Gefitinib-Sensitizing *EGFR* Mutations in Lung Cancer Activate Anti-Apoptotic Pathways
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Receptor tyrosine kinases of the EGFR family regulate essential cellular functions, including proliferation, survival, migration, and differentiation, and appear to play a central role in the etiology and progression of solid tumors (1, 2). EGFR is frequently overexpressed in breast, lung, colon, ovarian, and brain tumors, prompting the development of specific pharmacological inhibitors such as gefitinib (Iressa, AstraZeneca Pharmaceuticals), which disrupts EGFR kinase activity by binding the adenosine triphosphate (ATP) pocket within the catalytic domain (3). Gefitinib has induced substantial clinical responses in about 10% of patients with chemotherapy-refractory nonsmall cell lung cancers (NSCLCs) (4–7). Nearly all gefitinib-responsive lung cancers harbor somatic mutations within the EGFR kinase domain, whereas no mutations have been seen in nonresponsive cases (8, 9). These heterozygous mutations include small in-frame deletions and missense substitutions clustered or treat-
With use of transient transfections of mutant EGFRs, we showed previously that both types of mutations lead to increased EGF-dependent receptor activation as measured by autophosphorylation of Tyr1068 (Y1068), one of the prominent C-terminal phosphorylation sites of EGFR (8). To enable studies of qualitative differences in signaling by mutant EGFRs, we generated stable lines of nontransformed mouse mammary epithelial cells (NMuMg) expressing wild-type or mutant EGFRs (10) and analyzed EGF-mediated autophosphorylation of multiple tyrosine residues linked to activation of distinct downstream effectors (Fig. 1A) (1). Cell lines were generated that expressed either wild-type EGFR or one of two recurrent mutations detected in tumors from gefitinib-responsive patients: the missense mutation Leu858 → Arg858 (L858R) and the 18-base pair inframe deletion, delL747-P753insS (fig. S1). Notably different tyrosine phosphorylation patterns were observed between wild-type and the two mutant EGFRs at several C-terminal sites (Fig. 1B). EGF-induced phosphorylation of Y1045 and Y1173 was almost indistinguishable between wild-type and mutant EGFRs, whereas phosphorylation of Y992 and Y1068 was substantially increased in both mutants. Interestingly, Y845 was highly phosphorylated in the L858R missense mutant, but not in the wild-type or the deletion mutant, and hence appears to be unique in distinguishing between the two types of EGFR mutations. The differential EGF-induced tyrosine phosphorylation pattern seen with wild-type and mutant receptors was reproducible in transiently transfected COS7 cells, ensuring against potential cell type–specific effects (fig. S2). These observations suggest that the gefitinib-sensitive mutant EGFRs have the potential to transduce signals that are qualitatively distinct from those mediated by wild-type EGFR. These differences may result directly from structural alterations within the catalytic pocket affecting substrate specificity or from altered interactions with accessory proteins that modulate EGFR signaling.

The establishment of cell lines stably transfected with mutant EGFRs made it possible to compare the phosphorylation status of the major downstream targets of EGFR in a shared cellular background. EGF-induced activation of extracellular signal–regulated kinase 1 (Erk1) and Erk2 via Ras, of Akt via phospholipase C γ and phosphatidylinositol 3-kinase (PI3K), and

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**Fig. 1.** (A) Schematic representation of the autophosphorylation sites in the EGFR and the activation of the corresponding major signal transduction pathways. (B) Time course of ligand-induced phosphorylation of the various Tyr residues of the delL747-P753insS and L858R EGFR mutants, as compared with wild-type EGFR, after the addition of EGF (30 ng/ml) to stably transfected NMuMg. The phosphorylation of EGFR was determined by immunoblotting of whole cell lysates collected at the indicated times post-EGF treatment with antibodies that specifically recognize the phosphorylated Tyr845, Tyr992, Tyr1045, Tyr1068, and Tyr1173 of EGFR. Total phosphorylation of EGFR expressed in transfected cells was determined with the use of the py20 antibody. Total EGFR expression is also indicated.

