

ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines

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Therapies that target the EGF receptor (EGFR), such as gefitinib (IRESSA), are effective in a subset of patients with advanced non-small cell lung cancer (NSCLC). The differences in intracellular signaling networks between gefitinib-sensitive and -resistant NSCLCs remain poorly understood. In this study, we observe that gefitinib reduces phospho-Akt levels only in NSCLC cell lines in which it inhibits growth. To elucidate the mechanism underlying this observation, we compared immunoprecipitates of phosphoinositide 3-kinase (PI3K) between gefitinib-sensitive and -resistant NSCLC cell lines. We observe that PI3K associates with ErbB-3 exclusively in gefitinib-sensitive NSCLC cell lines. Gefitinib dissociates this complex, thereby linking EGFR inhibition to decreased Akt activity. In contrast, gefitinib-resistant cells do not use ErbB-3 to activate the PI3K/Akt pathway. In fact, abundant ErbB-3 expression is detected only in gefitinib-sensitive NSCLC cell lines. Two gefitinib-sensitive NSCLC cell lines with endogenous distinct activating EGFR mutations (L858R and Del747-749), frequently observed in NSCLC patients who respond to gefitinib, also use ErbB-3 to couple to PI3K. Down-regulation of ErbB-3 by means of short hairpin RNA leads to decreased phospho-Akt levels in the gefitinib-sensitive NSCLC cell lines, Calu-3 (WT EGFR) and H3255 (L858R EGFR), but has no effect on Akt activation in the gefitinib-resistant cell lines, A549 and H522. We conclude that ErbB-3 is used to couple EGFR to the PI3K/Akt pathway in gefitinib-sensitive NSCLC cell lines harboring WT and mutant EGFRs.

Akt | EGF receptor

Metastatic lung cancer is almost uniformly fatal, with a median survival of \approx 8–10 months when treated with the most active combination of conventional chemotherapies; only 2% of patients are still alive 5 years after diagnosis (1, 2). Recent therapeutic strategies have focused on the development of “targeted therapies” that aim to specifically disrupt critical oncogenic mechanisms. The EGF receptor (EGFR) is one such target, because it is known to promote growth of cells and function as an oncogene and is expressed in up to 80–90% of non-small cell lung cancer (NSCLC) (reviewed in ref. 3).

The EGFR is a member of four proteins in the ErbB family of receptor tyrosine kinases. The other members are ErbB-2/HER2, ErbB-3, and ErbB-4. These receptors homodimerize or heterodimerize upon ligand binding. Upon dimerization, there is transautophosphorylation of the dimer partner (see ref. 4 for review). The tyrosine-phosphorylated proteins then serve as docking molecules to initiate intracellular signaling pathways. ErbB-3 is unique among the ErbB family members in that it has been shown to have weak or no tyrosine kinase activity (5). However, it is believed to couple with other ErbB family members to activate intracellular signaling. In particular, ErbB-3 effectively couples to the phosphoinositide 3-kinase (PI3K)/Akt pathway. It has six tyrosine phosphorylation sites with YXXM motifs that serve as excellent binding sites for PI3K (6–8).

Gefitinib [brand name IRESSA (ZD1839), AstraZeneca, Wilmington, DE] is a small-molecule quinazoline derivative that was developed as a tyrosine kinase inhibitor (TKI) of the WT EGFR. Gefitinib has been tested as monotherapy for patients with relapsed NSCLC (9, 10). Patients treated with gefitinib have a response rate of \approx 10–20%, whereas another 20–30% of patients have stable disease for at least 2 months. Recent studies indicate that a majority of patients who respond to EGFR-TKI therapy contain somatic mutations in the EGFR tyrosine kinase domain that include EGFR L858R and EGFR with deletions in exon 19 (11–13). The mechanisms by which these mutations promote a cancer phenotype are incompletely defined. Experiments assessing overexpression of the EGFR mutants in fibroblasts and mammary epithelial cells suggest that the EGFR mutants may display different kinetics of activation and deactivation (11), as well as differential patterns of autophosphorylation and intracellular signaling cascades (14). Presumably, these mutations provide persistent stimulation of downstream pathways that drive cell transformation.

