The impact of human *EGFR* kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies

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Summary

To understand the role of human epidermal growth factor receptor (*hEGFR*) kinase domain mutations in lung tumorigenesis and response to EGFR-targeted therapies, we generated bitransgenic mice with inducible expression in type II pneumocytes of two common hEGFR mutants seen in human lung cancer. Both bitransgenic lines developed lung adenocarcinoma after sustained hEGFR mutant expression, confirming their oncogenic potential. Maintenance of these lung tumors was dependent on continued expression of the EGFR mutants. Treatment with small molecule inhibitors (erlotinib or HKI-272) as well as prolonged treatment with a humanized anti-hEGFR antibody (cetuximab) led to dramatic tumor regression. These data suggest that persistent EGFR signaling is required for tumor maintenance in human lung adenocarcinomas expressing EGFR mutants.

Introduction

EGFR is a tyrosine kinase receptor that plays an integral part in signaling pathways that control normal and malignant cell growth (Arteaga, 2003; Hynes and Lane, 2005). Because EGFR is overexpressed in many different tumor types, including more than 80% of non-small cell lung cancers (NSCLC), it was one of the first molecules to be selected for the development of targeted therapies (Hynes and Lane, 2005; Janne et al., 2005; Minna et al., 2005). However, it remains unclear whether overexpression of EGFR plays a crucial role in the malignant transformation of various cell types or is merely a secondary consequence of a selection process for growth advantage after tumor initiation. For EGFR-targeted therapy to be effective, the targeted tumor should ideally depend on EGFR activity for its malignant transformation and survival. Thus, the difference seen in the effectiveness of EGFR-targeted therapy in NSCLC patients with different EGFR status might be due to the heterogeneous roles that EGFR plays in the development of individual tumors.

Small molecule inhibitors of the EGFR enzymatic activity and antibodies against EGFR are the two major classes of agents that have been developed and used clinically for the treatment of cancers known to express EGFR (Giaccone, 2005; Hynes and Lane, 2005). While monoclonal antibodies such as cetuximab were designed to interfere with the binding of ligands to the hEGFR extracellular domain and to block downstream signaling, small molecules, including either reversible inhibitors

SIGNIFICANCE

The somatic *hEGFR* kinase domain mutations are highly correlated with the clinical response to gefitinib or erlotinib therapy in lung cancer patients. Our findings that these hEGFR kinase domain mutants are oncogenic in vivo and that their continual expression is necessary for tumor maintenance validate the importance of the mutated enzyme as a therapeutic target. EGFR-targeted therapy against murine cancers overexpressing hEGFR mutants is dramatically effective, suggesting that these mutants are directly involved in tumor maintenance. These inducible mouse models will be useful for evaluation of a new generation of EGFR inhibitors as well as other novel therapeutics prior to human clinical trials.

like gefitinib and erlotinib or the irreversible inhibitor HKI-272 (Kwak et al., 2005; Rabindran et al., 2004), are intracellular tyrosine kinase enzymatic inhibitors (TKI) that disrupt the kinase activity by binding the adenosine triphosphate (ATP) pocket within the catalytic domain (Giaccone, 2005; Hynes and Lane, 2005). Although the overall response rate of NSCLC patients to small molecule EGFR inhibitors was only 10% to 20% in several large clinical trials, somatic mutations in the kinase domain of *hEGFR* gene in NSCLC patients were subsequently found to be highly correlated with sensitivity (>80%) to gefitinib and erlotinib therapy (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004).

Emerging data also suggest that increased *EGFR* copy number is a predictor of response to gefitinib therapy, regardless of *hEGFR* kinase domain mutational status (Cappuzzo et al., 2005a). Increased copy number of *hEGFR*, however, was observed more frequently in patients bearing *hEGFR* mutations than in patients with wild-type (wt) *EGFR* (56% versus 22%), suggesting that the mutant alleles are selectively amplified. A similar finding has been observed in NSCLC cell lines (Gazdar et al., 2004; Sordella et al., 2004; Suzuki et al., 2005; Takano et al., 2005; Tracy et al., 2004). These findings have been construed to represent that the transformed cells exhibit an ''oncogene addiction'' to the EGFR mutants (Blagosklonny, 2004; Gazdar et al., 2004). Furthermore, in contrast to wt *hEGFR*, the *hEGFR* kinase domain mutations are mutually exclusive of *K-ras* mutation (Pao et al., 2005b; Shigematsu et al., 2005a; Soung et al., 2005). In addition, recent preclinical and clinical data suggest that cetuximab is not as effective as gefitinib or erlotinib in treating patients with lung cancer whose tumors harbor *hEGFR* kinase domain mutations (Mukohara et al., 2005; Tsuchihashi et al., 2005). These studies support the conclusion that hEGFR kinase domain mutants may have a different role in a tumor than wt hEGFR, the latter more likely being a secondary event in cancer progression due to a growth advantage selection, while the former may play a causal role in malignant transformation. A better understanding of the role of the hEGFR kinase domain mutants in the tumor initiation and maintenance in vivo will enhance our understanding and guide the use of EGFR-targeted therapy in patients whose tumors harbor these mutations.

Two of the most common kinase domain *hEGFR* mutations, accounting for approximately 80% of all known mutations in NSCLC, are the exon 19 deletion of the conserved four amino acid sequence LREA and the L858R point mutation in exon 21(Pao and Miller, 2005; Shigematsu et al., 2005a; Shigematsu et al., 2005b). In vitro studies using NIH3T3 and Ba/F3 cells showed that these hEGFR kinase domain mutants are transforming and confer sensitivity of these transformed cells to gefitinib and erlotinib growth inhibition (Greulich et al., 2005; Kobayashi et al., 2005b). Furthermore, most of the NSCLC cell lines harboring the *hEGFR* kinase domain mutations are highly sensitive to gefitinib or erlotinib (Amann et al., 2005; Paez et al., 2004; Tracy et al., 2004). Retrospective studies have demonstrated that NSCLC patients harboring the *hEGFR* kinase domain mutations treated with gefitinib have a significantly greater response (89%) and longer survival compared to those with wt *EGFR* (Mitsudomi et al., 2005; Takano et al., 2005). However, the precise roles these *hEGFR* kinase domain mutations play in the initiation, progression, and maintenance of NSCLC in vivo remains unclear. Additionally, the importance of these mutations in predicting response to EGFR-targeted therapies such as small molecule inhibitors and monoclonal antibodies remains a clinically important but unanswered question.

To this end, we generated inducible bitransgenic mice that express the two common hEGFR kinase domain mutants, the exon 19 deletion of the conserved region including LREA (referred to as *Del*) and the exon 21 substitution point mutation *L858R.* The expression of these two hEGFR mutants was targeted to lung type II pneumocytes using the *CCSP-rtTA* allele (Fisher et al., 2001). We demonstrated that the expression of either hEGFR mutant in the lungs of these bitransgenic mice is sufficient for the development of adenocarcinoma with bronchioloalveolar carcinoma (BAC) features. The histopathologic features of murine lung tumors were similar to features present in tumors from NSCLC patients harboring these *hEGFR* kinase domain mutations. Deinduction of the hEGFR mutant expression in established adenocarcinomas caused tumor regression, demonstrating that the activated EGFR pathway was necessary for tumor maintenance. Lastly, these lung tumors driven by the expression of hEGFR kinase domain mutants dramatically responded to the small molecule EGFR inhibitors erlotinib and HKI-272 as well as more prolonged treatment with the monoclonal anti-hEGFR antibody, cetuximab.