**Fig. 2.** Selective activation of Akt and STAT5 by mutant EGFRs. (A) Time course of ligand-induced activation of specific signal transduction pathway downstream of the delL747-P753insS and L858R EGFR mutants, as compared with wild-type EGFR in stably-transfected NMuMg cells, after the addition of 30 ng/ml of EGF to serum-starved cells. Phosphorylation of Erk1 and Erk2 was determined by immunoblotting with an antibody that specifically recognizes the phosphorylated Thr202 and Tyr204 residues. For Akt and STAT5, phospho-specific antibodies against Ser473 and Tyr547, respectively, were used. (B) Total Erk1, Erk2, Akt, and STAT5 from the corresponding cell lysates used in (A) was determined by immunoblotting with the relevant antibodies. (C and D) An analysis of NSCLC human cell lines that harbor either wild-type EGFR or heterozygous activating mutations in EGFR, analogous to that performed on the stable transfecants in (A) and (B). In both sets of experiments, the expression of mutant EGFRs is associated with selective activation of Akt and STAT5 but not of Erk1 and Erk2.
of signal transducer and activator of transcription 3 (STAT3) and STAT5 via Janus kinase 2 (JAK2) are essential downstream pathways mediating oncogenic effects of EGFR (1). EGF-induced Erk activation was essentially indistinguishable among cells expressing wild-type EGFR or either of the two activating EGFR mutants (Fig. 2, A and B). In contrast, phosphorylation of both Akt and STAT5 was substantially elevated in cells expressing either of the mutant EGFRs (Fig. 2, A and B). Increased phosphorylation of STAT3 was similarly observed in cells expressing mutant EGFRs (11). The unaltered Erk activation by the mutant EGFRs is consistent with the absence of increased phosphorylation of Y1173, an important docking site for the Shc and Grb-2 adaptors that leads to Ras activation and subsequent Erk phosphorylation (1). The increased Akt and STAT phosphorylation after activation of the mutant EGFRs is consistent with the increase in Y992 and Y1068 phosphorylation, both of which have been previously linked to Akt and STAT activation (1). Thus, the selective EGF-induced autophosphorylation of C-terminal tyrosine residues within EGFR mutants is correlated well with the selective activation of downstream signaling pathways.

To extend these observations to lung cancer cells in which EGFR mutations appear to drive tumorigenesis, we studied lines derived from five NSCL tumors. NCI-H1975 carries the recurrent heterozygous missense mutation L858R and NCI-H1650 has the in-frame deletion delE746-A750, whereas NCI-358, NCI-H1666, and NCI-H1734 express wild-type EGFR (fig. S3). As in transfected cells, EGF-induced autophosphorylation of Y992 and Y1068 was markedly elevated in the two lines with endogenous EGFR mutations, as was phosphorylation of Akt and STAT5 but not Erk (Fig. 2, C and D, and fig. S4).

The oncogenic activity of EGFR reflects the activation of signals that promote both cell proliferation and cell survival (12). Although these pathways exhibit overlap, Ras-mediated activation of the Erk kinases contributes substantially to the proliferative activity of EGFR, whereas activation of Akt and STATs is largely linked to an anti-apoptotic function (12–17). The two lung cancer cell lines harboring EGFR mutations exhibited increased cell number over time relative to cells expressing wild-type EGFR when maintained in the presence of EGF in low serum concentration (Fig. 3A). However, the proliferation rate and cell density at confluence were comparable at normal serum concentrations (11). In contrast, apoptotic pathways were markedly different in lung cancer cells with mutant EGFRs: Small interfering RNA (siRNA)–mediated specific inactivation of mutant EGFR in these cell lines resulted in rapid and massive apoptosis. About 90% of NCI-H1975 cells transfected with L858R-specific siRNA died within 96 hours, as did NCI-H1650 cells transfected with delE746-

