Tumorigenesis and Neoplastic Progression

Src-Family Kinases Are Activated in Non-Small Cell Lung Cancer and Promote the Survival of Epidermal Growth Factor Receptor-Dependent Cell Lines

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The role of Src-family kinases (SFKs) in non-small cell lung cancer (NSCLC) has not been fully defined. Here we addressed this question by examining SFK phosphorylation in NSCLC biopsy samples and using genetic and pharmacological approaches to inhibit SFK expression and activity in cultured NSCLC cells. Immunohistochemical analysis of NSCLC biopsy samples using a Tyr416 phosphorylation-specific, pan-SFK antibody revealed staining in 123 (33%) of 370 tumors. Because c-Src is known to be both an upstream activator and downstream mediator of epidermal growth factor receptor (EGFR), we next investigated SFK phosphorylation in a panel of NSCLC cell lines, including ones that depend on EGFR for survival. The EGFR-dependent NSCLC cell lines HCC827 and H3255 had increased phosphorylation of SFKs, and treatment of these cells with an SFK inhibitor (PP1 or SKI-606) induced apoptosis. PP1 decreased phosphorylation of EGFR, ErbB2, and ErbB3 and strikingly enhanced apoptosis by gefitinib, an EGFR inhibitor. HCC827 cells transfected with c-Src short hairpin RNA exhibited diminished phosphorylation of EGFR and ErbB2 and decreased sensitivity to apoptosis by PP1 or gefitinib. We conclude that SFKs are activated in NSCLC biopsy samples, promote the survival of EGFR-dependent NSCLC cells, and should be investigated as therapeutic targets in NSCLC patients. (*Am J Pathol* 2007, 170:366–376; DOI: 10.2353/ajpath.2007.060706)

Recent studies have shown that a subset of patients with non-small cell lung cancer (NSCLC) have tumors that require activation of epidermal growth factor receptor (EGFR) for cell survival.^{1,2} NSCLC cells that depend on EGFR for survival constitutively activate the receptor through a combination of activating mutations in the EGFR kinase domain and overexpression of EGFR, its dimerization partners (ErbB2 and ErbB3), and their ligands.³ Treatment of these patients with EGFR-specific tyrosine kinase inhibitors (TKIs), such as gefitinib or erlotinib, leads to rapid and sustained shrinkage of tumor burden and improved patient survival.^{4,5} However, the initial tumor response is typically followed by disease recurrence, which has been associated with the outgrowth of tumor cells that have acquired additional EGFR mutations.⁶ The problem of disease recurrence has not been obviated by the addition of standard chemotherapeutic agents to EGFR TKIs.⁷ Thus, improvement in the clinical outcome of this subset of patients will require the identification of prosurvival molecules other than EGFR that, when inhibited, can enhance the proapoptotic effects of EGFR TKIs.

Potentially important in this subset of patients are the Src family of kinases (SFKs), which include at least nine nonreceptor tyrosine kinases that function as gatekeepers for many signaling pathways that regulate cancer progression from initiation to metastasis.^{8,9} Overexpression or hyperactivity of SFKs is common in human epithelial tumors, including NSCLCs.¹⁰ One SFK, c-Src, has been functionally linked with EGFR. Concurrent overexpression of c-Src and EGFR has been found in ~70% of breast cancers, and the biological synergy between these two tyrosine kinases has been demonstrated in

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human breast cancer tissues and cell lines.¹¹ c-Src becomes transiently activated on association with activated EGFR and phosphorylates multiple downstream targets, including EGFR itself.¹² EGFR can be phosphorylated on multiple sites by c-Src, most notably Tyr845.¹¹ Tumors with activated EGFR have enhanced c-Src kinase activity, and inhibition of c-Src can reverse the transformed properties of cells overexpressing EGFR.¹³ In cancer cells that express high EGFR, inhibition of c-Src expression induces apoptosis by decreasing activation of signal transducer and activator of transcription (STAT) 3, a downstream mediator of c-Src, and the prosurvival molecule Bcl-X_L.¹³ Thus, EGFR and c-Src interact bidirectionally and synergistically, and c-Src may be an important prosurvival mediator of EGFR.

Given the importance of EGFR in maintaining NSCLC cell survival and the role of interactions between c-Src and EGFR in maintaining the survival of other tumor types, in this study we sought to examine the role of SFKs in NSCLC cells, which has not been fully defined. We analyzed SFK phosphorylation in NSCLC biopsy samples, using a large repository of tissues annotated for relevant histological and clinical variables. We subsequently investigated SFK phosphorylation in NSCLC cell lines with activating mutations in *EGFR* and the role of SFKs in the survival of these cells by using genetic and pharmacological approaches to inhibit SFK expression and activity. We conclude that SFKs are phosphorylated in tumors from a subset of NSCLC patients, contribute to the survival of EGFR-dependent NSCLC cells, and should be investigated as therapeutic targets in NSCLC patients.

Materials and Methods

Antibodies

We purchased rabbit polyclonal antibodies against Tyr1068-phosphorylated EGFR (PY1068-EGFR), Tyr1086-phosphorylated EGFR (PY1086-EGFR), PY416-pan-SFK (P-SFK), total SFK (SC-18), poly(ADP-ribose) polymerase (PARP), caspase 3, PY877-ErbB2, PY1289-ErbB3, PY705-STAT3, PS473-AKT, total AKT, PT202/204-ERK (extracellular signal-regulated kinase), and anti-rabbit and anti-mouse secondary antibodies from Cell Signaling Technologies (Beverly, MA); polyclonal antibodies against total ErbB2, ErbB3, and pan-SFKs and monoclonal antibody against total ERK1/2 from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibody against total EGFR from NeoMarkers (Fremont, CA); rabbit polyclonal antibody against pY845-EGFR from Biosource International (Camarillo, CA); and mouse monoclonal antibody against β -actin from Sigma Chemicals (St. Louis, MO).

