We isolated mouse *EGFR* from liver cDNA by PCR and deleted 15 nucleotides encoding five amino acids (*EGFR*$^{de1748\text{-}752}$), which is equivalent to the *EGFR*$^{delE\ N746\text{-}A750}$ (Fig. 1a) mutation found in lung cancer patients. We then generated a mutant *EGFR* construct consisting of the SP-C promoter, open reading frame of the mutated *EGFR*, SV40 small T intron, and SV40

doi: 10.1111/j.1349-7006.2008.00875.x

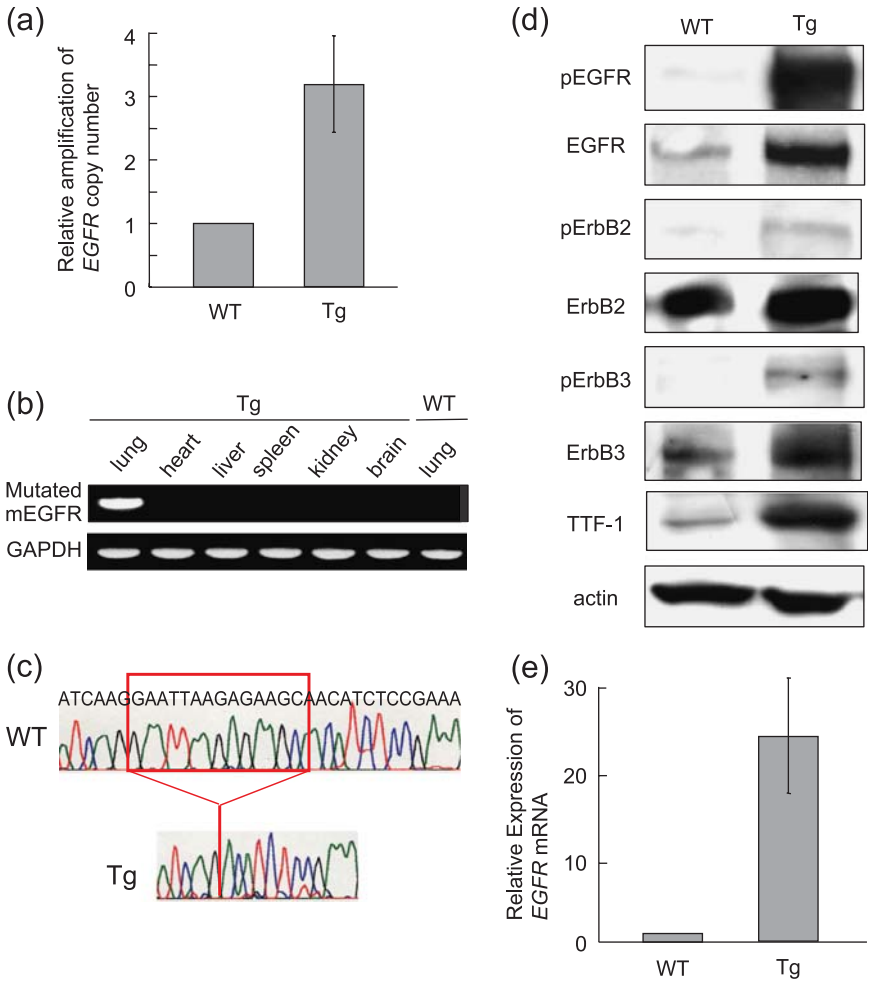

Fig. 2. Analyses of transgene copy number and expression. (a) Analysis of the *EGFR* copy number in the lungs of the transgenic mice. Genomic DNA extracted from the tails of the mice was subjected to TaqMan real-time polymerase chain reaction (PCR). (b) Lung tissue–specific expression of the transgene. Total RNA was extracted from various mouse tissues and subjected to mutant *EGFR*-specific reverse transcription (RT)–PCR analysis as described in 'Materials and Methods'. (c) Direct sequencing of the RT-PCR products. Sequence traces of wild-type (upper) and mutant (lower) *EGFR* are compared. (d) Western blots of EGFR, ErbB2, ErbB3, and TTF-1 expression in the lungs of 2-week-old tumor-free transgenic mice and control mice. (e) *EGFR* mRNA expression was significantly higher in the lungs of the transgenic mice with lung cancer than in the control mice. The cDNA was synthesized from total lung RNA and subjected to TaqMan real-time PCR.

polyadenylation signal (Fig. 1b). Following injection of the construct into fertilized eggs, 47 pups were born and examined for integration of the transgene by PCR using tail genomic DNA. Of these, nine mice were positive and three (#6-5, #6-3, and #7-9) later developed lung adenocarcinoma. Subsequently, line #6-5 was able to produce offspring, all of which developed lung tumors at 7 weeks of age or later. Unfortunately, mice #6-3 and #7-9 were unable to produce offspring because of cancer death before mating; they died at 15 and 19 weeks of age, respectively. No lung tumors were detected in any of the other mice. We attempted to produce transgenic mice expressing mouse $EGFR^{L860R}$, which corresponds to human $EGFR^{L858R}$, but failed to generate a founder.