Results

Generation of Tet-op-hEGFR L858R-Luc and Tet-op-hEGFR Del-Luc alleles

To generate mice with inducible expression of two common *hEGFR* mutants, an exon 19 deletion mutant (*Del*) and exon 21 *L858R* mutant (*L858R*) in murine lung epithelial cells, we constructed a 6.0 kb DNA segment consisting of seven direct repeats of the tetracycline (tet)-operator sequence, an *hEGFR L858R* or *hEGFR Del* cDNA, and internal ribosome entry site (*IRES*) followed by *luciferase* cDNA and the *SV40* poly (A) (Figure S1A in the Supplemental Data available with this article online, and Experimental Procedures). Expression of the luciferase protein linked to IRES allowed us to monitor the concurrent expression of hEGFR mutants in the affected cell populations through in vivo noninvasive imaging. These constructs were injected into FVB/N blastocysts and the progeny was screened using Southern blot and PCR strategy (data not shown). Eight *hEGFR L858R* founders and 12 *hEGFR Del* founders, referred to as *Tet-op-hEGFR L858R-Luc* and *Tet-op-hEGFR Del-Luc*, respectively, were identified from these analyses and then crossed to Clara cell secretory protein (*CCSP*)-*rtTA* mice, harboring an allele that had been shown specifically to target the expression of the reverse tetracycline transactivator protein (rtTA) in type II alveolar epithelial cells (Fisher et al., 2001; Perl et al., 2002). This allowed us to generate bitransgenic mouse cohorts harboring both the activator and the responder transgenes to test the inducibility of the transgene with doxycycline administration (Fisher et al., 2001; Perl et al., 2002). Two tightly inducible *hEGFR Del* (#19 and #23) founders and three *hEGFR L858R* founders (#12, #14, and #27) were identified by RT-PCR analysis, with low or no baseline expression of transgene that can be induced up to 10-fold after 2 weeks of doxycycline administration (see below). Additionally, the copy numbers from individual founders were determined by quantitative real-time PCR (Figure S1B). The *Del* (#19 and #23) founders and *L858R* founders (#14 and #27) with similar transgene copy numbers were chosen for the following experiments.

Inducibility of both hEGFR mutants L858R and Del in lung tissues

The inducibility of the two human *EGFR* kinase domain mutant transgenes was evaluated in the lungs at both RNA and protein levels. RT-PCR with transgene-specific primers was performed to determine the *hEGFR* mutant RNA level in the lungs of the bitransgenic mouse *CCSP-rtTA/Tet-op-hEGFR L858R-Luc* and *CCSP-rtTA/Tet-op-hEGFR Del-Luc* cohorts for each potential founder before and after 2 weeks of doxycycline administration. All mice had normal lung histology (data not shown). The *hEGFR* mutant transcripts were undetectable from both nontransgenic and the bitransgenic mice without doxycycline treatment, but readily detectable after 2 weeks of doxycycline administration in both *Del* and *L858R* mice lines (Figure 1A). To confirm that the induction of hEGFR mutants also occured at the protein level, immunoblotting was performed on lung lysates from the bitransgenic mice before and after doxycycline administration. Typical induction in total EGFR protein levels was shown from bitransgenic mice from both the *Del* and *L858R* lines (Figure 1B). This subsequently led to the activation of EGFR by the phosphorylation of three important tyrosine residues, 992, 1068, and 1173, which are associated with both cell proliferation and survival signaling (Figure 1B) (Sordella et al., 2004). Since normal lungs express a fairly low level of mouse endogenous EGFR, no phosphorylation of EGFR was observed in nontransgenic wt mice (Figure 1B). Therefore, the EGFR phosphorylation and activation most likely resulted from the induction of hEGFR kinase domain mutant expression.

To further confirm the in vivo induction of the transgenes, we took advantage of the noninvasive bioluminescent imaging to detect the activity of coexpressed luciferase (Zhang et al., 2004). The bitransgenic mice from both *L858R* and *Del* lines (3 each group) were imaged on day 1 before doxycycline administration to confirm the lack of luciferase expression at baseline (Figure S2A). After 5 days of doxycycline administration, these mice showed high luciferase activity specifically over the regions of the lungs, indicating the tissue-specific induction of hEGFR mutants (Figure S2B). Withdrawal of doxycycline from the diet was started on day 5, and these mice were then subsequently imaged on days 6, 8, and 10. The luciferase activity rapidly declined on day 6 after 1 day of doxycycline withdrawal, and completely disappeared on day 10 (Figures S2C–S2E). Correspondingly, we showed an extinction of hEGFR mutant expression with similar kinetics upon doxycycline withdrawal (see below). Thus, these data support the specific expression of the transgenes in the lung and their temporal regulation by doxycycline administration.

Overexpression of the hEGFR L858R and hEGFR Del mutants drive initiation and progression of the lung adenocarcinoma with BAC features

To determine if overexpression of the hEGFR kinase domain mutants can initiate lung tumorigenesis, bitransgenic mice from both the *L858R* and *Del* cohorts on continuous doxycycline administration were sacrificed at various time points for histological examination of the lungs. In contrast to the normal histology in untreated mice (Figure 2A), early precancerous atypical adenomatous hyperplasia (AAH) lesions started to appear after 3–4 weeks of doxycycline treatment (Figure 2B). After 5–6 weeks of continuous doxycycline administration, multiple tumor foci of BAC were present in both lungs of bitransgenic mice (Figure 2C).

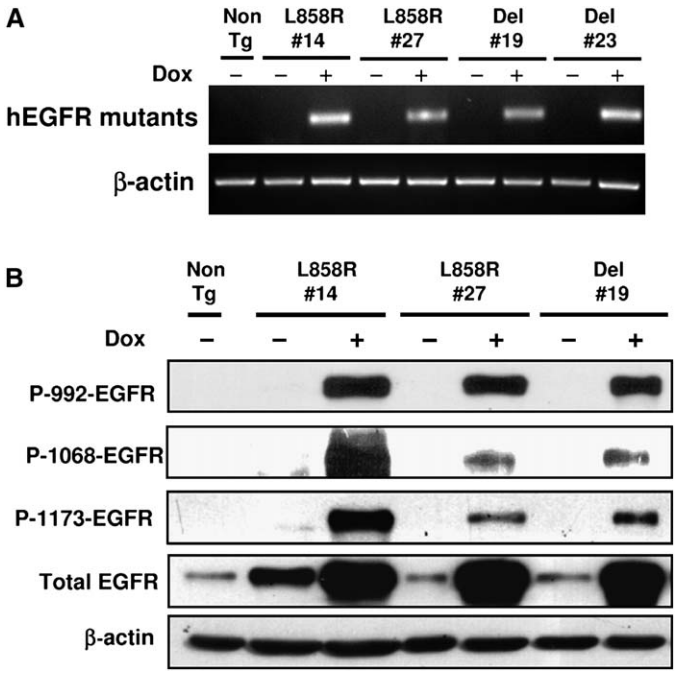

Figure 1. Induction of *hEGFR* kinase domain mutants at both RNA and protein levels in bitransgenic mice from different founders of *L858R* and *Del* lines

A: Bitransgenic mice from indicated lines were divided into two groups and given a diet with or without doxycycline. After 2 weeks of doxycycline treatment, the transcriptional levels of *hEGFR* mutants in the lungs from these two groups were evaluated by RT-PCR, as described in the Experimental Procedures.

B: Protein levels of both mouse and human total EGFR as well as phosphorylated EGFR at different tyrosine residues were analyzed by immunoblotting. β-actin serves as loading control. Representative data are shown from three independent experiments.

Tumors diffusely involved the lung parenchyma, the peripheral spaces surrounding the airways and the subpleural regions (Figure 2C). Benign adenoma was also notable at this stage (Figure 2D). Invasive adenocarcinomas with acinar, papillary, and solid features were present after 8–10 weeks (Figure 2E). The tumors were positive for phospho-EGFR Y1068, indicating that the induction of hEGFR mutants leads to the activation of EGFR signaling (Figure 2F). This EGFR activation is accompanied with phosphorylation and activation of downstream signaling molecules, including Erk1/2 (Figure 2G) and Akt (Figure 2H).