Tissue Microarrays

All samples were obtained from patients who had undergone lung resection at The University of Texas M. D. Anderson Cancer Center for removal of NSCLC. Based

on pathological staging, the majority (84%) of patients had early-stage (I or II) disease. None had received neoadjuvant radiotherapy or chemotherapy. Only patients for whom signed informed consent and pertinent clinical data were available were included. Grading of histological change was performed as previously described by a single investigator (I.W.).¹⁴ Tissue microarrays were prepared from formalin-fixed, paraffin-embedded blocks of resected samples from 370 patients with NSCLC using triplicate 1-mm-diameter cores per tumor, obtaining tissues from central, intermediate, and peripheral tumor areas.

Immunohistochemical Analysis

After paraffin removal and hydration, slides were steamed for 15 minutes (Handy Steamer Plus; Black & Decker, Towson, MD) to induce epitope retrieval. Endogenous peroxidase was quenched by immersing the slides in 3% hydrogen peroxide for 5 minutes at room temperature. The slides were rinsed three times with Tris-buffered saline/Tween 20, and protein blocker (DAKO, Carpinteria, CA) was applied for 20 minutes. After being rinsed another three times, the slides were incubated with primary antibody against PY416-SFK overnight at 4°C, rinsed three times with phosphate-buffered saline (PBS)/Tween 20, and then incubated with DakoEnvision Dual Link System Plus (k4063; DAKO) for 30 minutes, rinsed, and incubated in the substrate peroxide and the chromogen diaminobenzidine per the manufacturer's instructions (DAKO). The slides were then rinsed, counterstained with hematoxylin for 30 seconds, rinsed in deionized water, dehydrated through graded ethanol solutions, cleared in xylene, and mounted. Blocking peptide and absence of primary antibody were used as negative controls for the antibodies. P-SFK staining was quantified based on the percentages of cells staining positively in cytoplasmic, membranous, or both compartments. For each tumor, the P-SFK score was defined as the average of the three core samples. A tumor was considered positive if at least 10% of the cells demonstrated staining.

Cell Lines

The NSCLC cell lines HCC827, HCC2279, H3255, H1975, H1819, and H1299 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA). These cell lines were treated with the SFK inhibitors PP1 [4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; Tocris Pharmaceuticals, Ellisville, MO] and SKI-606 (4-anilino-3-quinolinecarbonitrile; Wyeth Pharmaceuticals, Philadelphia, PA) or the EGFR TKI gefitinib (AstraZeneca, Wilmington, DE).

Western Blotting

Mid-log phase NSCLC cells were seeded at 50 to 70% confluence, cultured for 48 hours, serum-starved for 24

hours, and treated with PP1 (0, 0.1, 1, and 10 $\mu\text{mol/ml}$) for 60 minutes followed by 50 ng/ml of EGF for 60 minutes (for HCC827, H3255, HCC2279, and H1819) or 5 minutes (for H1299), which were the time points at which EGF maximally increased EGFR phosphorylation in these cell lines. As a control, cells were treated with the same concentration of the vehicle dimethyl sulfoxide (DMSO). Cells were then washed with ice-cold PBS and solubilized in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L sodium fluoride, 1 mmol/L phenylmethyl sulfonyl fluoride, phosphatase inhibitor cocktail I (P-2850; Sigma), and phosphatase inhibitor cocktail II (P-5726; Sigma). This suspension was frozen at -80°C , thawed, and then subjected to brief sonication.

Protein concentrations were estimated with a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL), and equal amounts of protein were denatured and reduced with a sample buffer containing 1% sodium dodecyl sulfate and 2.5% 2-mercaptoethanol. After boiling for 5 minutes, aliquots of the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 6% or 8% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The fractionated proteins were transferred onto Immobilon-Blot polyvinylidene fluoride membrane (Bio-Rad, Richmond, CA) at 300 mA for 3 hours in 0.5 $\times$ transfer buffer (12.5 mmol/L Tris, 100 mmol/L glycine, and 20% methanol, pH 8.3) with a semidry transfer cell unit (Bio-Rad). After the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (125 mmol/L NaCl, 20 mmol/L Tris, and 0.1% Tween 20, pH 7.5) for 1 hour at room temperature, the membrane was probed with a 1:1000 dilution of primary antibodies at 4°C overnight. This was followed by incubation with anti-rabbit (1:2000) or anti-mouse (1:4000) secondary antibodies for 1 hour at room temperature and visualization by an ECL or ECL Plus Western blotting detection system (Amersham, Piscataway, NJ).

Cell Proliferation Assays

Cells were seeded at a density of 1000 to 5000 cells per well in 96-well plates and allowed to attach for 24 hours. The cells were then treated with the gefitinib, PP1, or both in the presence of 10% serum for 5 days, at the end of which the cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was added to each well, as specified by the supplier (Roche, Nutley, NJ). After a 4-hour incubation, the percentages of cell densities for each treatment group, relative to the cell densities in control (DMSO-treated) cultures, were determined by measuring WST-1 absorbance at 450 nm. All cell proliferation data shown are the means of four measurements and are representative of three independent experiments.