Analysis of copy number and expression of the transgene. Real-time PCR using tail genomic DNA from heterozygous transgenic mice yielded approximately three-fold higher signal levels from the transgene compared to endogenous *EGFR*. This suggests that four copies of the transgene were integrated in our model mice (Fig. 2a). The mRNA expression of the transgene was examined in multiple tissues from 7-week-old transgenic (#6-5 line) and control mice by mutant-specific RT-PCR. We observed that mutant *EGFR* expression was restricted to the lung tissue of the transgenic mice (Fig. 2b). In addition, the presence of the deletion was confirmed by direct sequencing of the RT-PCR products (Fig. 2c).

We then evaluated phosphorylated and total EGFR expression in the lungs of 2-week-old transgenic mice showing no overt signs of lung tumors using Western blot analysis. Significantly greater phosphorylated and total EGFR expression was observed in the lung tissue of the transgenic mice than in that of the controls (Fig. 2d). In addition, we consistently detected numerous alveolar epithelial cells expressing high levels of EGFR in the lungs of 3-week-old transgenic mice by immunohistochemical examination (Fig. 3Aa,b). We also determined the level of *EGFR* mRNA expression in the lungs of transgenic mice with multiple tumors by TaqMan real-time PCR. More than 20-fold overexpression of *EGFR* was observed compared to the level in the normal lungs of non-transgenic mice (Fig. 2e). These findings likely reflect remarkable expansion of the cells expressing mutated *EGFR* by carcinogenesis. Next we evaluated phosphorylated and total ErbB2, ErbB3 expression and thyroid transcription factor-1 (TTF-1) expression in the same fashion. The definitely greater phosphorylated ErbB2, ErbB3, and TTF-1 expression were observed in the transgenic mice (Fig. 2d).

Histology of the lungs of the transgenic mice. To determine the oncogenic effect of the activating *EGFR* mutation in the lungs, we sacrificed transgenic (#6-5 line) and control mice at various time points for pathological examination (Fig. 4a–4h). Histological changes consistent with AAH and diffuse bronchioloalveolar carcinoma (BAC) developed at 3–4 weeks and 4–5 weeks of age, respectively (Fig. 4c,d). At around 7 weeks of age, adenocarcinoma with solid features was observed (Fig. 4e,f) and the tumor mass further progressed (Fig. 4g,h). The tumors displayed strong immunoreactivity with anti-EGFR antibodies (Fig. 3a). Ultimately, the mice died at around 14 weeks after birth if left untreated. Macroscopically multiple tumor nodules were obsereved on the lung surface (Fig. 4i), and these tumor formations were not found in other organs but only in the lungs. In contrast, none of