Still, some lung cancers that respond to EGFR-TKI therapy have the WT EGFR (11, 13). A substantial proportion of patients with prolonged stable disease after treatment with EGFR-TKIs also possess WT EGFR (P.A.J., J. Lee, X. Zhao, N. Lindeman, T.M., B. Halmos, T. Pham, P. Fidiias, D. Eberhard, M. Meyerson, and B.E.J., unpublished data). Therefore, if mutations in EGFR were used as the single criteria to determine who should receive anti-EGFR therapy, a significant population of patients who would derive clinical benefit from the EGFR-TKIs might be excluded. An understanding of the intracellular pathways that mediate EGFR-dependent and gefitinib-sensitive transformation would provide a mechanism for predicting which patients are likely to respond to this drug and may also suggest other drug targets for treating NSCLC patients who become resistant to anti-EGFR therapies.

In this study, we observe that the PI3K/Akt pathway is down-regulated in response to gefitinib only in NSCLC cell lines that are growth-inhibited by gefitinib. Comparison of PI3K immunoprecipitates (IPs) between gefitinib-sensitive and -resistant NSCLC cell lines reveals that ErbB-3 couples to PI3K only in gefitinib-sensitive NSCLC cell lines harboring either WT or mutant EGFRs [L858R and Del747-749 (exon 19)]. Further, inhibition of ErbB-3 expression by means of ErbB-3 short hairpin RNA (shRNA) decreases Akt activation in gefitinib-sensitive cell lines harboring WT or mutant EGFR. In contrast to gefitinib-sensitive NSCLC cell lines, we do not detect significant ErbB-3 expression in resistant NSCLC cell lines. Thus,

Abbreviations: EGFR, EGF receptor; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide 3-kinase; shRNA, short hairpin RNA; TKI, tyrosine kinase inhibitor; IPs, immunoprecipitates; PTyr, phosphotyrosine.

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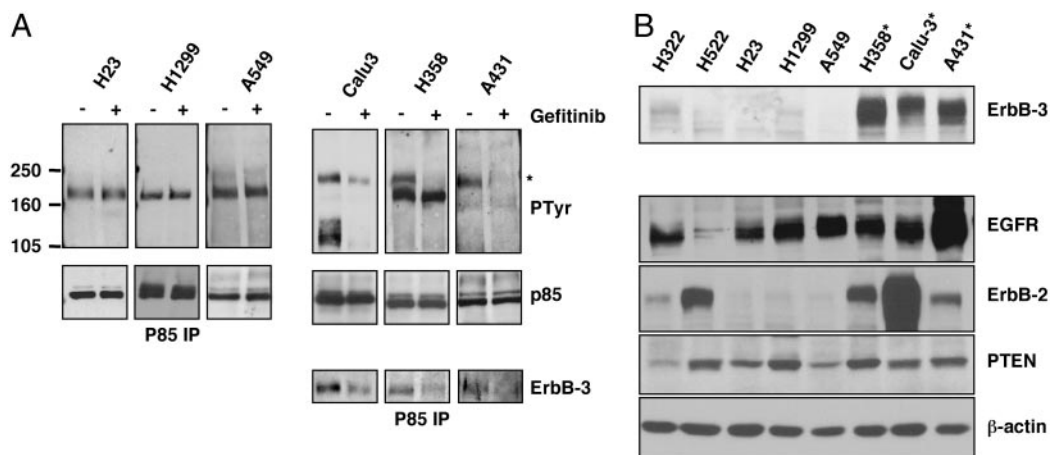