Apoptosis Assays

Mid-log phase HCC827 and H3255 cells at 80% confluence were treated with gefitinib (5 nmol/L), PP1 (1 $\mu\text{mol/L}$),

both, or vehicle for 2 days. The adherent and floating cells were then pooled and evaluated for induction of apoptosis by Western analysis to detect PARP and caspase-3 cleavage and by flow cytometric analysis of cells subjected to a terminal dUTP nick-end labeling (TUNEL)-based assay (Apo-BRDU kit; Phoenix Flow Systems, San Diego, CA). For the TUNEL assay, the cells were fixed in 1% paraformaldehyde containing PBS and then in 70% ethanol, washed, and labeled with bromodeoxyuridine. After additional washing, labeled cells were treated with fluorescein-labeled anti-bromodeoxyuridine monoclonal antibody and propidium iodide/RNase solution before flow cytometric analysis (BD Calibur, San Jose, CA) to determine the percentage of apoptotic cells. The data shown are representative of three independent experiments.

Transfection of c-Src Short Hairpin RNA (shRNA)

pRS (retroviral-silencing) c-Src shRNAs were purchased from OriGene, Rockville, MD. Sh-Src target sequences were: A: 5'-AGAGGGCGGGCCCGCTGGCCG-3'; C: 5'-CTTAGACCTGAGGGACCCTTC-3'; D: 5'-GGGGACCCCTGGCTCTGGGCC-3'. PT67 fibroblasts (Becton Dickson, Mountain View, VA) were transfected with a single Src shRNA construct (A, C, or D) and selected for 3 to 4 weeks in 2 $\mu\text{g/ml}$ of puromycin to generate mass populations of retrovirus-producing cells. Viral particles in supernatants from PT67 cells were concentrated using a 0.45- μm filter. HCC827 cells were exposed to retrovirus expressing Src shRNA (A, C, or D) or empty vector in the presence of 5 $\mu\text{g/ml}$ polybrene (Sigma) for 4 to 6 hours. The media were then replaced with fresh media containing retrovirus, and the transfectants were incubated overnight. This transfection process was repeated three times to increase the transfection efficiency. Transfectants were then selected with 1 $\mu\text{g/ml}$ puromycin for 3 ~ 4 weeks, and mass populations were used in experiments.

Transfection of EGFR and c-Src Constructs

H1299 cells were transfected with EGFR constructs (wild-type or mutant $\Delta 746-750$), dominant-active mutant c-Src (Y527F), or empty vector (pCDNA3.1 and pLHCX for EGFR and c-Src, respectively). Stable transfectants were selected in 500 $\mu\text{g/ml}$ G418 for 3 to 4 weeks, and single-cell subclones were isolated and expanded for use in experiments.

Statistical Analysis

For the immunohistochemical studies, the primary objective was to correlate P-SFK membrane scores with clinical variables (gender, pathological stage, smoking history, duration of survival, and tumor histology). Included in the analysis were 370 patients. Summary statistics were used to summarize clinical factors and biomarker measurements. Fisher's exact test was used to correlate

Figure 1. SFK Tyr416 phosphorylation in NSCLC biopsy samples. **A:** Western blotting of H1299 cells stably transfected with dominant active mutant c-Src (DA-Src) or empty vector (H1299). **B:** Optimization of P-SFK immunohistochemical staining conditions so that staining was absent without primary antibody (1° Ab) or in the presence of blocking peptide (peptide). **C:** Examples of tumors staining positively for P-SFK (ADC, adenocarcinoma; SCC, squamous cell carcinoma; BAC, bronchioloalveolar cell carcinoma). **D:** Example of bronchial epithelial staining positively for P-SFK. **Arrow** points to a positive cell in basal layer.

membranous and cytoplasmic staining. Wilcoxon rank sum test was used for the two-way comparisons. Statistical analysis was performed with SAS Release 8.02 (SAS Institute, Cary, NC), and graphs were produced using S-Plus version 6.1 (Insightful Corp., Seattle, WA). *P* values of less than 0.05 were considered significant.

For studies examining the anti-proliferative effects of kinase inhibitors on cell lines, cell density values were calculated from five replicate wells per condition, and data illustrated were representative of three independent experiments. IC₅₀ values were determined from a sigmoidal dose-response curve fit to data (percent inhibition versus the log of the concentration). *P* values were calculated using the two-way analysis of variance test. To examine synergistic interactions between PP1 and gefitinib in cell lines, the Chou and Talalay¹⁵ combination index analysis was used. A combination index score of 1 is defined as the expected additive effect of two drugs. Synergism is defined as a combination index score of less than 1. CalcuSyn software (Cambridge, UK) was used to calculate the combination index.

Results

SFKs Are Phosphorylated in NSCLC

SFKs are recruited to the cytoplasmic membrane by receptor tyrosine kinases where they undergo activation through a series of phosphorylation and dephosphorylation events.^{8,9} Tyr416 is an autophosphorylation site that is highly conserved among Src family members.^{8,9} Phosphorylation at this site is reported to initiate a conformational reorganization of the kinase activation loop, leading

to relief of the steric barrier for substrate binding and activation of Src tyrosine kinase.¹⁶ To investigate SFK activation in NSCLC, we first performed immunohistochemical analysis of NSCLC biopsy samples using a pan-SFK antibody that detects Tyr416 phosphorylation (P-SFK) and quantified staining in the cytoplasmic and membranous compartments. For these studies, we used a tissue microarray constructed from 370 randomly collected NSCLC biopsy samples. The tumors had been annotated for patients' clinical variables (age, gender, smoking status, disease stage, and duration of survival) and tumor histological features (adenocarcinoma, squamous cell carcinoma, or bronchioloalveolar cell carcinoma).