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**Fig. 3.** Expression of mutant EGFRs provides an essential cell survival signal in NSCLC. (A) Growth curves for two NSCLC cell lines expressing only wild-type EGFR (left) and two lines with the indicated heterozygous EGFR mutations (right) maintained in the presence or absence of 100 ng/ml of EGF. The scale of the graph to the left is expanded in order to visualize each of the curves. (B) siRNA–mediated specific knockdown of the mutant EGFR allele in NSCLC cell lines results in rapid and massive apoptosis. H1975 cells transfected with siRNA targeting the endogenous L858R missense transcript, but not those transfected with siRNA against the delE746A-750 transcript, showed 90% decreased viability relative to untreated cells (control) as measured by the MTT assay within 96 hours of transfection. Similarly, H1650 cells expressing the delE746A-750 mutant were susceptible to siRNA targeting the endogenous mutation but not the L858R transcript. H358 cells expressing wild-type EGFR were unaffected by the mutant-specific siRNAs, and siRNA directed against wild-type EGFR (which also targets the mutants) had no detectable effect on cells expressing only wild-type EGFR but effectively induced apoptosis in lines expressing mutant EGFR. Each column reflects the average of four different experiments, each performed in triplicate. Error bars indicate standard deviation. (C) The decreased number of viable cells after siRNA treatment in (B) is due to an increase in apoptosis as revealed by immunostaining of fixed cells with an antibody directed specifically against cleaved caspase-3. Cells were co-stained with 4',6-diamidino-2-phenylindole (DAPI) to reveal nuclei. (D) The H1650 and H1975 lung cancer cell lines, which express endogenous EGFR kinase domain mutations, exhibit comparably increased drug sensitivity with respect to Erk as well as STAT5 phosphorylation. The activity of Erk1, Erk2, and STAT5 was determined by immunoblotting with phospho-specific antibodies. Cells were untreated or pretreated for 3 hours with increasing concentrations of gefitinib and then stimulated with 30 ng/ml of EGF for 15 min. Total Erk1, Erk2, and STAT5 protein were also determined by immunoblotting and are shown in the lower panels.
A750-specific siRNA (Fig. 3, B and C). SiRNAs specific for either EGFR mutation had no effect on cells expressing the alternative mutation, and siRNA that targets both wild-type and mutant EGFR had minimal effect on the viability of untreated cells (control). (B and C) NSCL tumor lines harboring EGFR kinase domain mutations exhibit increased sensitivity to pharmacological inhibitors of anti-apoptotic signaling mediated by the PI-3K–Akt pathway (B) and the Jak-STAT pathway (C). The PI-3K inhibitor Ly294002 (Eli Lilly) was used at the indicated concentrations to disrupt Akt activation, and the Jak inhibitor AG490 was used at the indicated concentrations to disrupt STAT activation. (D to F) NSCL tumor lines harboring EGFR kinase domain mutations exhibit significantly increased resistance to the chemotherapeutic agents cisplatin (D) and doxorubicin (E) as well as the pro-apoptotic Fas ligand (F) relative to NSCL tumor lines expressing wild-type EGFR. Cells were treated with increasing concentrations of cisplatin, doxorubicin, or Fas ligand in the presence of 100 ng/ml of EGF, and their viability was determined after 96 hours with use of the MTT assay. Error bars represent standard deviation.

Oncogene addiction has been proposed to explain the apoptosis of cancer cells after suppression of a proliferative signal on which they have become dependent. (18) Interestingly, imatinib mesylate (Gleevec, Novartis)
efficiently triggers cell death in chronic myeloid leukemias expressing the BCR-ABL translocation product and in gastrointestinal stromal tumors expressing activating c-Kit mutations, both of which frequently exhibit constitutive STAT activation that is effectively inhibited by the drug (19, 20). Similarly, in lung cancer cells with EGFR kinase mutations, gefitinib responsiveness may result in large part from its effective inhibition of essential anti-apoptotic signals transduced by the mutant receptor.

References and Notes
10. Materials and methods are available as supporting material on Science Online.
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