Fig. 2. PI3K regulatory subunit p85 binds ErbB-3 in a gefitinib-dependent manner exclusively in NSCLC cell lines sensitive to gefitinib. (A) Three NSCLC cell lines resistant to gefitinib (H23, A549, and H1299) and three cell lines sensitive to gefitinib (H358, Calu-3, and A431) were grown in the presence or absence of gefitinib ($1 \mu\text{M}$) for 6 h. Extracts ($\approx 1 \text{ mg}$) were immunoprecipitated with an anti-p85 antibody and subjected to immunoblot assay with anti-PTyr, anti-p85, and anti-ErbB-3 antibodies. * marks a PTyr protein observed in p85 coprecipitations in gefitinib-sensitive cells whose association is significantly diminished in the presence of gefitinib. (B) 50 micrograms of extracts from the cell lines shown were probed with antibodies against ErbB-3, EGFR, ErbB-2, PTEN, and β -actin. * denotes cells that have an IC_{50} of $< 1 \mu\text{M}$ to gefitinib (gefitinib-sensitive). Note that H322 and H522 cells are resistant to $1 \mu\text{M}$ gefitinib (data not shown).

resistant cells, there is no detectable major ≈ 210 -kDa PTyr protein interacting with p85, and gefitinib has no effect on p85-associated PTyr proteins. In Calu-3 cells, there is also a ≈ 110 -kDa PTyr protein that interacts with p85 in a gefitinib-sensitive manner. Of note, there is an ≈ 160 -kDa PTyr protein that interacts with p85 in both the gefitinib-sensitive and -resistant NSCLC cell lines. This association is not interrupted by gefitinib in any of the NSCLC cell lines tested. The identity of this protein is currently unknown.

The ≈ 210 -kDa PTyr protein that coprecipitates with p85 is of particular interest because it associates with p85 in a gefitinib-labile manner only in the gefitinib-sensitive NSCLC cell lines. To help determine the identity of this PTyr protein, the SCANSITE matrix (21, 22) was used to search for proteins in the SWISS-PROT database of the molecular mass range 140–200 kDa that contain potential p85 SH2 binding sites. Six of the top 15 hits in this molecular weight range were from the same protein, ErbB-3 (Table 1). Therefore, the p85 IPs were probed with anti-ErbB-3 antibodies. In the gefitinib-sensitive NSCLC cell lines, there is indeed a gefitinib-labile interaction between p85 and ErbB-3 (Fig. 2A Bottom Right). The ErbB-3 band comigrates with the PTyr band of ≈ 210 kDa (data not shown). No ErbB-3 was

identified in p85 coprecipitations from the gefitinib-resistant NSCLC cell lines (data not shown).

This finding led us to evaluate NSCLC cell lines for the expression of ErbB-3 (Fig. 2B). High expression of ErbB-3 was observed in only the gefitinib-sensitive NSCLC cell lines (denoted by *). We also probed NSCLC cell line extracts with antibodies against EGFR and ErbB-2. EGFR expression did not correlate with sensitivity to gefitinib as has been demonstrated by other studies (23, 24). ErbB-2 expression is detected in all three gefitinib-sensitive cell lines, as well as in the gefitinib-resistant cell lines H322 and H522. As has been reported previously, the ErbB-2 locus is amplified in Calu-3 cells, leading to overexpression of this protein.

A previous report had shown that a cell line without a functional PTEN tumor suppressor was unable to be inhibited by gefitinib, presumably because PTEN-deficient cell lines maintain Akt activity in the presence of EGFR inhibition (18). As shown in Fig. 2B, PTEN expression is present in the cell lines resistant to gefitinib. Thus, its absence does not explain resistance to gefitinib in these cell lines.

EGF Stimulates PI3K Association with ErbB-3 in a Gefitinib-Sensitive NSCLC Cell Line. To determine whether the association of p85 with ErbB-3 is enhanced by EGF ligand in a gefitinib-sensitive

Table 1. SCANSITE search for p85 SH2 binding proteins of molecular mass 140–200 kDa