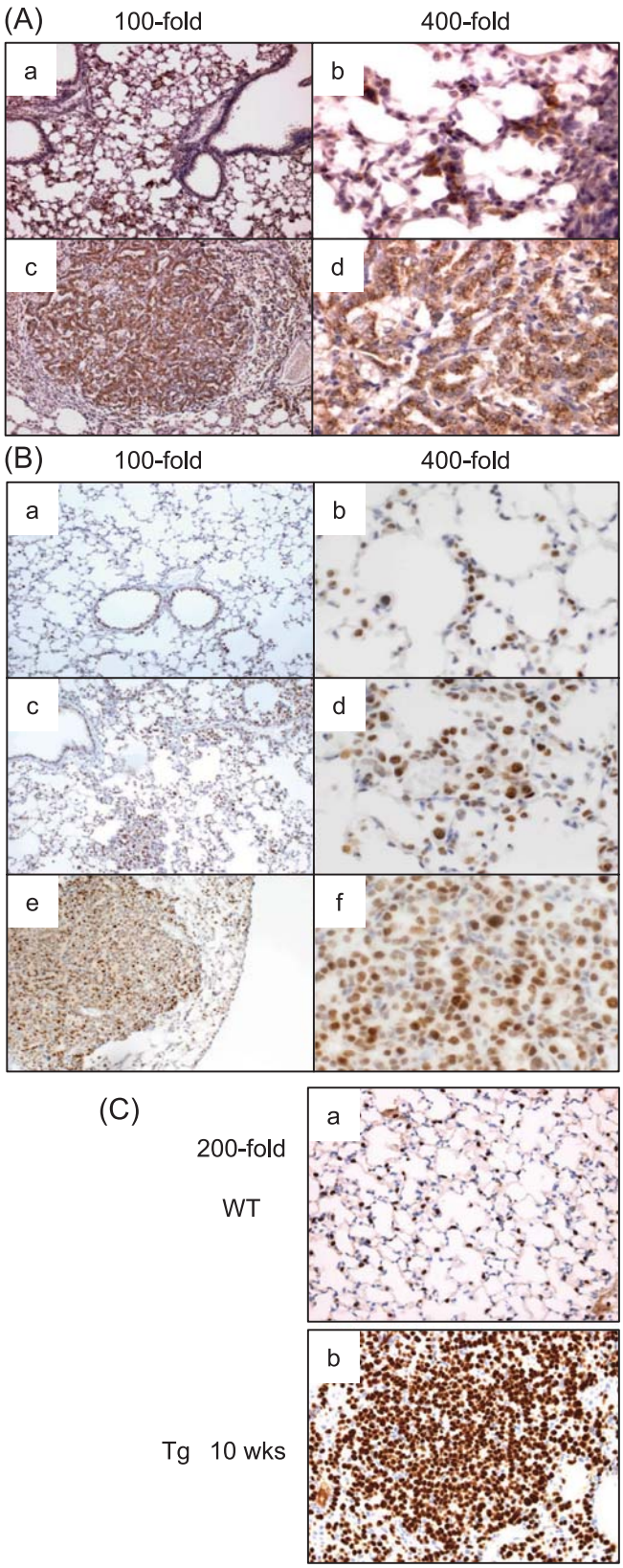

Fig. 3. (A) EGFR staining of lung tissue from the transgenic at 3 weeks of age (a, 100× and b, 400×) and at 14 weeks of age (c, 100× and d, 400×). (B) PCNA staining of lung tissue at 2 weeks of age (a, 100×, b, 400×), at 7 weeks of age (c, 100×, d, 400×), and at 14 weeks of age (e, 100×, f, 400×) in the transgenic mice. (C) TTF-1 staining of normal lung tissue in the wild-type and an invasive tumor at 10 weeks of age in the transgenic mice.