Relevant positive controls (Western blotting of cells transfected with dominant-active mutant c-Src) and negative controls (immunohistochemical staining of tissues in the absence of primary antibody and preincubation of primary antibody with blocking peptide) confirmed the specificity of the antibody for P-SFK (Figure 1, A and B). The staining varied in intensity and extent among tumor biopsy samples and was detected in both cytoplasmic and membranous compartments (examples of staining for P-SFK are shown in Figure 1C). Of the 370 tumors examined, 123 (33%) stained positively, 105 (28%) were positive both in cytoplasm and membrane, four (1%) were positive in cytoplasm only, 14 (3.8%) were positive in membrane only, and 247 (67%) were negative for staining. Staining in membrane and cytoplasm was highly correlated (*P* < 0.001). Staining was associated with several clinical variables (Table 1), including male gender (*P* = 0.001), active smoking (*P* = 0.03), and squamous cell subtype (*P* < 0.001) but not ethnicity, patho-

Table 1. Correlation of p-SFK Staining with Clinical Variables in NSCLC Patients

	p-SFK-positive n/total (%)	P value
Gender		
Female	46/190 (24.2)	0.001
Male	68/168 (40.5)	
Smoking status		
Never/former	23/97 (23.7)	0.03
Active	89/258 (35.9)	
Histology		
ADC	44/213 (20.7)	<0.001
SCC	53/109 (48.6)	
BAC	4/18 (22.2)	

ADC, adenocarcinoma; SCC, squamous cell carcinoma; BAC, bronchioloalveolar cell carcinoma.

logical stage of disease, or duration of survival (data not shown). Thus, P-SFK staining was detected in a subset of NSCLC biopsy samples.

For comparison, P-SFK staining was examined in normal bronchial epithelium adjacent to tumors in the tissue microarray. Staining was observed only in the basal layer of the epithelium (Figure 1D). Of the 46 normal bronchi examined, P-SFK was detected in 5 (10.8%), which was significantly less than the staining frequency in tumor cytoplasm ($P = 0.01$) and membrane ($P = 0.005$). We then correlated staining frequency with bronchial size. P-SFK was detected in none of the terminal bronchioles (0 of 11) and in 4.3% (1 of 23) of the small bronchi (defined as having a smooth muscle layer but no cartilage), whereas 33.3% (4 of 12) of the large bronchi (defined as having a cartilage layer) had detectable P-SFK. Thus, the frequency of epithelial P-SFK staining varied with bronchial size.

Increased SFK Phosphorylation in EGFR-Dependent NSCLC Cell Lines

Based on the above findings, we postulated that a subset of NSCLC cell lines has increased phosphorylation of SFKs. We investigated SFK phosphorylation in a panel of NSCLC cell lines that included ones that have constitutive EGFR activation and are dependent on EGFR for survival (HCC827, H3255, HCC2279, and H1975).^{3,17} The other cell lines in the panel (H1299 and H1819) are relatively resistant to EGFR TKIs and have wild-type *EGFR*.³ Cells were grown in the absence of serum for 48 hours to remove exogenous activators of SFKs. Under these conditions, SFK phosphorylation was robust in HCC827 cells, H3255 cells, and HCC2279 cells, was detected at lower levels in H1975 cells, and was undetectable in H1299 cells and H1819 cells (Figure 2A). Thus, among the cell lines examined here, SFK phosphorylation was increased in EGFR-dependent cells.

One possible mechanism by which SFK phosphorylation was enhanced in EGFR-dependent cell lines is through the secretion of peptide growth factors, which, if present, would be enriched in a time-dependent manner after serum withdrawal. Supporting this possibility, in the presence of serum the cell lines in the panel had similar levels of SFK phosphorylation, whereas in serum-free

Figure 2. Increased SFK phosphorylation in EGFR-dependent NSCLC cell lines. **A:** Western blotting of P-SFK and total SFKs in NSCLC cell lines. Actin was used as a loading control. **B:** Serum withdrawal enhanced SFK phosphorylation in EGFR-dependent cell lines. Western analysis of NSCLC cell lines in the presence of serum (+SFK) or subjected to serum withdrawal for 48 hours (–SFK). **C:** SFK phosphorylation increased in a time-dependent manner after serum withdrawal. Western analysis of HCC827 cells lysed before ($t = 0$) or at the indicated time points after serum withdrawal. Actin was used as a loading control.

conditions SFK phosphorylation was higher in EGFR-dependent cell lines than in H1299 cells (Figure 2B). We examined time-dependent changes in SFK phosphorylation and found that the increase was detected 2 hours after serum withdrawal in HCC827 cells (Figure 2C).

SFK Inhibitors Induce Apoptosis of NSCLC Cell Lines

We next examined the sensitivity of these cell lines to treatment with the SFK inhibitors PP1^{18–20} or SKI-606.²¹

Figure 3. SFKs are required for the survival of EGFR-dependent NSCLC cells. Western blotting of NSCLC cell lines treated with PP1. **A:** WST-1 assays of NSCLC cells treated with PP1 or SKI-606. Values expressed relative to control cells treated with DMSO, which were set at 1. **B** and **C:** Western blotting of NSCLC cells treated with PP1 (10 $\mu\text{mol/L}$), SKI-606 (2 $\mu\text{mol/L}$), or vehicle (**C**). SFK inhibition was assessed by detection of P-SFK and apoptosis by caspase-3 and PARP cleavage. Actin was used as a loading control.