Protein	Sequence	Position	Score
ErbB-3	LLSPSSGYMPMNQGN	1054	0.152
ErbB-3	GTPDEDYEYMNRRQ	1260	0.195
ErbB-3	EEDEDEEYEMNRR	1197	0.212
Disco-interacting protein 2 homolog	GPDPPTVYVDMRALR	1334	0.244
Collagen $\alpha 2$ (XI) chain precursor	YDYEPYVDVMTTGT	280	0.264
ALK tyrosine kinase receptor	KNCPGPVYRIMTQCW	1359	0.299
ErbB-4	GHSPPPAYTPMSGNQ	1056	0.299
Met receptor	EYCPDPLYEVMLKWCW	1313	0.313
Receptor-type protein-tyrosine phosphatase U	ISAPSFYADMPSP	589	0.338
ErbB-3	SSLEELGYEYMDVGS	1222	0.346
Brain-specific angiogenesis inhibitor 3	ERMESDYIVMPRSS	1308	0.354
Kinesin-like protein KIFIA	IYMTLSAYIEMENCT	1271	0.354
Neuronal nitric oxide synthase	LETGCTEYICMGSIM	334	0.354
ErbB-3	CPASEQGYEEMRAFQ	1289	0.368
ErbB-3	QICTIDVYYMVMVKC	941	0.376

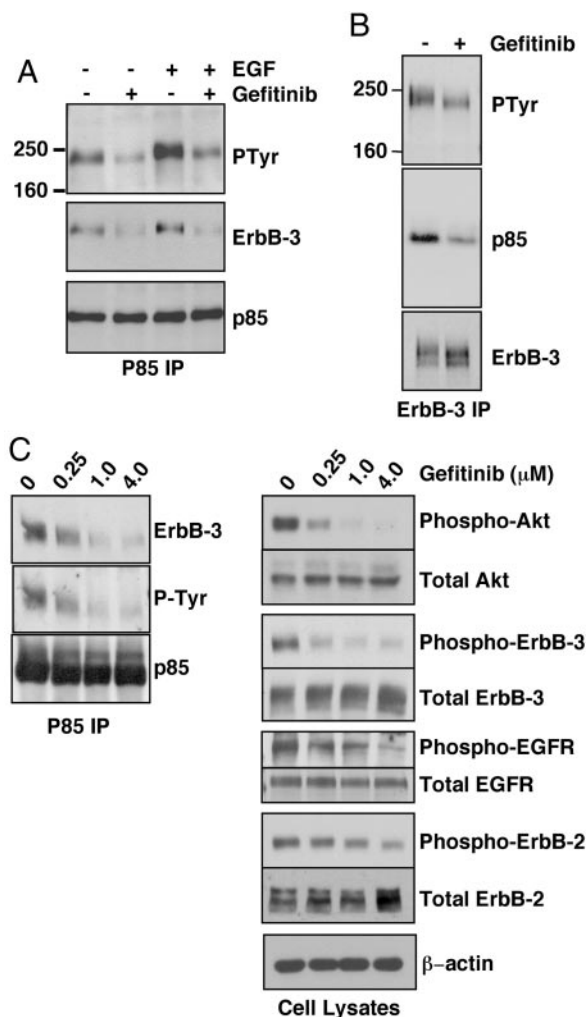


Fig. 3. EGF stimulates PI3K association with ErbB-3 in a gefitinib-sensitive NSCLC cell line. (A) Calu-3 cells were incubated in propagation medium in the presence or absence of gefitinib (1 μ M) for 6 h followed by stimulation with or without EGF (60 ng/ml) for 10 min. Extracts were immunoprecipitated with anti-p85 antibodies and then subjected to immunoblot analysis with antibodies against PTyr, ErbB-3, and p85. (B) Calu-3 cells were exposed or not exposed to gefitinib for 3 h. Extracts were immunoprecipitated with anti-ErbB-3 antibodies and subjected to immunoblot analysis with anti-PTyr, anti-ErbB-3, and anti-p85 antibodies. (C) Calu-3 cells were incubated in the presence of different concentrations of gefitinib (0, 0.25, 1, and 4 μ M). Cell extracts were prepared, and anti-p85 IPs were performed as in Fig. 2. IPs were probed with antibodies against ErbB-3, PTyr, and p85. Fifty micrograms of extracts prepared from these Calu-3 cells were probed with the indicated antibodies.