Tumors driven by the expression of these hEGFR kinase domain mutants stained positive for prosurfactant protein C (SP-C) (Figure 2I) but negative for the Clara cell marker CCSP (Figure 2J). Although these data suggest a type II pneumocyte tumor cell origin, it is also possible that these tumors initiated from progenitor/stem cells located in the terminal airways that are double-positive for CCSP and SPC, with the ability to differentiate into single-positive cells dependent on the location (Kim et al., 2005). None of the monotransgenic *hEGFR* mutant mice (>5 mice in each cohort) from both lines (*L858R* #27 and *Del* #19) and the *CCSP-rtTA* monotransgenic mice (5 mice) developed lung tumors or other lung pathological changes despite doxycycline administration for up to 32 weeks. These data confirm the nonleakiness of the bitransgenic regulatory system in these founders and that doxycycline exposure does not play a crucial role in lung tumorigenesis in our system (Seftor et al.,

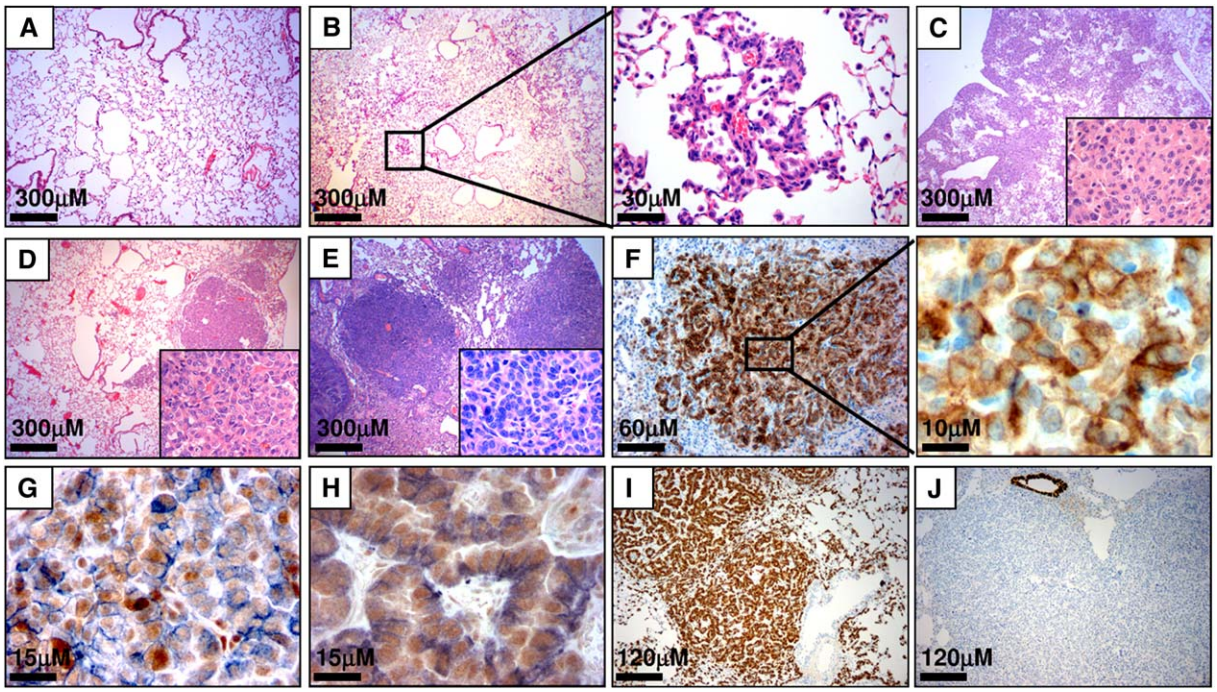

Figure 2. Expression of hEGFR mutants leads to the development of lung adenocarcinoma with BAC feature

A: Representative photomicrographs of a cross-sectional view from the *L858R* line of mouse lung illustrating histopathologic assessment of lung carcinoma. At least 4 mice at each time point were examined for histology. Bitransgenic mice up to 30 weeks old without doxycycline treatment have normal histopathologic features.

B: Early precancerous AAH lesions started to appear after 3 to 4 weeks of doxycycline treatment.

C and D: Multiple tumor foci of BAC (**C**) and benign adenoma (**D**) are present after 5–6 weeks of continuous doxycycline administration.

E: Invasive adenocarcinoma appears after 8 or more weeks of continuous doxycycline treatment.

F–H: Tumors are positive for phospho-EGFR (**F**), stained in blue/gray, associated with downstream activation of Erk1/2 (**G**) and Akt (**H**), stained in brown.

I and J: The tumors are positive for SPC staining (**I**) but negative for CCSP staining (**J**).

1998). Bitransgenic mice from the two founders of the *hEGFR Del* allele (#19 and #23) and three founders of the *hEGFR L858R* allele (#12, #14, and #27) all developed indistinguishable adenocarcinoma with BAC features after 8 or more weeks of sustained doxycycline induction (data not shown).

The induction of hEGFR mutant in the lungs in the bitransgenic mice as a function of time on doxycycline treatment was further analyzed molecularly at both RNA and protein levels (Figure 3). In both *L858R* and *Del* bitransgenic lines, the *hEGFR* transcripts appeared after 2 weeks of doxycycline treatment and increased with time for 8 or more weeks of doxycycline treatment (Figure 3A). This was further confirmed by quantitative real-time RT-PCR. After 2 weeks of doxycycline treatment, a significant increase of total human and mouse *EGFR* mRNA was observed, approximately 8-fold in the *L858R* line (#27) and 10-fold in the *Del* line (#19) (Figure 3B). Continuous doxycycline treatment for 8 or more weeks was accompanied by even higher *EGFR* levels, with a 32-fold increase in *L858R* line and a 77-fold increase in *Del* line (Figure 3B). This increase in expression likely represents an accumulation of *EGFR* mutant-expressing cells at the later time point, rather than increased expression of the transgene on a per-cell basis. Consistent with this observation, total EGFR protein level increased with time on doxycycline treatment (Figure 3C), which was associated with an increase in EGFR phosphorylation and activation of the downstream signaling molecules Akt and Erk1/2 (Figure 3C). A baseline level of phospho-Akt and phospho-Erk1/2 in the lungs from either normal wt or single transgenic mice or the bitransgenic mice without doxycycline administration was consistently observed. Sequencing analyses of these lung tumors showed that they are wild-type for *K-ras*, and real-time PCR data demonstrated that there was no amplification of the *hEGFR* mutant transgene or the mouse endogenous *EGFR* locus during the process of tumorigenesis (data not shown). No difference in the *EGF* and *TGF-*α transcript levels in the lungs from bitransgenic mice before and after sustained doxycycline administration was observed (data not shown), although it remains formally possible that other EGFR ligands such as *amphiregulin*, *HB-EGF*, or *betacellulin* might be differentially affected (Hynes and Lane, 2005). These data indicate that overexpression of EGFR mutant driven by the high copy of transgenes itself may be sufficient to drive lung tumorigenesis and progression.