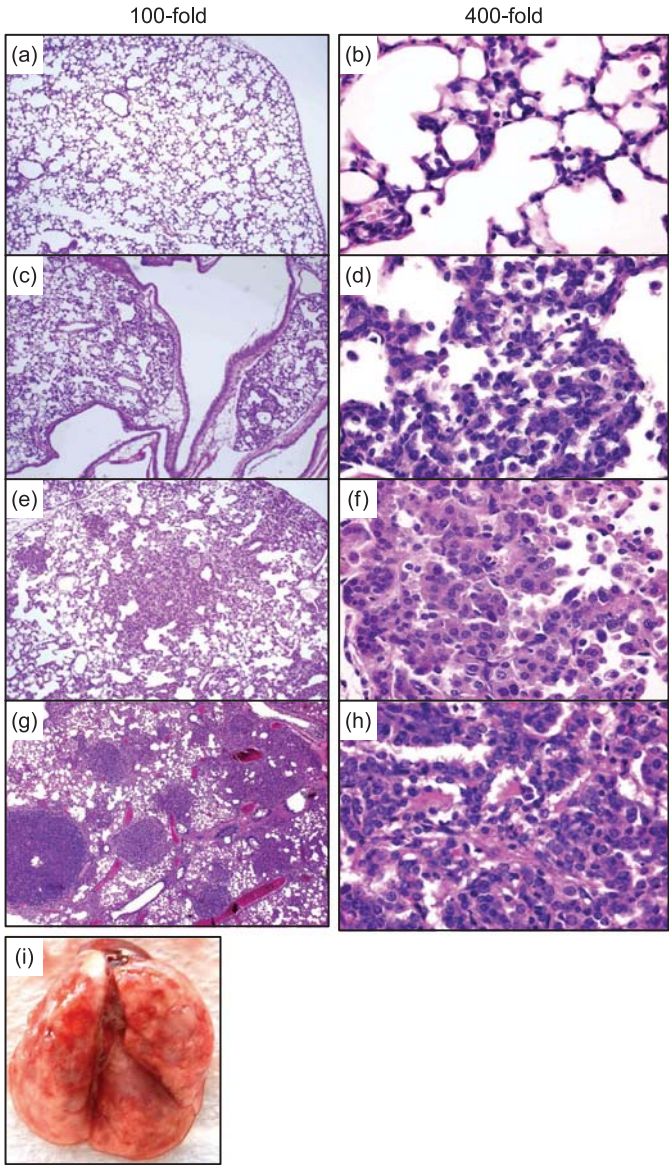

Fig. 4. Lung cancer in the transgenic mice. Histological examination of lung tissue from the transgenic mice with hematoxylin–eosin (HE) staining. Normal respiratory unit at 3 weeks of age (a, 100×, b, 400×); atypical cuboidal cells replacing the lining of alveoli, with rarely mitotic figures, at 5 weeks of age (c, 100×, d, 400×); small tumor foci multifocally developed in the lung (e, 100×), increase of atypical cells with scattered mitotic figures at 8 weeks of age (f, 400×); multicentric invasive foci of the tumors occupied in the lung (g, 100×), high per view of the tumor focus occupied with dense prolification of cancer cells at 14 weeks of age (g, 100×, h, 400×) were found in the transgenic mice. Macroscopically multiple tumors were seen on the surface of both lungs at 14 weeks of age (i).

the control mice developed lung tumors and all of the animals 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 difference in the number of PCNA-positive cells was observed between the wild-type and transgenic mice prior to lung tumor development at 2 weeks of age (Fig. 3Ba,b and data not shown). As lung tumors developed at 4 weeks or later, the number of PCNA-positive cells, most of which seemed to be tumor cells,

doi: 10.1111/j.1349-7006.2008.00875.x

was markedly increased in the transgenic mice (Fig. 3Bc,d). Moreover, the observed tumor mass at 14 weeks was largely PCNA-positive (Fig. 3Be,f).

The expression of TTF-1 on the lung tumors in the transgenic mice at 10 weeks of age was extremely higher than that in wild type mice (Fig. 3C).

Inhibitory effect of gefitinib on lung adenocarcinoma induced by an activating EGFR mutation. To investigate the sensitivity of the tumors induced by the activating *EGFR* mutation to EGFR TKIs, gefitinib or solvent alone was administered by gavage as a 5 mg/kg suspension to 7-week-old transgenic mice carrying lung tumors. After 7 days, the mice were sacrificed and their lungs were examined by Western blotting. The total and phosphorylation levels of EGFR in the lungs of the gefitinib-treated transgenic mice were remarkably decreased compared to the levels observed in the non-transgenic control mice (Fig. 5a). The level of EGFR in the lungs of the control solvent alone mice was not decreased by gefitinib treatment. Importantly, no lung tumors were detected in the gefitinib-treated mice ($n = 3$), whereas every untreated control mouse carried extensive lung tumors ($n = 3$; Fig. 5b). Thus, EGFR inhibition led to rapid tumor regression. It is interesting to note that gefitinib treatment diminished the level of not only phosphorylated EGFR, but also total EGFR, suggesting that most, if not all, pneumocytes expressing high levels of the mutant EGFR were transformed to become cancerous and that these cells were subsequently killed by gefitinib treatment, leading to the loss of EGFR expression. Furthermore, identical results were obtained in 2-week-old mice without lung tumors. These data suggest that before overt tumorigenesis, the pneumocytes expressing the transgene were already dependent on EGFR signaling (i.e. oncogene addiction; Fig. 5c).

We next investigated whether the effect of gefitinib on tumor regression was temporary. Transgenic mice treated with gefitinib for 1 week were examined after a follow-up period of 1 week ($n = 3$). Interestingly, we noticed extensive lung tumors in the mice, similar to the untreated transgenic mice, which is consistent with rapid re-expansion of the tumor cells (data not shown).

Effect of gefitinib on the survival of the transgenic mice. Because gefitinib induced remarkable regression of lung adenocarcinoma in our transgenic mice, we examined the effect of gefitinib on survival. One group of transgenic mice ($n = 6$) received gefitinib orally each day from 10 weeks of age to death, whereas a second group (control, $n = 5$) was left untreated. Survival curves were plotted for the treated and untreated groups (Fig. 6a). Gefitinib treatment significantly enhanced the survival of the transgenic mice (log-rank test, $P < 0.01$); the median survival time of the gefitinib-treated mice was 34.9 weeks, whereas that of the untreated mice was only 14.1 weeks (Fig. 6a). Additionally we evaluated whether apoptosis occurred in lung tumors by gefitinib. Approximately 30% of the cancer cells in the non-treated transgenic mice were positive for the cleaved caspase-3 antibody, whereas over 80% of the cancer cells were revealed to be the cleaved caspase-3-positive in gefitinib-treated transgenic mice (Fig. 6b).