NSCLC cell line, Calu-3 cells were grown in the presence or absence of gefitinib before stimulation with EGF. As shown in Fig. 3A, EGF stimulated an association between p85 and ErbB-3 (also observed as the 210-kDa PTyr protein). This association was blocked by exposing the cells to 1 μ M gefitinib. Assessment of ErbB-3 immunoprecipitations reveals that gefitinib leads to a decrease in ErbB-3 tyrosine phosphorylation (Fig. 3B Top) and the amount of coprecipitated p85 (Fig. 3B Middle).

As mentioned earlier, Calu-3 cells possess amplification of the ErbB-2 locus. Previous studies have shown that breast cancer cell lines with overexpression of ErbB-2 are sensitive to gefitinib (25, 26). The studies we have presented thus far demonstrate that Calu-3 cellular growth and Akt activation are inhibited by 1 μ M gefitinib but do not demonstrate whether these effects are due to inhibition of EGFR or off-target inhibition of ErbB-2. We

performed a dose-response analysis of the p85–ErbB-3 interaction as well as the phosphorylation status of Akt, EGFR, ErbB-2, and ErbB-3. As shown in Fig. 3C, phosphorylated Akt levels are dramatically reduced by 0.25 μ M gefitinib. At this concentration, we also observe a reduction in the amount of ErbB-3 associated with p85 and decreased levels of phosphorylated ErbB-3 and phosphorylated EGFR. In contrast, phosphorylated ErbB-2 levels are not significantly reduced until concentrations of 4 μ M are used. Of note, previous reports showed that the IC_{50} for inhibition of ErbB-2 by gefitinib is >100-fold higher than that for EGFR *in vitro* (24) and that an exogenously expressed chimeric protein containing the ErbB-2 intracellular domain is not significantly inhibited by 1 μ M gefitinib (26). Thus, although our experiments do not clearly demonstrate that gefitinib's effects on PI3K activation are due to inhibition of EGFR and not ErbB-2, this explanation seems more plausible given this collection of observations.

NSCLC Cell Lines Harboring Activating EGFR Mutations Demonstrate a Gefitinib-Labile Interaction Between p85 and ErbB-3. The experiments presented thus far have evaluated NSCLC cell lines with WT EGFR. However, it appears that the majority of cancers with dramatic responses to EGFR tyrosine kinase inhibition harbor a somatic mutation in the EGFR (11–13). Therefore, we evaluated two patient-derived NSCLC cell lines that have mutant EGFR: H3255 and DFCILU-011 cells. H3255 cells contain an EGFR with a point mutation (L858R) (12) and DFCILU-011 contains a deletion mutation in exon 19 (Del1747-749). Both cell lines are very sensitive to gefitinib, with an IC_{50} of \approx 50 nM (12) (T.M., J.A.E., N. Hanna, S. Kobayashi, N. Lindeman, B. Halmos, J.P., L.C.C., D. Tenen, B.E.J., and P.A.J., unpublished data). As shown in Fig. 4A, Akt activity is diminished in both cell lines when they are grown in full serum in the presence of gefitinib. Interestingly, we observe an interaction between p85 and ErbB-3 that is disrupted by gefitinib in both cell lines (Fig. 4B), suggesting that the mutant EGFRs activate the PI3K/Akt pathway by means of ErbB-3 as well. In the H3255 cells, PI3K also associates with an \approx 110-kDa PTyr protein in a gefitinib-sensitive manner. Indeed, both the DFCILU-011 and H3255 cells demonstrate significant expression of ErbB-3, analogous to the gefitinib-sensitive NSCLC cell lines with WT EGFR (Fig. 4C). These results suggest that the mutant EGFRs also use ErbB-3 for activation of PI3K.

To determine whether ErbB-3 cooperates with mutant EGFRs to activate PI3Ks, EGFR WT and EGFR mutant (L858R) cells were transfected with or without ErbB-3 into CHO cells, a cell line that has no detectable EGFR or ErbB-3. In cells transfected with the L858R mutant (in the absence of ErbB-3), Akt activity returned to near baseline by 240 min after EGF stimulation (Fig. 4D Left). In contrast, cotransfection of ErbB-3 with the L858R mutant prolongs the strong activation of Akt up to 240 min after EGF stimulation (Fig. 4D Right). ErbB-3 expression appears to enhance basal activation of the Akt pathway by both the mutant and WT EGFR.