Expression of the hEGFR mutants is essential for tumor maintenance

To determine whether the continued expression of hEGFR kinase domain mutants is required for tumor maintenance and cell survival, doxycycline was withdrawn from the diet of tumor-bearing mice to deinduce the mutated *hEGFR* transgene expression. These tumor-bearing mice were serially analyzed molecularly, radiographically, and histopathologically before and after doxycycline withdrawal. RT-PCR was performed to determine the *hEGFR* kinase domain mutant expression level on the pathologic lungs of bitransgenic mouse cohorts on doxycycline with tumors and after doxycycline withdrawal. After withdrawal of doxycycline from the diet for only 3 days, the *hEGFR*

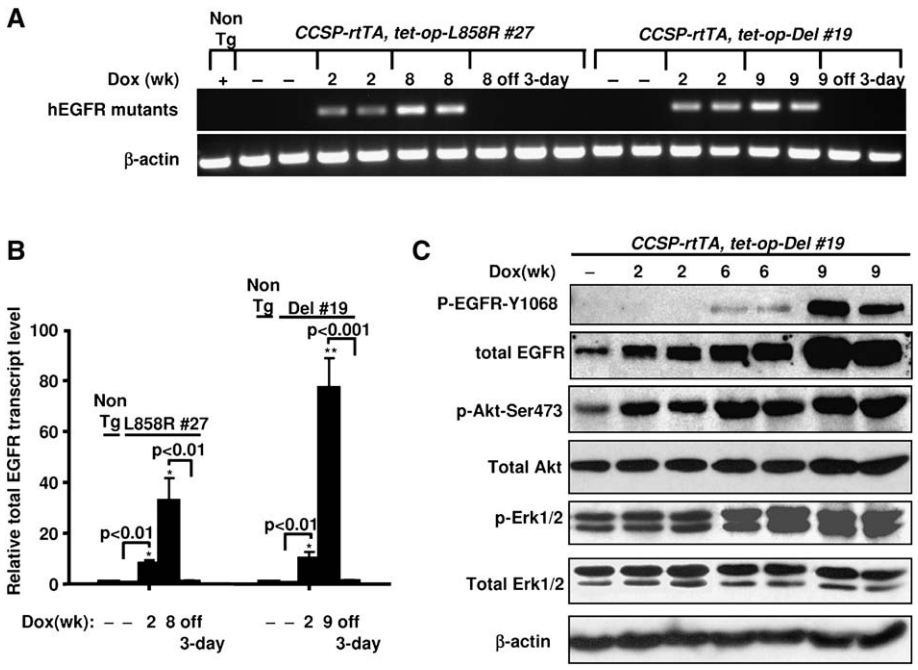

Figure 3. Molecular analysis of the induction of hEGFR kinase domain mutants with time on doxycycline administration and activation of downstream signaling

A: Induction and deinduction of *hEGFR* kinase domain mutant mRNA transcript by feeding the bitransgenic mice a diet with or without doxycycline as indicated. β-actin transcriptional level serves as negative control.

B: Quantitative RT-PCR analysis of total *EGFR* transcript induction and deinduction in bitransgenic mice from both *L858R* and *Del* lines. Each sample was amplified in triplicate for detection of both total *EGFR* and β-*actin* transcripts. The endogenous mouse *EGFR* level from nontransgenic mice was arbitrarily designated as 1 and used to derive the standard curve. Data were analyzed by relative quantitation using the comparative Ct method and normalization to β-*actin*, and error bars correspond to mean ± standard deviation. Statistical analyses were preformed using Student's exact t test.

C: Bitransgenic mice from *Del* lines were treated with doxycycline for the indicated time, and lung lysates were subjected to immunoblotting for total EGFR, phospho-EGFR, and downstream phospho-Akt and phospho-Erk1/2 protein levels. β-actin serves as loading control. Representative data are shown from three independent experiments.

mutant RNA levels in the lungs from both two lines returned to levels similar to those from untreated control mice (Figure 3A). This deinduction of expression was further confirmed by quantitative real-time RT-PCR (Figure 3B).

To demonstrate that continued hEGFR kinase domain mutant expression is necessary for lung tumor maintenance, bitransgenic *CCSP-rtTA/Tet-op-L858R-Luc* and *CCSP-rtTA/Tet-op-Del-Luc* mice (4 mice per group) treated with 8 weeks doxycycline were imaged with a baseline MRI scan to document the lung tumor burden in each mouse. Doxycycline was then removed from the diet for 1 week, and animals were then subjected to MRI reimaging. In all these mice, the lung tumors consistently regressed dramatically (with a decrease of 93.4% ± 2.9% in the *Del* line and 91.3% ± 2.1% in the *L858R* line) as documented by MRI (Figure 4A). These observations established the essential role of persistent expression of hEGFR mutants on lung tumor maintenance. To test if tumors recur after complete regression, we performed the long-term doxycycline withdrawal experiments. After 3 weeks of doxycycline withdrawal, a complete regression of the lung tumors was observed (Figure 4B), and there were no tumor recurrences with an additional 6 weeks of doxycycline withdrawal (Figure 4B). This further confirms the essential role of EGFR mutant expression in lung tumor maintenance and validates EGFR mutants as good targets for cancer therapies.

In comparison with the tumors from mice that remained on doxycycline (Figure 5A), there was a dramatic decrease in tumor density and cellularity after 1 week of doxycycline withdrawal (Figure 5B). There were foci of mildly increased interstitial thickness and cellularity that likely represented the remnants of the tumors after doxycycline withdrawal (Figure 5B). These histologic responses correlated with the MRI analysis of tumor regression. No residual tumors were found in lungs from three bitransgenic mice after doxycycline was removed from the diet for more than 3 weeks (Figure 5C). Concomitant with the rapid tumor regression after 1 week of doxycycline withdrawal, we observed a 23-fold decrease of Ki-67-positive tumor cells (Figures 5D, 5E, and 5H). To determine if the tumor regression was also associated with apoptosis, we performed TUNEL assays using tumor samples from bitransgenic mice before and after doxycycline withdrawal (Figures 5F, 5G, and 5I). We noted a 20-fold increase of TUNEL-positive cells after 1 week of doxycycline withdrawal (Figure 5I). Consistent with this, Western blot analyses using the whole lung lysate showed that after doxycycline withdrawal, a dramatic reduction of both total EGFR and activated EGFR levels was observed in the lungs of these bitransgenic mice (Figure 5J). The rapid decrease of EGFR protein level likely reflects both decreased transcription of mutant EGFR and the significant reduction in the number of mutant EGFR-expressing tumor cells as shown from histological analyses (Figure 5B). These data demonstrate that a marked reduction of tumor cell proliferation and increase in tumor cell apoptosis is associated with deprivation of hEGFR mutant expression, demonstrating the requirement of hEGFR mutant expression for tumor maintenance.

The hEGFR mutant-driven lung tumors are sensitive to treatment with erlotinib or HKI-272 as well as a prolonged course of cetuximab treatment

To investigate the sensitivity of these hEGFR kinase domain mutant-driven lung tumors to different EGFR-targeted therapies, we serially imaged the tumor-bearing bitransgenic mice before and after treatment with either erlotinib, a reversible EGFR inhibitor, or HKI-272, an irreversible EGFR inhibitor (Kwak et al., 2005; Rabindran et al., 2004). After 8 weeks of doxycycline treatment, bitransgenic *CCSP-rtTA/Tet-op-hEGFR L858R-Luc* and *CCSP-rtTA/Tet-op-hEGFR Del-Luc* mice were imaged with MRI to document the baseline tumor burden. Tumor-bearing mice were then treated orally with erlotinib (6 mice from both *Del* and *L858R* lines), HKI-272 (4 mice from *Del* and 5 from *L858R* lines), or placebo (3 mice per line). Both compounds were given by gavage at a dose of 50 mg/kg for 1 week, and all mice were