When the mice treated with or without gefitinib died, we investigated the extent of lung adenocarcinoma and lung histology. Multiple recurrent lung adenocarcinomas, but no metastatic tumors to other oranges, were found in the dead transgenic mice. No interstitial lung diseases were found in the mice treated with gefitinib.

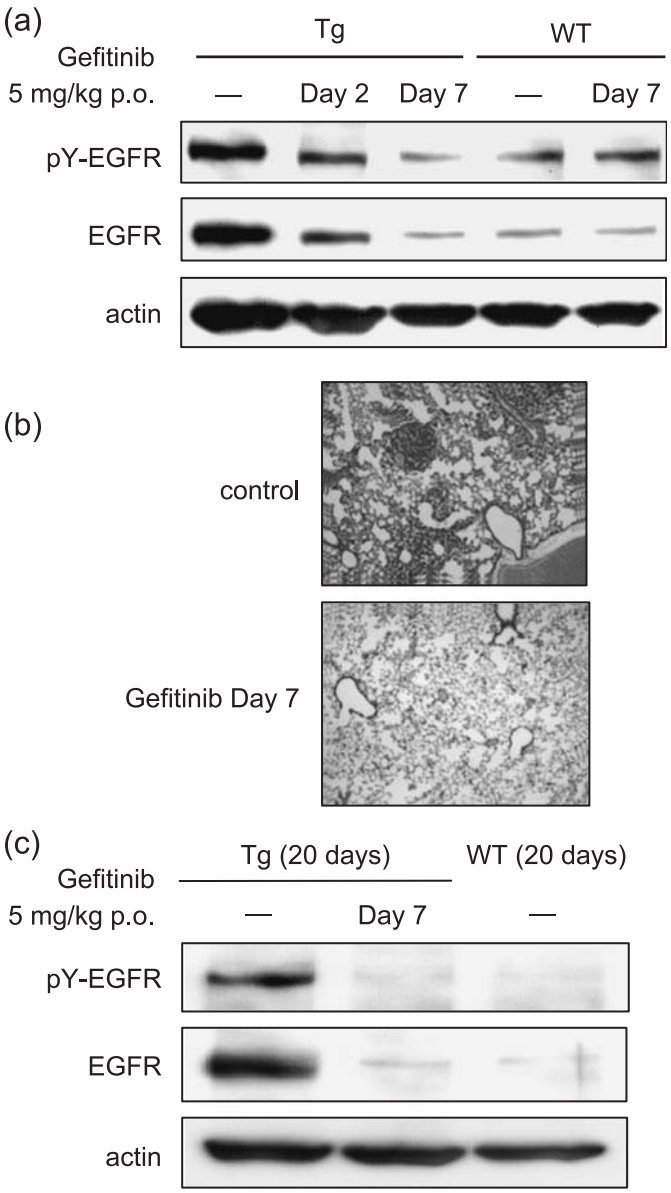

Fig. 5. Response of the transgenic mice to gefitinib treatment. (a) Total and phosphorylated EGFR levels in the lungs of 7-week-old transgenic mice with lung cancer (Tg) or wild-type mice (WT). The mice were sacrificed on the indicated day of the gefitinib treatment course, and total cellular lysates were prepared. Phosphorylated EGFR (pY-EGFR) was detected using a specific antibody against phospho-EGFR Y1068. (b) The effects of gefitinib on lung cancer. Seven-week-old gefitinib-treated and -untreated transgenic mice were sacrificed and their lungs were examined by hematoxylin–eosin (HE) staining. (c) The total and phosphorylated EGFR levels in lung tissue from the transgenic mice prior to lung tumor development (2 weeks of age) following 1 week of treatment with gefitinib.

Discussion

We generated *EGFR*$^{de1748\text{-}752}$ transgenic mice (corresponding to *EGFR*$^{delE\ N746\text{-}A750}$ in NSCLC patients). Previously, groups at the Dana-Farber Cancer Institute and Memorial Sloan-Kettering Cancer Center have established activating *EGFR* transgenic mice.[20,21] Their mice developed lung adenocarcinoma using a tetracycline on system. We also succeeded transgenic mice expressing the delE748-A752 *EGFR* mutation using the SP-C promoter. Similarly, our mice invariably developed multifocal lung adenocarcinomas of varying sizes at between 5 and 6 weeks of age, and they died from tumor progression approximately 2 months later.