PP1 decreased P-SFK levels (Figure 3A) and cell numbers (Figure 3B) in a dose-dependent manner, with 50% inhibitory concentration (IC_{50}) values ranging from 1.2 to 8.6 $\mu\text{mol/L}$ (a 7.2-fold difference). HCC827 cells and H3255 cells had the lowest PP1 IC_{50} values, and their sensitivities differed significantly ($P < 0.001$) from those of H1299, H2279, H1819, and H1975 cells. The pattern of sensitivity to SKI-606 was similar to that of PP1: HCC827 cells and H3255 cells were the most sensitive, H1299 cells were the most resistant, and the other cell lines demonstrated intermediate sensitivity (Figure 3B). Because both of these compounds inhibit c-Abl, we sought to exclude the involvement of c-Abl in maintaining the survival of HCC827 cells and H3255 cells by treating them with imatinib, a potent c-Abl inhibitor. When administered at doses up to 10 $\mu\text{mol/L}$, imatinib had no effect on cell proliferation (data not shown), suggesting that c-Abl is not required for the survival of HCC827 cells and

H3255 cells. We next examined NSCLC cell lines for evidence of apoptosis after treatment with SFK inhibitors. Treatment with PP1 or SKI-606 resulted in cleavage of caspase-3 and PARP in HCC827 cells, H3255 cells, and HCC2279 cells but not H1299 cells (Figure 3C). Thus, NSCLC cell lines with constitutive phosphorylation of SFKs underwent apoptosis after treatment with these SFK inhibitors.

c-Src Depletion Diminishes Sensitivity to PP1

The proapoptotic effects of PP1 raised the possibility that SFKs promote the survival of these cells. However, these findings did not exclude the possibility that PP1 induced apoptosis through SFK-independent mechanisms. To address this question, we depleted c-Src from HCC827 cells by stably transfecting them with c-Src shRNA to

Figure 4. c-Src depletion in HCC827 cells diminishes sensitivity to PP1. **A:** Western blotting of HCC827 cells stably transfected with Src shRNA vectors (A, C, or D), empty vector (vector), or nothing (HCC827). **B:** WST-1 assays to examine the proliferation of transfectants (shRNA or pRS empty vector) and parental cells (HCC827) treated with PP1. Values for shRNA transfectants were expressed relative to values for parental cells (HCC827) treated with DMSO, which were set at 1. As an additional control, we compared parental cells and pRS transfectants with respect to effects of PP1, which were indistinguishable in these cells (top left).

derive clones that do not require c-Src for survival. We chose to target c-Src as opposed to other SFKs because it is the Src family member previously reported to regulate the phosphorylation of EGFR,^{8,9} a crucial prosurvival mediator in HCC827 cells. We used several shRNA constructs (A, C, and D) targeting different c-Src coding sequences to examine whether c-Src depletion by differ-

ent shRNA sequences induced consistent biological effects.

Four HCC827 transfectants (three shRNA constructs and one empty vector control) were selected for further study. Relative to its expression in control cells, c-Src was strikingly reduced in the three c-Src shRNA transfectants (Figure 4A), whereas expression of other SFKs (Lyn, Fyn, Yes) was unchanged (data not shown). P-SFK expression was reduced in c-Src shRNA transfectants (Figure 4A), but the residual P-SFK in c-Src-depleted cells is consistent with expression of other SFKs. Using this model, we investigated whether c-Src was required for the sensitivity of HCC827 cells to PP1. PP1 treatment decreased the numbers of c-Src-depleted cells, but their sensitivity was diminished relative to that of controls (Figure 4B; $P < 0.001$ for shRNA A, C, or D versus parental cells). Thus, PP1 mediated its effects through c-Src-dependent and -independent mechanisms in HCC827 cells.

PP1 Inhibits ErbB Phosphorylation

The two cell lines (HCC827 and H3255) we found to be most sensitive to SFK inhibitors are highly dependent on EGFR for survival,³ raising the possibility that PP1 induced apoptosis through effects on EGFR, its dimeric partners (ErbB2 and ErbB3), or both. To test this, we examined whether PP1 decreased the phosphorylation of Src substrates on EGFR (Y845 and Y1068) and ErbB2 (Y877).^{22–24} PP1 prominently decreased the phosphorylation of EGFR and ErbB2 at these sites (Figure 5A). We also examined the phosphorylation of ErbB3 (Y1289), a docking site for phosphatidylinositol 3-kinase,²⁵ which is phosphorylated by EGFR or ErbB2 through dimeric interactions with ErbB3. PP1 decreased ErbB3 phosphorylation at this site in HCC827 cells and H3255 cells (Figure 5A). In contrast, PP1 minimally decreased phosphorylation of these sites in H1819 cells (Figure 5A), HCC2279 cells, H1975 cells, and H1299 cells (data not shown). Thus, PP1 prominently inhibited ErbB phosphorylation in

Figure 5. SFKs regulate phosphorylation of EGFR, ErbB2, and ErbB3. **A:** Western analysis of ErbB family members in NSCLC cell lines treated with PP1. Actin was used as a loading control. **B:** Western blotting of HCC827 cells stably transfected with Src shRNA vectors (A, C, or D), empty vector (vector), or nothing (HCC827).

the two NSCLC cell lines that were most highly sensitive to PP1.