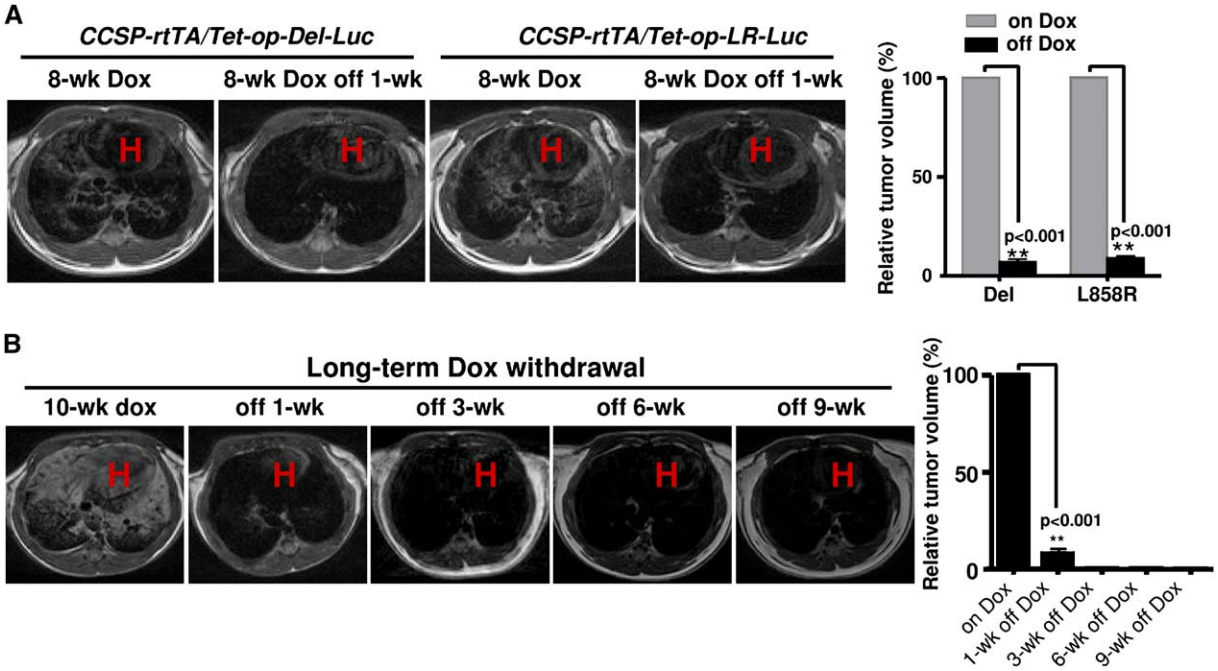

Figure 4. Radiographical analyses of the essential roles of hEGFR kinase domain mutants for tumor maintenance

A: Bitransgenic *CCSP-rtTA/Tet-op-hEGFR L858R-Luc* or *CCSP-rtTA/Tet-op-hEGFR Del-Luc* mice were given a doxycycline diet for 8 weeks, which was then replaced with a normal diet for 1 week.

B: For long-term doxycycline withdrawal experiments, the three bitransgenic *CCSP-rtTA/Tet-op-hEGFR Del-Luc* mice were given doxycycline diet for 9 week, which was then replaced with a normal diet for a long period.

All mice were MRI imaged before and after doxycycline withdrawal at indicated time points. Hearts are indicated as H. Bar diagram expressed as mean ± standard deviation illustrates the tumor regression measured by MRI, and statistical analyses were performed using Student's exact t test.

kept on doxycycline throughout the study. After 1 week of treatment, bitransgenic mice from both *L858R* and *Del* lines receiving either erlotinib or HKI-272 demonstrated a significant tumor burden reduction (81% ± 7% for erlotinib and 85% ± 8% for HKI-272) as documented by restaging MRI (Figure 6). These data demonstrate that inhibition of EGFR activity using TKIs is a highly effective therapy in these tumors, since the hEGFR kinase domain mutants are directly involved in the tumor initiation, cell survival, and tumor maintenance.

Cetuximab is a humanized monoclonal antibody designed to interfere with the binding of EGF or other ligands to the extracellular domain of EGFR (Giaccone, 2005; Hynes and Lane, 2005; Minna et al., 2005). In order to determine the efficacy of cetuximab monotherapy in an in vivo model, we treated tumor-bearing bitransgenic mice (4 mice from *L858R* line) with 1.0 mg cetuximab by intraperitoneal (I.P.) injection every two days. After 1 week of treatment, these mice underwent reimaging and were sacrificed for histological analysis. The targeting of cetuximab in the lung tumors driven by hEGFR kinase domain mutant expression was confirmed by positive fluorescence immunostaining using anti-human IgG-FITC in comparison with the negative staining in those tumors treated with placebo (data not shown). In contrast to HKI-272-treated or erlotinib-treated mice, cetuximab therapy was less effective (46% ± 23%) than erlotinib as measured by MRI imaging after 1 week of treatment (Figure 6).

Histological analyses of the lungs from the mice treated with erlotinib or HKI-272 for 1 week confirmed the dramatic reduction of tumor burden seen in the imaging scans (Figure 7A). Furthermore, in contrast to the group treated with cetuximab for 1 week, the lungs treated with erlotinib or HKI-272 were grossly normal and microscopically similar to that seen in the mice after doxycycline withdrawal (Figure 7A and data not shown). In the areas likely representing the remnants of tumors treated with either erlotinib or HKI-272, a significantly lower number of Ki-67-positive cells were visible compared with tumors from cetuximab-treated mice (Figures 7A and 7B). Positive TUNEL staining was also notable in remnant tumor areas, suggesting that the treatment of either erlotinib or HKI-272 results in tumor resolution associated with an apoptotic process (Figures 7A and 7C). In contrast, the tumors treated with cetuximab for 1 week retained similar pathologic features to those from untreated animals showing high Ki-67 staining and low TUNEL staining. In accord with these findings, treatment with erlotinib or HKI-272 for 1 week decreased both the total EGFR and activated EGFR levels in the lungs similar to those observed after doxycycline withdrawal, whereas the effects of 1 week of cetuximab treatment on total and activated EGFR were considerably more modest (Figure 7D). Thus, these data demonstrate that pharmacologically effective EGFR inhibition leads to rapid tumor regression associated with decreased tumor proliferation and increased apoptosis of tumor cells.

As 1 week of cetuximab treatment was not as effective as erlotinib or HKI-272, we sought to determine the relative efficacy of these agents to longer-term treatment. To this end, we performed the treatment using either erlotinib or cetuximab for 4 weeks (3 mice each from *L858R* line). Interestingly, after 2 weeks of cetuximab treatment, we began to observe a larger decrease of tumor burden (75% ± 5%), and after 4 weeks of treatment, a progressive 94% ± 4% shrinkage of the tumors, comparable to the durable tumor response seen with 4 weeks of erlotinib