A significant advantage of our transgenic model is that our mice do not need tetracycline to induce lung tumors. The adenocarcinomas emerged spontaneously, and the mice died due

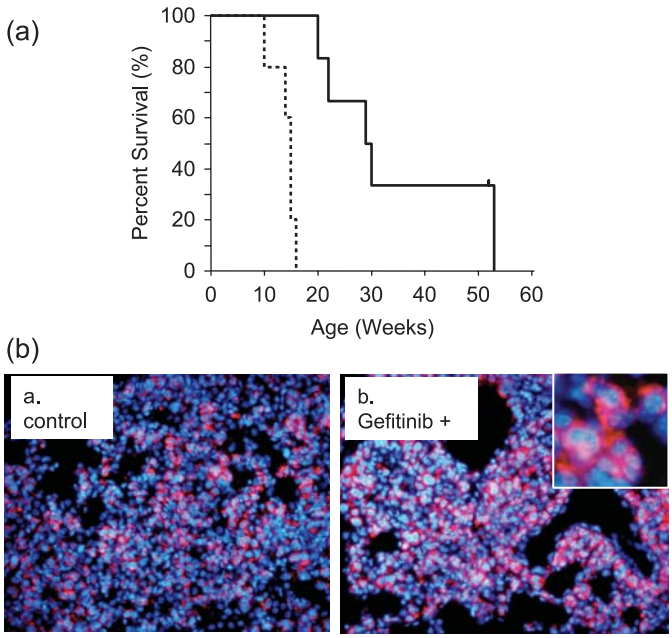

Fig. 6. (a) Survival curves of gefitinib-treated (solid line) and -untreated (dotted line) *EGFR*$^{de1748\text{-}752}$-transgenic mice. +indicates the censored mouse. (b) Active caspase-3 in the cytoplasm was clearly stained to red, and nuclei was counterstained to blue by bisBenzamid. Active caspase-3 increased at 10 weeks of age in the transgenic mice treated with gefitinib for 2 days (b, 200×) when compared to that without gefitinib (a, 200×). High magnification was presented in the right upper portion of (b) (800×).

to the lung tumors. Consequently, in chemoprevention trials, we do not have to consider the interactions between tetracycline and chemopreventive agents, because doxycycline might have a substantial biological effect.[23]

In our transgenic mice, adenocarcinomas strikingly regressed after gefitinib treatment, and cessation of gefitinib treatment led to regrowth of the tumors. These observations indicate that *EGFR*$^{de1748\text{-}752}$ is essential for the growth and maintenance of lung cancer in our mouse NSCLC model. It is possible that overexpression of the transgene may have contributed to carcinogenesis in our model; however, we were unable to detect additional amplification of the transgene in the tumors by real-time PCR using genomic DNA (data not shown). These data support the notion that the mutation is sufficient for carcinogenesis and that it may be the initiating event in NSCLC. Cells expressing the mutated *EGFR* did not proliferate rapidly until 4–5 weeks of age in our model, suggesting that another pivotal change (i.e. a second hit) is necessary to invoke full transformation. However, we favor the possibility that the *EGFR* mutation was necessary and sufficient for full development of lung cancer in our model because most, if not all, cells expressing the transgene seemed to become cancerous in the transgenic mice (Fig. 3A).

What causes activating *EGFR* mutations in patients is unclear; however, such mutations are more common in those who have never smoked,[12] Asians, and females.[4] The carcinogens in tobacco do not seem to prevent activating *EGFR* mutations because approximately 20% of patients with a history of heavy smoking have activating *EGFR* mutations.[24] Activating *EGFR* mutations might be related to host susceptibility to the carcinogens in tobacco. Recent reports have indicated that irradiation and chemical agents can induce mutations in *EGFR*; in particular, point mutations may be induced by tobacco-specific nitrosamines in 8-oxoguanine DNA glycosylase 1 (Ogg1)–knockout mice.[25] Ogg1 participates in the repair of oxidative DNA damage via base excision. In addition, a single nucleotide polymorphism (SNP) has been identified in human Ogg1 at codon 326, which involves an amino acid change (Ser326Cys). The reduced ability of OGG1–326Cys to prevent mutagenesis has been reported.[26,27] The frequency of this SNP is much higher in Asians than Caucasians (>40% *versus* <20%, respectively).[28] Taken together, a defect in DNA repair may explain why some *EGFR* mutations are detected more frequently among Asians. In addition, the higher occurrence of *EGFR* mutations in females suggests that hormonal or lifestyle factors affect tumorigenesis in this type of NSCLC. In our model, the development of NSCLC in males and females was identical. This suggests that the carcinogenic factor(s) that favor females promote mutations in *EGFR* (initiation stage), and not subsequent disease development (promotion or progression stage).