Using the HCC827 c-Src shRNA-transfectants, we examined the role of c-Src in the phosphorylation of ErbB family members in HCC827 cells. c-Src depletion reduced the phosphorylation of Y845-EGFR and Y877-ErbB2 (Figure 5B) but not Y1068-EGFR or Y1289-ErbB3 (data not shown). Thus, in HCC827 cells, c-Src regulated the phosphorylation of two known Src substrates (Y845-EGFR and Y877-ErbB2) but not Y-1068-EGFR, which is reported to be a Src substrate in glioblastoma cells.²³ We conclude that c-Src depletion recapitulated some, but not all, of the effects of PP1 on ErbB phosphorylation.

PP1 and Gefitinib Are Synergistic in NSCLC Cells

Based on the above finding that PP1 induced apoptosis in NSCLC cells that are highly sensitive to EGFR TKIs, we postulated that combined treatment with gefitinib enhances apoptosis induced by PP1. Consistent with this hypothesis, the combination caused more striking reductions in cell numbers than the effects of either drug alone in HCC827 cells and H3255 cells (Figure 6A). In fact, these compounds had synergistic effects, with combination index values of 0.6 and 0.91 for HCC827 cells and H3255 cells, respectively. In contrast, there was no synergy observed in the other four cell lines examined (data not shown). Using low doses of gefitinib and PP1 that were insufficient to induce apoptosis when administered singly, the combination was sufficient to induce apoptosis of HCC827 cells by TUNEL staining and cleavage of caspase-3 and PARP (Figure 6, B and C). Relative to the effect of either drug alone, the combination more potently suppressed phosphorylation of ERK, STAT3, and cyclin D1, which are known downstream mediators of EGFR and c-Src (Figure 6D).^{25–29} Thus, the combination of PP1 and gefitinib had synergistic effects in HCC827 cells.

Lastly, based on the above finding that c-Src depletion reduced the phosphorylation of certain ErbB family members, we hypothesized that c-Src depletion reduces EGFR dependence and thereby diminishes gefitinib sensitivity. Consistent with this hypothesis, gefitinib treatment decreased cell numbers to a greater degree in controls than it did in c-Src-depleted cells (Figure 7; $P < 0.001$ for shRNA A, C, or D versus parental cells). Thus, c-Src contributed to EGFR-dependence in HCC827 cells.

Discussion

Here we performed the first analysis of SFK phosphorylation in tumor specimens from a large cohort of NSCLC patients and found immunohistochemical evidence of SFK activation more frequently in tumor cells than in adjacent normal bronchial epithelium. Staining correlated with male gender, active smoking, and squamous cell tumors, a histological subtype previously observed to have high Src expression.^{30–32} In a panel of six NSCLC cell lines, four had high SFK activity, and treatment of

these cell lines with SFK inhibitors (PP1 or SKI-606) induced apoptosis. Thus, SFKs are activated and are able to maintain cell survival in a subset of NSCLCs.

Our findings differ from that of Masaki and colleagues,¹⁰ who found SFK activation to be more common in lung adenocarcinomas than in squamous cell carcinomas. These studies have several important differences that may have contributed to their divergent outcomes. First, Masaki and colleagues¹⁰ used NSCLC specimens from patients of Asian descent, whereas the patients in our study were primarily Western in origin. Recent findings have shown that ethnicity plays an important role in determining the biology of NSCLC. For example, *EGFR* mutations are more common in adenocarcinomas arising in patients from Asian background than in adenocarcinomas derived from Western patients.³³ Based on our findings, the presence of *EGFR* mutations is associated with high SFK activity. Thus, one conceivable explanation for the correlation of SFK activation with different histological subtypes in the two studies may be related to the studies' relative frequencies of *EGFR*-mutant tumors. Second, our tissue microarray contained more squamous cell tumors than the Masaki and colleagues¹⁰ cohort did (109 versus 12, respectively); thus, our study may have had greater power than that of Masaki and colleagues to detect a subset of squamous cell tumors with high SFK activity.

Several lines of evidence reported here support cooperative effects between EGFR and SFKs in NSCLC cell survival. First, the cell lines that required SFKs for survival were also EGFR-dependent. Second, depletion of c-Src from HCC827 cells reduced sensitivity to the anti-proliferative effects of PP1 or gefitinib. These findings extend previous reports that EGFR and c-Src cooperate in cancer cells. For example, cells genetically engineered to overexpress EGFR and c-Src have enhanced DNA synthesis, soft agar colony formation, and tumorigenicity in nude mice relative to cells that overexpress only one of the two molecules.³⁴ Further, EGFR and c-Src are both overexpressed in a subset of breast cancer cells, which have enhanced EGFR-dependent signaling and tumorigenicity relative to other breast cancer cells that do not overexpress both proteins.^{35,36} Thus, a growing body of evidence supports the hypothesis that c-Src and EGFR cooperate in cellular transformation and in maintaining cell survival.

Several studies have reported that SFKs are upstream activators of ErbB complexes.^{8,9} In support of these findings, here we demonstrated phosphorylation of known Src substrates on ErbB family members in NSCLC cell lines with constitutive SFK phosphorylation, and PP1 treatment of HCC827 cells and H3255 cells decreased the phosphorylation at these sites. In support of a role for c-Src in PP1 actions, c-Src depletion in HCC827 cells decreased the phosphorylation of Y845-EGFR and Y877-ErbB2. However, in c-Src depleted cells we did not detect a decrease in the phosphorylation of Y1068-EGFR, which differs from a previous report that Y1068-EGFR is a c-Src substrate in glioblastoma cells,²³ raising the possibility that, in HCC827 cells, a Src kinase family member other than c-Src phosphorylates Y1068-EGFR and that the role of c-Src at EGFR Y1068 is dependent on the

cellular context. However, we have not excluded the possibility that our experimental conditions were not sufficiently sensitive to detect a change in Y1068-EGFR

phosphorylation; for example, detection of change might be enhanced by the use of single cell subclones of c-Src shRNA transfectants (as opposed to mass transfectant