ErbB-3 Expression Is Necessary for Activation of the PI3K/Akt Pathway in Gefitinib-Sensitive NSCLC Cell Lines. The data generated in this study suggest that ErbB-3 is used to activate the PI3K/Akt pathway in gefitinib-sensitive NSCLC cell lines, whether the EGFR is WT or mutant. To test this idea, we interfered with ErbB-3 expression by employing RNA interference using a lentiviral-based shRNA expression system. The Calu-3 and H3255 cells were infected with ErbB-3 shRNA and control virus. For controls, the cell lines A549 and H522, which do not express ErbB-3, were also infected with the same viruses. As shown in Fig. 5, the ErbB-3 shRNA efficiently down-regulated ErbB-3 in both the Calu-3 and H3255 cells, leading to a marked reduction in phospho-Akt levels and demonstrating that ErbB-3 couples to

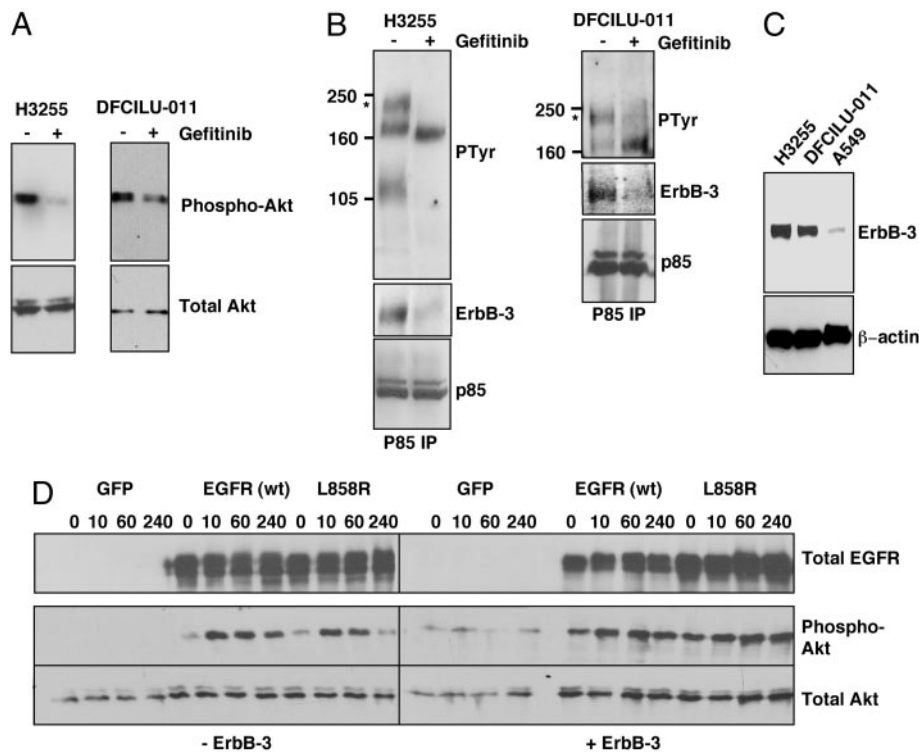


Fig. 4. NSCLC cell lines harboring activating EGFR mutations demonstrate a gefitinib-labile interaction between p85 and ErbB-3. (A) H3255 cells (L858R) and DFCILU-011 cells (Del747-749) were grown in the presence of gefitinib ($1 \mu\text{M}$) or vehicle control (DMSO) for 6 h. Fifty micrograms of extract was probed with an antibody against phospho-Akt (Ser-473) and total Akt. (B) The cells were treated exactly as in A. Extracts ($\approx 1 \text{ mg}$) were immunoprecipitated with an anti-p85 antibody and subjected to immunoblot assay with anti-PTyr, anti-p85, and anti-ErbB-3 antibodies. (C) Total extracts ($50 \mu\text{g}$) were probed with antibodies specific for ErbB-3 and β -actin. Equal amounts of extracts for A549 cells were included for control. (D) CHO cells were transiently transfected with EGFR (WT), EGFR L858R, or GFP control with either ErbB-3 or GFP control. About 30 h after transfection, cells were serum-starved overnight, followed by stimulation with EGF (60 ng/ml) for the indicated number of minutes (0, 10, 60, or 240). Cells were lysed, and $40 \mu\text{g}$ of extracts were assessed for expression of EGFR, phospho-Akt, and total Akt. SDS/PAGE gels from the -ErbB-3 and +ErbB-3 conditions were treated identically, incubated together in primary and secondary antibody solutions, and developed together on the same piece of film.