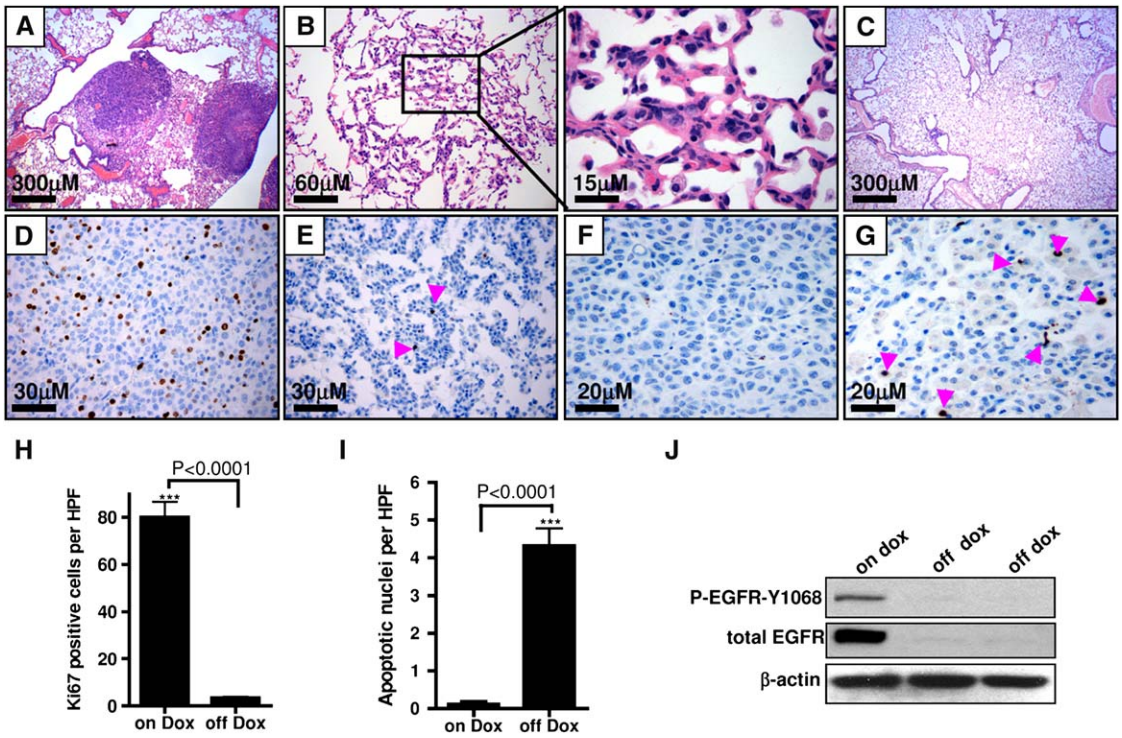

Figure 5. Molecular and histopathological analysis of the essential roles of *hEGFR* kinase domain mutants for tumor maintenance

A–C: Bitransgenic *CCSP-rtTA/Tet-op-hEGFR L858R-Luc* or *CCSP-rtTA/Tet-op-hEGFR Del-Luc* mice with lung tumors documented by MRI imaging were analyzed by histopathologic examination before (**A**) and 1 week (**B**) and 3 weeks (**C**) after doxycycline withdrawal.

D–G: In contrast to those tumors under continuous doxycycline treatment (**D**, Ki-67 staining and **F**, TUNEL staining), dramatically decreased Ki-67-positive cells (**E**) and increased TUNEL-positive cells (**G**), indicated by arrows, are present in lung tumors from the bitransgenic mice after one week of doxycycline withdrawal.

H and I: Bar diagrams expressed as mean ± standard deviation illustrating the proliferative (**H**) and apoptotic (**I**) indices in lung tumors before and 1 week after doxycycline withdrawal were determined from at least 200 high-power fields (HPF). Statistical analyses were preformed using Student's exact t test.

J: Protein levels of total EGFR and phospho-EGFR Y1068 were analyzed by immunoblotting. β-actin served as loading control. Data shown are representative of three independent experiments.

treatments (Figure 8). These data suggest that although 1 week of cetuximab treatment is not as efficient as small TKI treatment, with a prolonged treatment, the efficacy of cetuximab is comparable to that of erlotinib treatment.

Discussion

We have demonstrated that the two common hEGFR kinase domain mutants are oncogenic in vivo and that their expression is essential for tumor initiation, survival, and maintenance, validating these hEGFR kinase domain mutants as therapeutic targets for cancer therapy. Similar results have been obtained by another group that also generated inducible bitransgenic mice expressing the human EGFR kinase domain mutants specifically in the lungs (Politi et al., 2006).

Consistent with both in vitro human NSCLC cell lines and clinical studies, the mouse lung tumors driven by overexpression of hEGFR kinase domain mutants are very sensitive to either erlotinib or HKI-272 EGFR-targeted therapy in both short-term and long-term treatment. In contrast, short-term (1 week) treatment with cetuximab was less effective, although the prolonged treatment (4 weeks) did prove to be effective as well. Tumor regression caused by EGFR inhibitor treatment is associated with proliferative arrest and apoptosis. These results are consistent with the dramatic clinical responses to erlotinib or gefitinib treatment in NSCLC patients bearing tumors with these hEGFR kinase domain mutations.

Mutations of the *hEGFR* kinase domain usually occur in two regions of *EGFR*: exon 19 deletion of the amino acid sequence LREA and the exon 21 point mutation *L858R*. Initial studies demonstrated that these two hEGFR mutants are more potent stimulators of the phosphoinositide-3 kinase (PI3K)/Akt survival signaling than wt hEGFR (Sordella et al., 2004). However, several other studies suggested that the exon 19 deletion mutant may function differently from L858R (Pao et al., 2004). When overexpressed in 293T cells, the hEGFR exon 19 deletion mutant had less autophosphorylation activity than either the wt EGFR or L858R mutant (Pao et al., 2004). Unlike the L858R mutant, the exon 19 deletion mutant had similar sensitivity to gefitinib as wt EGFR in these studies (Pao et al., 2004). The different findings observed between these sets of studies may reflect the use of different cell lines, each with a distinct complement of ErbB family members, for the various experiments (Janne et al., 2005; Pao et al., 2004; Sordella et al., 2004). Interestingly, there are emerging clinical data suggesting that lung cancer patients whose tumor harbored the *EGFR* exon 19 deletion and who were treated with erlotinib or gefitinib had a longer median survival than erlotinib- or gefitinib-treated patients with the *EGFR* L858R point mutation (Riely et al., 2006). In our study, we have demonstrated that both the exon 19 deletion mutant and the L858R mutant are equally transforming in vivo without notable phenotypic difference, as both caused the

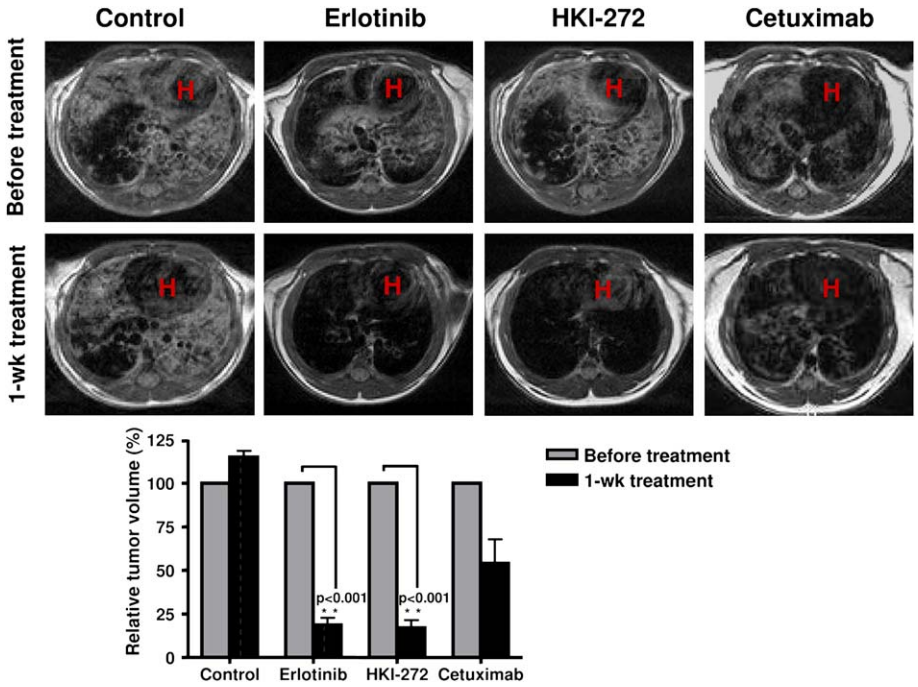

Figure 6. Differential response of lung tumors driven by hEGFR kinase domain mutant expression to 1-week treatment with erlotinib, HKI-272, or cetuximab