Figure 7. c-Src depletion in HCC827 cells diminishes sensitivity to gefitinib. WST-1 assays to examine the proliferation of transfectants (shRNA or pRS empty vector) and parental cells (HCC827) treated with gefitinib. Values for shRNA transfectants were expressed relative to values for parental cells (HCC827) treated with DMSO, which were set at 1. As an additional control, we compared parental cells and pRS transfectants with respect to effects of gefitinib, which were indistinguishable in these cells (top left).

populations). Given the ability of PP1 to inhibit the phosphorylation of EGFR and ErbB2, we next examined Y1289-ErbB3, which is phosphorylated by EGFR or ErbB2 through dimeric interactions with ErbB3. PP1 decreased phosphorylation of Y1289-ErbB3 in HCC827 cells and H3255 cells, and this effect was not recapitulated in HCC827 cells by c-Src depletion. Together, these findings suggest that PP1 mediated its effects through c-Src-dependent and -independent mechanisms. However, we have not excluded the possibility that PP1 and SKI-606 have direct inhibitory activity against EGFR or other ErbB family members in cells.

Other studies have shown that SFKs are downstream mediators of ErbBs. For example, in squamous-cell carcinoma cell lines, EGFR activation increases SFK phosphorylation, which is required for EGFR-induced phosphorylation of STATs and calveolin-1, cleavage of EGFR proligands, and tumor cell proliferation and invasion.^{29–31} Further, ErbB2 is reported to activate c-Src.^{36,37} Consistent with these reports, we found that NSCLC cell lines with constitutive EGFR activity also had high P-SFK phosphorylation. However, stable transfection of H1299 cells with a mutant *EGFR* expression construct ($\Delta 746-750$) that is identical to the mutation in HCC827 cells was not sufficient to increase SFK phosphorylation (data not shown), indicating that other signals are required. Elucidating the upstream activators of SFKs in EGFR-dependent NSCLC cells will require further study.

One of the clinical implications from this study is that SFK inhibitors may be useful in the treatment of NSCLC patients. The cell lines most sensitive to treatment with SFK inhibitors had high P-SFK expression and were dependent on EGFR for survival, raising the possibility that assays to measure the phosphorylation of SFKs and EGFR in tumor biopsies may predict efficacy to treatment

with SFK TKIs in NSCLC patients. Combined treatment with PP1 and gefitinib demonstrated synergy in HCC827 cells, supporting combination strategies to target both SFKs and EGFR in NSCLC patients as a way to enhance the efficacy of EGFR TKI alone, which heretofore has not been curative even in patients with EGFR-dependent tumors. However, in our study, synergy between PP1 and gefitinib was not observed in all EGFR-dependent NSCLC cell lines, indicating that combined treatment with SFK and EGFR TKIs may not be effective in all NSCLC patients with EGFR-dependent tumors. Consistent with our findings, dasatinib, a Src TKI that is currently under investigation in patients with a variety of tumor types, was recently reported to induce apoptosis in NSCLC cell lines that depend on EGFR for survival.³⁸

In patients who have an activating *EGFR* mutation and initially benefit from EGFR TKI, the disease typically recurs and is characterized by the emergence of TKI-resistant clones that carry an additional *EGFR* mutation at T790, also called the gatekeeper residue, which is known to be an important determinant of inhibitor binding in the context of multiple kinases.²⁰ In H1975 cells, which have an activating *EGFR* mutation as well as a T790M mutation, the addition of PP1 only modestly enhanced the efficacy of gefitinib (data not shown), suggesting that this combination may not be an effective salvage regimen in patients who experience recurrence after EGFR TKI. These questions will require further investigation by performing clinical trials in NSCLC patients to test the efficacy of strategies that inhibit SFKs, EGFR, and the combination.

The characteristics of patients we found to most frequently have high SFK phosphorylation in the tissue microarray (squamous cell, male, active smoker) differed from those of patients with EGFR-dependent NSCLC (adenocarcinoma, female, non- or former smoker). Although its frequency of detection varied among the different subgroups, SFK phosphorylation was detected in all three histological subtypes examined (adenocarcinoma, squamous cell, and bronchioloalveolar cell), in both genders, and in all smoking categories. Further, based on our findings in EGFR-dependent NSCLC cell lines, this subgroup of adenocarcinomas had high SFK phosphorylation and depended on SFKs for survival. We conclude that the role of SFKs should be investigated in all NSCLCs that have high SFK phosphorylation.

References

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004, 350:2129–2139
2. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004, 304:1497–1500
3. Fujimoto N, Wislez M, Zhang J, Iwanaga K, Dackor J, Hanna AE, Kalyankrishna S, Cody DD, Price RE, Sato M, Shay JW, Minna JD, Peyton M, Tang X, Massarelli E, Herbst R, Threadgill DW, Wistuba II,