The status of other ErbB family members has been reported as predicting gefitinib sensitivity.[29–32] Phosphorylated ErbB2, ErbB3, and total-ErbB2, ErbB3 were also up-regulated in our transgenic mice compared with those in wild-type mice. Fujimoto *et al*. reported that EGFR up-regulated the expression of ErbB3.[31] The ErbB2 and ErbB3 in our transgenic mouse might be up-regulated by mutated EGFR signaling. Additionally, Yatabe *et al*. reported that *EGFR* mutations were specifically detected in terminal-respiratory-unit-type adenocarinoma, which constitute a major subset of adenocarcinoma based on their distinctive morphologies, expression of TTF-1, and surfactant protein.[33] Interestingly, TTF-1 expressed significantly higher in our mouse model than that in wild-type mouse. Recently, TTF-1 was crucial for the survival of a subset of lung adenocarcinoma.[34] Phospholylated ErbB2, ErbB3, and TTF-1 might play some roles in carcinogenesis and progression of lung tumors in our transgenic mice.

Contrary to the expected result, gefitinib did not show a survival benefit in unselected patients in the ISEL study.[35] Furthermore, maintenance treatment with gefitinib produced a significant decrease in survival after chemoradiotherapy followed by docetaxel in unselected patients.[36] However, it has been suggested that gefitinib may provide a significant survival benefit in patients selected based on their clinical characteristics (e.g. gender, smoking, and ethnicity) or *EGFR* mutation status. In support of this notion, we observed a significant survival benefit of gefitinib treatment in our NSCLC model. Unfortunately, the majority of patients treated with gefitinib eventually relapse, and the median response duration ranges from 8 to 13 months.[37] Roughly half of this acquired resistance occurs as a result of a second mutation in *EGFR*, T790M.[38,39] It was recently reported that acquired *cMET* amplification and the loss of *PTEN* also led to gefitinib resistance in lung cancer patients.[40–42] In all other cases, the source of resistance is unknown. Following treatment with 5 mg/kg gefitinib, our transgenic mice also developed resistance to gefitinib and died of lung tumors within 15 months. We are currently exploring the mechanism of acquired gefitinib resistance in our model mice.

In conclusion, the development of lung adenocarcinoma in our mutant-*EGFR* transgenic mice confirmed the critical role of *EGFR* mutations in NSCLC oncogenesis. The model presented here may be useful in elucidating the mechanisms of carcinogenesis, chemoprevention, and acquired gefitinib resistance in activating *EGFR* mutation-related lung cancer without doxycycline.

Acknowledgments

We thank Masayo Kimura, Hiromi Nakashima, and Yoko Sato for expert technical support; Kyoko Takahashi and Mutsumi Maruyama for secretarial assistance and AstraZeneca for providing gefitinib. This work was supported in part by grant no. 19590895 from the Ministry of Education, Culture, Sports, Science, and Technology (KK).