Bitransgenic *CCSP-rtTA/Tet-op-hEGFR L858R-Luc* or *CCSP-rtTA/Tet-op-hEGFR Del-Luc* mice were orally treated with either erlotinib or HKI-272 at 50 mg/kg daily or cetuximab I.P. at 1 mg per dose every two days. These mice were MRI imaged before and after 1 week of treatment. Empty vehicle was used as control. Representative imaging were shown from *L858R* line. Hearts are indicated as H. Bar diagram expressed as mean ± standard deviation illustrates the tumor regression measured by MRI, and statistical analyses were performed using Student's exact t test.

development of lung adenocarcinomas with BAC features in a similar time period with similar histology. Furthermore, in our mouse models, no notable differences were observed between these two mutants in their abilities to activate EGFR signaling, to initiate malignant transformation, or to confer sensitivity of tumors to short-term erlotinib or HKI-272 treatment. As highlighted by the in vitro studies, the presence of other ErbB family members may influence the efficacy of EGFR inhibition (Cappuzzo et al., 2005b; Engelman et al., 2005; Hirata et al., 2005). Additional studies are needed to determine the role of ErbB2 and ErbB3 in our murine models in lung tumorigenesis and in possible differential responses of the two *hEGFR* kinase domain mutations to long-term EGFR inhibition treatment.

EGFR-targeted therapies in lung tumors with overexpression of hEGFR kinase domain mutants are very effective in mice, consistent with the human clinical observation that most NSCLC patients with *hEGFR* kinase domain mutations respond to either erlotinib or gefitinib. Our mouse model data suggest that the lung tumors harboring *hEGFR* kinase domain mutations are dependent on the activated EGFR signaling for survival and thus provide a biological explanation for marked tumor responses caused by inhibition of this pathway. However, the *EGFR* kinase domain mutations are present in only 10% of lung adenocarcinomas in Caucasians and 30% of East Asian patients (Pao and Miller, 2005; Shigematsu et al., 2005a). There also appear to be NSCLC patients with wt *EGFR* who clinically benefit from gefitinib and erlotinib therapy by stabilizing disease and preventing further progression (Engelman and Janne, 2005; Giaccone, 2005). In vitro studies have suggested that expression of wt hEGFR in NSCLC might not play a prominent role in malignant transformation or survival pathways, but instead be involved in cell proliferation pathways (Adjei, 2005; Sordella et al., 2004). Consistent with these in vitro observations, thus far, we have failed to observe lung tumor development in 2 founders (more than 6 mice per founder cohort) with overexpression of wt hEGFR in lung compartments after 26 weeks of doxycycline administration (data not shown). It remains possible that lung tumors might develop with a much longer latency period and/or that other concurrent genetic alterations involving *p53* or *PTEN* are necessary for these wt EGFR-overexpressing mice to develop lung cancer.

HKI-272 is an irreversible inhibitor that covalently binds to the EGFR kinase domain cleft, whereas erlotinib and gefitinib are reversible EGFR inhibitors. Almost all NSCLC patients whose tumors harbor *hEGFR* kinase domain mutations initially respond to gefitinib or erlotinib, but eventually acquire resistance to these inhibitors. Molecular analyses of some of the relapsed tumors have shown a secondary *hEGFR T790M* mutation (Kobayashi et al., 2005a; Kwak et al., 2005; Pao et al., 2005a). HKI-272 in vitro is able to overcome that resistance (Kwak et al., 2005). Recently, we have also shown that HKI-272 is effective in treatment of EGFRvIII-dependent mouse lung tumors; *EGFRvIII*, a well characterized activating *EGFR* mutation harboring an in-frame deletion of exon 2 to 7, was found to be present in a small percentage of human squamous cell lung cancers (Ji et al., 2006). Here, we demonstrate that HKI-272 is as effective as erlotinib in the treatment of tumors driven by expression of two major groups of hEGFR kinase domain mutants. It would be of interest to determine the potential efficacy of HKI-272 relative to erlotinib and gefitinib in patients who have not been exposed to EGFR inhibitors and whether or not a different spectrum of resistance mutations would develop after chronic treatment with either class of EGFR inhibitors or in combination.

Our results with cetuximab treatment in mice lung cancers driven by overexpression of hEGFR kinase domain mutants differ somewhat from recent publications of EGFR mutant cell lines studies in vitro (Mukohara et al., 2005). The in vitro studies suggest that cetuximab was less effective than gefitinib at inhibiting the growth of EGFR mutant cell lines. These studies were performed over a 72 hr exposure period, and in fact are similar to the short term (1 week) in vivo cetuximab treatment in the lung tumors bearing EGFR kinase domain mutants in mice. The

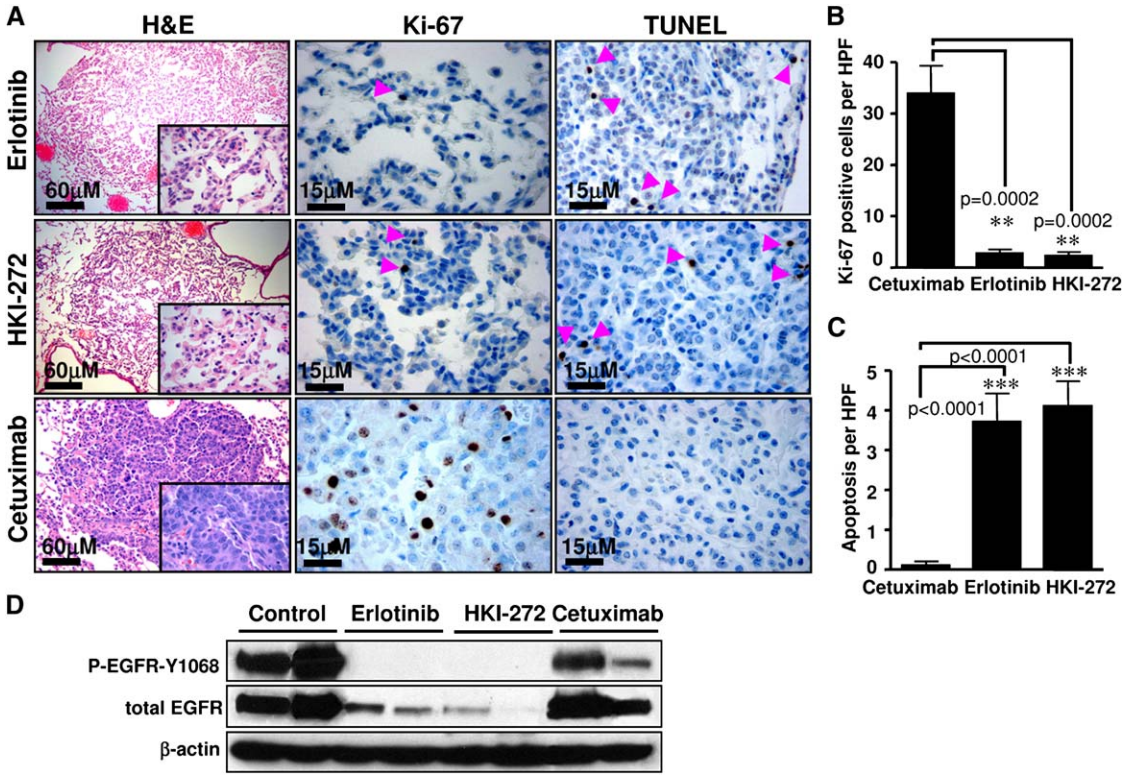

Figure 7. Histopathological and molecular analyses of the lung tumors after 1 week of treatment with erlotinib, HKI-272, or cetuximab

A: Bitransgenic mice bearing lung tumors treated with either erlotinib or HKI-272 or cetuximab as documented by MRI scan were further analyzed histologically. Tumors from mice treated with either erlotinib or HKI-272 had dramatic decrease of interstitial thickness and cellularity, consistent with tumor regression detected by the MRI analysis. These findings are associated with a decrease in Ki-67-positive tumor cells and an increase in TUNEL-positive staining as indicated by arrows, in contrast to the lung tumors treated with cetuximab.