the PI3K/Akt pathway in these cells. In contrast, the amount of phospho-Akt was unchanged in the A549 and H522 cell lines infected with ErbB-3 shRNA. As shown in Fig. 2B, there is no appreciable expression of ErbB-3 detected in A549 or H522 cells.

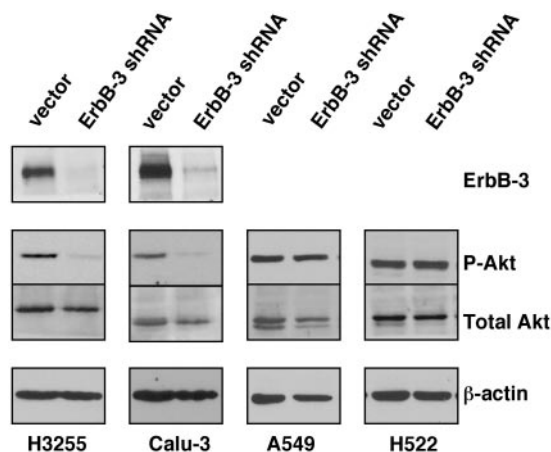


Fig. 5. ErbB-3 shRNA down-regulates phospho-Akt levels in the gefitinib-sensitive cell lines, H3255 and Calu-3. The Calu-3 and H3255 cell lines were infected with ErbB-3 shRNA or control lentiviral vector. Five days after infection, protein extracts were prepared and probed with antibodies against ErbB-3, phospho-Akt (Ser-473), total Akt, and β -actin. The A549 and H522 cell lines, which do not express ErbB-3 and are not sensitive to gefitinib, were infected by identical methods to serve as controls.

Discussion

In this study, we observed that although the PI3K/Akt pathway is activated in all NSCLC cell lines investigated, Akt activity is reduced in response to gefitinib selectively in NSCLC cell lines whose growth is inhibited by gefitinib. To our knowledge, the findings in this manuscript are the first to compare p85 IPs of several gefitinib-resistant and -sensitive cell lines and correlate their sensitivity to gefitinib with the existence of p85-ErbB-3 interactions. Furthermore, this study demonstrates that the recently discovered mutant EGFRs also use ErbB-3 to activate the PI3K pathway. Additionally, we observe that inhibition of ErbB-3 expression leads to diminished Akt activation specifically in gefitinib-sensitive NSCLC cell lines.

It has been known for almost a decade that ErbB-3 is a very potent link between EGFR activation and PI3K activity (7, 8). However, the role of ErbB-3 in tumorigenesis is currently evolving. For example, in a subclone of NIH 3T3 cells that does not express endogenous ErbB receptors, EGFR expression alone is unable to promote cellular transformation. However, coexpression with ErbB-3 coupled with stimulation with heregulin (not EGF) was sufficient to induce cellular transformation (27). It also appears that ErbB-3 likely promotes ErbB-2/HER2-dependant breast carcinogenesis; down-regulation of ErbB-3 leads to G_1 arrest and decreased Akt phosphorylation in SKBR3 cells (28). Those observations, together with the ones in this study, suggest that cancer cell lines driven by a member of the ErbB receptor system often couple to ErbB-3 to activate the PI3K/Akt pathway and promote the cancer phenotype. Thus, antibodies that inhibit ErbB-3's heterodimerization with other