- Kurie JM: 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, 65:11478–11485
4. 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, 64:8919–8923
5. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L: Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005, 353:123–132
6. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005, 2:e73
7. Herbst RS, Prager D, Hermann R, Fehrenbacher L, Johnson BE, Sandler A, Kris MG, Tran HT, Klein P, Li X, Ramies D, Johnson DH, Miller VA: TRIBUTE: a phase III trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non-small-cell lung cancer. *J Clin Oncol* 2005, 23:5892–5899
8. Bromann PA, Korkaya H, Courtneidge SA: The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* 2004, 23:7957–7968
9. Ishizawa R, Parsons SJ: c-Src and cooperating partners in human cancer. *Cancer Cell* 2004, 6:209–214
10. Masaki T, Igarashi K, Tokuda M, Yukimasa S, Han F, Jin YJ, Li JQ, Yoneyama H, Uchida N, Fujita J, Yoshiji H, Watanabe S, Kurokohchi K, Kuriyama S: pp60c-src activation in lung adenocarcinoma. *Eur J Cancer* 2003, 39:1447–1455
11. Biscardi JS, Ishizawa RC, Silva CM, Parsons SJ: Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res* 2000, 2:203–210
12. Osherov N, Levitzki A: Epidermal-growth-factor-dependent activation of the src-family kinases. *Eur J Biochem* 1994, 225:1047–1053
13. Karni R, Jove R, Levitzki A: Inhibition of pp60c-Src reduces Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* 1999, 18:4654–4662
14. Hirsch FR, Varella-Garcia M, McCoy J, West H, Xavier AC, Gumerlock P, Bunn Jr PA, Franklin WA, Crowley J, Gandara DR: Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. *J Clin Oncol* 2005, 23:6838–6845
15. Chou TC, Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984, 22:27–55
16. Xu W, Doshi A, Lei M, Eck MJ, Harrison SC: Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell* 1999, 3:629–638
17. Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, Treiber DK, Milanov ZV, Atteridge CE, Biggs III WH, Edeen PT, Floyd M, Ford JM, Grotzfeld RM, Herrgard S, Insko DE, Mehta SA, Patel HK, Pao W, Sawyers CL, Varmus H, Zarrinkar PP, Lockhart DJ: Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci USA* 2005, 102:11011–11016
18. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA: Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 1996, 271:695–701
19. Bain J, McLauchlan H, Elliott M, Cohen P: The specificities of protein kinase inhibitors: an update. *Biochem J* 2003, 371:199–204
20. Karni R, Mizrahi S, Reiss-Sklan E, Gazit A, Livnah O, Levitzki A: The pp60c-Src inhibitor PP1 is non-competitive against ATP. *FEBS Lett* 2003, 537:47–52
21. Golas JM, Arndt K, Etienne C, Lucas J, Nardin D, Gibbons J, Frost P, Ye F, Boschelli DH, Boschelli F: SKI-606, a 4-anilino-3-quinolinecarboxylic acid dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. *Cancer Res* 2003, 63:375–381
22. Stover DR, Becker M, Liebetanz J, Lydon NB: Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. *J Biol Chem* 1995, 270:15591–15597
23. Amos S, Martin PM, Polar GA, Parsons SJ, Hussaini IM: Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase Cdelta/c-Src pathways in glioblastoma cells. *J Biol Chem* 2005, 280:7729–7738
24. Segatto O, Lonardo F, Pierce JH, Bottaro DP, Di Fiore PP: The role of autophosphorylation in modulation of erbB-2 transforming function. *New Biol* 1990, 2:187–195
25. Engelman JA, Janne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, Cichowski K, Johnson BE, Cantley LC: ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci USA* 2005, 102:3788–3793
26. Walker F, Kato A, Gonez LJ, Hibbs ML, Pouliot N, Levitzki A, Burgess AW: Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors results in cell survival but not proliferation. *Mol Cell Biol* 1998, 18:7192–7204
27. Sinibaldi D, Wharton W, Turkson J, Bowman T, Pledger WJ, Jove R: Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: role of activated STAT3 signaling. *Oncogene* 2000, 19:5419–5427
28. Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, Arteaga CL: PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat Med* 2002, 8:1145–1152
29. Zhang Q, Thomas SM, Xi S, Smithgall TE, Siegfried JM, Kamens J, Gooding WE, Grandis JR: SRC family kinases mediate epidermal growth factor receptor ligand cleavage, proliferation, and invasion of head and neck cancer cells. *Cancer Res* 2004, 64:6166–6173
30. Xi S, Zhang Q, Dyer KF, Lerner EC, Smithgall TE, Gooding WE, Kamens J, Grandis JR: Src kinases mediate STAT growth pathways in squamous cell carcinoma of the head and neck. *J Biol Chem* 2003, 278:31574–31583
31. Kim YN, Dam P, Bertics PJ: Caveolin-1 phosphorylation in human squamous and epidermoid carcinoma cells: dependence on ErbB1 expression and Src activation. *Exp Cell Res* 2002, 280:134–147
32. Maa MC, Leu TH, McCarley DJ, Schatzman RC, Parsons SJ: Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proc Natl Acad Sci USA* 1995, 92:6981–6985
33. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF: Clinical and biological features associated with epidermal growth factor gene mutations in lung cancers. *J Natl Cancer Inst* 2005, 97:339–346
34. Xing C, Imagawa W: Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF. *Carcinogenesis* 1999, 20:1201–1208
35. Salh B, Marotta A, Matthewson C, Ahluwalia M, Flint J, Owen D, Pelech S: Investigation of the Mek-MAP kinase-Rsk pathway in human breast cancer. *Anticancer Res* 1999, 19:731–740
36. Muthuswamy SK, Siegel PM, Dankort DL, Webster MA, Muller WJ: Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol Cell Biol* 1994, 14:735–743
37. Muthuswamy SK, Muller WJ: Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene* 1995, 11:1801–1810
38. Song L, Morris M, Bagui T, Lee FY, Jove R, Haura EB: Dasatinib (BMS-354825) selectively induces apoptosis in lung cancer cells dependent on epidermal growth factor receptor for survival. *Cancer Res* 2006, 66:5542–5548