B and C: Bar diagrams expressed as mean ± standard deviation illustrating the proliferative (**B**) and apoptotic (**C**) indices in lung tumors before and after 1 week of treatment with erlotinib, HKI-272, or cetuximab, determined from at least 200 high-power fields (HPF).

D: Immunoblotting analysis showed decreased levels of both total EGFR and phosphor-EGFR Y1068 in the lungs from the bitransgenic mice treated for 1 week with either erlotinib or HKI-272, but not cetuximab. β-actin served as loading control. Data shown are representative of at least 3 independent experiments. Representative data were shown from the *L858R* line.

prolonged exposures of cetuximab were not examined in in vitro cell line studies. Interestingly, our study of prolonged cetuximab treatment in vivo (>2 weeks) demonstrates that cetuximab is effective in treatment of lung tumors driven by hEGFR mutant overexpression as well (Figure 8). This difference in the time course of tumor response between cetuximab and TKIs from in vivo studies might be explained in several ways, including that cetuximab is a less effective inhibitor of EGFR mutant signaling or that the large molecular weight of cetuximab requires a longer period to reach inhibitory levels in tumor tissues.

In summary, we have generated an informative model of human lung adenocarcinomas harboring the *hEGFR* kinase domain mutations that predict clinical response to EGFR-targeted therapies. We have shown that expression of hEGFR mutants is essential for tumor maintenance in these lung cancers and that small molecule EGFR inhibitors and the humanized anti-EGFR antibodies demonstrate activity in this model, albeit with different kinetics. These unique lung cancer mouse models will be useful for future testing to determine the mechanism of tumor regression caused by EGFR inhibition and to determine the potency of newer-generation EGFR inhibitors or other novel therapeutics prior to human clinical testing. In addition, these mouse models will serve as platforms on which to layer additional oncogenic or tumor suppressor alleles to determine their genetic interactions on tumor initiation and progression, as well as their impact on sensitivities to therapeutic interventions.

Experimental procedures

Mouse cohorts

The generation of *Tet-op-hEGFR L858R-Luc* and *CCSP-rtTA/Tet-op-hEGFR Del-Luc* mice is described in the Supplemental Data. Three founders from *L858R* (#12, #14, and #27) and 2 founders from *Del* (#19 and #23) with tight regulation of *hEGFR* mutant expression were identified as described in the Supplemental Data for further studies.

The *CCSP-rtTA* mice were generously provided by Dr. Jeffery Whitsett at University of Cincinnati (Fisher et al., 2001). All mice were housed in a pathogen-free environment at the Dana-Farber Cancer Institute. All mice were handled in strict accord with good animal practice as defined by the Office of Laboratory Animal Welfare, and all animal work was done with DFCI IACUC approval. Genotyping protocols are supplied in the Supplemental Data.

Histology and immunohistochemistry

Mice were sacrificed and the left lungs were dissected and snap-frozen for biochemical analysis. The remainder of the lungs were then inflated with neutral buffered 10% formalin for 10 min and then fixed in 10% formalin overnight at room temperature, washed once in PBS and put in 70% ethanol, embedded in paraffin, and sectioned at 5 μm. Hematoxylin and eosin (H&E) stains were performed in the Department of Pathology in Brigham and Women's Hospital. Details for immunohistochemistry and antibody information are listed in the Supplemental Data.

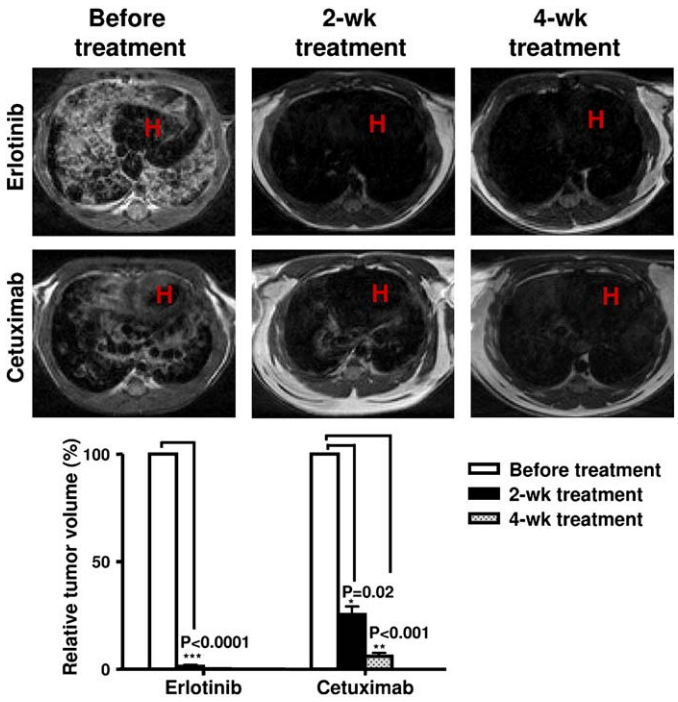

Figure 8. Prolonged treatment of lung tumors driven by hEGFR kinase domain mutant expression using either cetuximab or erlotinib

Bitransgenic *CCSP-rtTA/Tet-op-hEGFR L858R-Luc* mice (3 per group) were treated with either erlotinib orally at 50 mg/kg daily or cetuximab I.P. injection at 1 mg every two days for 4 weeks. These mice were MRI imaged before and after 2-week and 4-week treatment. Hearts are indicated as H. Bar diagram expressed as mean ± standard deviation illustrates the tumor regression measured by MRI, and statistical analyses were performed using Student's exact t test.

Doxycycline withdrawal and targeted therapy using the hEGFR inhibitors erlotinib, HK-272, or cetuximab in vivo

After sustained doxycycline treatment, the bitransgenic mice were subjected to MRI imaging to document the lung tumor burden. For doxycycline withdrawal experiment, the mice were given normal diet and MRI reimaged at indicated time points. For targeted therapies, either erlotinib (Biaffin GmbH & Co. KG, Kassel, Germany) or HKI-272 (Wyeth Pharmaceuticals, Pearl River, NY) formulated in 0.5% methocellulose-0.4% polysorbate-80 (Tween 80, Sigma-Aldrich) was given to mice by gavage at 50 mg/kg daily. Cetuximab (BMS pharmaceuticals, NJ) was given by I.P. injection into mice at 1 mg per dose every two days. After treatment, the same mice were MRI imaged at different time points to determine the tumor volume reduction. The mice were sacrificed and subjected to histological and biochemical analysis.

RT-PCR and quantitative PCR

Total RNA samples were prepared as described (Tonon et al., 2005) and retrotranscribed into first-strand cDNA using the first-strand synthesis system following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in an ABI 7700 sequence detection system (Perkin Elmer Life Sciences, Shelton, CT). Additional details are supplied in the Supplemental Data.

Western blot analysis

The lungs were homogenized in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitors (EMD Biosciences, San Diego, CA) and subjected to Western blot. Antibody information is listed in the Supplemental Data.

MRI imaging and tumor volume measurement

MRI measurements were performed as described in the Supplemental Data. Using the RARE sequence scans, volume measurements of the tumors were preformed using in-house custom software, and statistical analysis was performed using Student's exact t test (Sun et al., 2004).

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and two supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/6/485/DC1/.

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