doi: 10.1111/j.1349-7006.2008.00875.x

References

1 Herbst RS, Fukuoka M, Baselga J. Gefitinib – a novel targeted approach to treating cancer. *Nat Rev Cancer* 2004; **4** (12): 956–65.
2 Giaccone G. Epidermal growth factor receptor inhibitors in the treatment of non-small-cell lung cancer. *J Clin Oncol* 2005; **23** (14): 3235–42.
3. Fujiwara K, Kiura K, Ueoka H, Tabata M, Hamasaki S, Tanimoto M. Dramatic effect of ZD1839 ('Iressa') in a patient with advanced non-small-cell lung cancer and poor performance status. *Lung Cancer* 2003; **40** (1): 73–6.
4 Shigematsu H, Lin L, Takahashi T *et al*. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 2005; **97** (5): 339–46.
5 Toyooka S, Matsuo K, Shigematsu H *et al*. The impact of sex and smoking status on the mutational spectrum of epidermal growth factor receptor gene in non small cell lung cancer. *Clin Cancer Res* 2007; **13** (19): 5763–8.
6 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.
7 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.
8 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 USA* 2004; **101** (36): 13306–11.
9 Shepherd FA, Rodrigues Pereira J, Ciuleanu T *et al*. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005; **353** (2): 123–32.
10 Tsao MS, Sakurada A, Cutz JC *et al*. Erlotinib in lung cancer – molecular and clinical predictors of outcome. *N Engl J Med* 2005; **353** (2): 133–44.
11 Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* 2004; **64** (24): 8919–23.
12 Tokumo M, Toyooka S, Kiura K *et al*. The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. *Clin Cancer Res* 2005; **11** (3): 1167–73.
13 Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA. Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. *Cancer Res* 2004; **64** (20): 7241–4.
14 Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 2004; **305** (5687): 1163–7.
15 Tang X, Shigematsu H, Bekele BN *et al*. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res* 2005; **65** (17): 7568–72.
16 Kozuki T, Hisamoto A, Tabata M *et al*. Mutation of the epidermal growth factor receptor gene in the development of adenocarcinoma of the lung. *Lung Cancer* 2007; **58** (1): 30–5.
17 Greulich H, Chen TH, Feng W *et al*. Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. *Plos Med* 2005; **2** (11): e313.
18 Jiang J, Greulich H, Janne PA, Sellers WR, Meyerson M, Griffin JD. Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. *Cancer Res* 2005; **65** (19): 8968–74.
19 Yun CH, Boggon TJ, Li Y *et al*. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* 2007; **11** (3): 217–27.
20 Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes Dev* 2006; **20** (11): 1496–510.
21 Ji H, Li D, Chen L *et al*. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell* 2006; **9** (6): 485–95.
22 Korfhagen TR, Glasser SW, Wert SE *et al*. Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. *Proc Natl Acad Sci USA* 1990; **87** (16): 6122–6.
23 Wang-Gillam A, Siegel E, Mayes DA *et al*. Anti-tumor effect of doxycycline on glioblastoma cells. *J Cancer Mol* 2007; **3** (5): 147–53.
24 Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. *Cancer Sci* 2007; **98** (12): 1817–24.
25 Igarashi M, Yoshida M, Watanabe M *et al*. Lung carcinogenesis induced by NNK in Ogg1 knockout mice. Proceedings 66th Annual Meeting of the Japanese Cancer Association.
26 Kohno T, Shinmura K, Tosaka M *et al*. Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. *Oncogene* 1998; **16** (25): 3219–25.
27 Kohno T, Kunitoh H, Toyama K *et al*. Association of the OGG1-Ser326Cys polymorphism with lung adenocarcinoma risk. *Cancer Sci* 2006; **97** (8): 724–8.
28 Sugimura H, Kohno T, Wakai K *et al*. hOGG1 Ser326Cys polymorphism and lung cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* 1999; **8** (8): 669–74.
29 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** (22): 5007–18.
30 Hirata A, Hosoi F, Miyagawa M *et al*. HER2 overexpression increases sensitivity to gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, through inhibition of HER2/HER3 heterodimer formation in lung cancer cells. *Cancer Res* 2005; **65** (10): 4253–60.
31 Fujimoto N, Wislez M, Zhang J *et al*. High expression of ErbB family members and their ligands in lung adenocarcinomas that are sensitive to inhibition of epidermal growth factor receptor. *Cancer Res* 2005; **65** (24): 11478–85.
32 Engelman JA, Jänne PA, Mermel C *et al*. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci USA* 2005; **102** (10): 3788–93.
33 Yatabe Y, Kosaka T, Takahashi T, Mitsudomi T. EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. *Am J Surg Pathol* 2005; **29** (5): 633–9.
34 Tanaka H, Yanagisawa K, Shinjo K *et al*. Lineage-specific dependency of lung adenocarcinomas on the lung development regulator TTF-1. *Cancer Res* 2007; **67** (13): 6007–11.
35 Thatcher N, Chang A, Parikh P *et al*. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 2005; **366** (9496): 1527–37.
36 Kelly K, Chansky K, Gaspar LE *et al*. Updated analysis of swog 0023: a randomized phase III trial of gefitinib versus placebo maintenance after definitive chemoradiation followed by docetaxcel in patients with locally advaanced stage III non-small cell lung cancer. *ASCO Annual Meeting Proceedings* 2007.
37 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 (The IDEAL 1 Trial). *J Clin Oncol* 2003; **21** (12): 2237–46.
38 Kobayashi S, Boggon TJ, Dayaram T *et al*. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005; **352** (8): 786–92.
39 Pao W, Miller VA, Politi KA *et al*. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *Plos Med* 2005; **2** (3): e73.
40 Engelman JA, Zejnullahu K, Mitsudomi T *et al*. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007; **316** (5827): 1039–43.
41 Bean J, Brennan C, Shin JY *et al*. *MET* amplification occurs with or without *T790M* mutations in *EGFR* mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci USA* 2007; **104** (52): 20932–7.
42 Yamasaki F, Johansen MJ, Zhang D *et al*. Acquired resistance to erlotinib in A-431 epidermoid cancer cells requires down-regulation of MMAC1/PTEN and up-regulation of phosphorylated Akt. *Cancer Res* 2007; **67** (12): 5779–88.