

# Src-Family Kinases Are Activated in Non-Small Cell Lung Cancer and Cooperate with Epidermal Growth Factor Receptor to Maintain Cell Survival

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## ABSTRACT

Epidermal growth factor receptor (EGFR) is constitutively activated in a subset of non-small-cell lung cancers (NSCLCs). In these tumors, EGFR activation maintains cell survival, but the prosurvival mediators of EGFR have not been fully defined. Here we investigated the role of the Src family of non-receptor tyrosine kinases (SFKs), which can function as both downstream mediators and upstream activators of EGFR, in the survival of NSCLC cells with constitutively active EGFR. In NSCLC biopsy samples, Tyr418 phosphorylation of SFKs was increased in a subset of tumors and correlated highly with EGFR autophosphorylation, and SFKs were constitutively phosphorylated in NSCLC cell lines with activating mutations in the EGFR kinase domain. Treatment with PP1, a SFKs inhibitor, decreased SFKs and EGFR phosphorylation and induced apoptosis in those cells. In addition, PP1 enhanced the induction of apoptosis by Gefitinib, an EGFR inhibitor. Combined treatment with PP1 and Gefitinib suppressed EGFR and SFKs phosphorylation and the activation of downstream signals to a greater extent than did treatment with either inhibitor alone. Depletion of c-Src expression and SFKs activation by specific c-Src shRNA diminished sensitivity to Gefitinib in HCC827 cells, suggesting that SFKs activation contributes to the sensitivity to Gefitinib in HCC827 cells. Thus, SFKs and EGFR cooperate in NSCLC to maintain cell survival, and therapeutic strategies to inhibit both EGFR and SFKs may be more efficacious than strategies to inhibit EGFR alone.

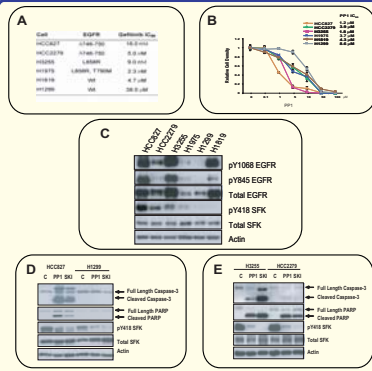


Figure 2. NSCLC cell lines with EGFR mutations have high SFK phosphorylation and undergo apoptosis in response to PP1 treatment. A, EGFR mutational status and Gefitinib IC50 values in panel of NSCLC cell lines used in this study. B, Effect of SFK inhibitor PP1 on the number of NSCLC cells. Values expressed relative to control cells treated with DMSO, which were set at 1. C, EGFR and SFK phosphorylation in NSCLC cells with or without EGFR mutations. Actin was used as a loading control. D and E, Induction of apoptosis by PP1 (10  $\mu$ M), Src666 (2  $\mu$ M), or control (C) in NSCLC cells. Apoptosis was assessed by detection of caspase-3 and PARP cleavage. Actin was used as a loading control.

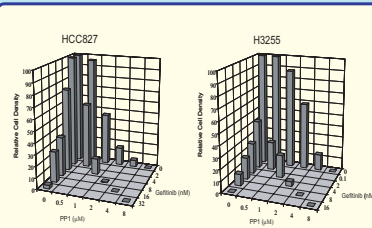


Figure 3. Analysis of synergistic effects of PP1 and Gefitinib by treatment with or without various concentrations of these agents alone and in combination. Values were expressed relative to control cells treated with DMSO, which were set at 1.

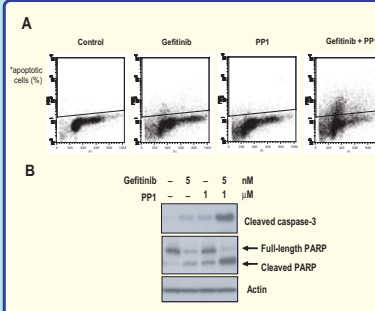


Figure 4. TUNEL assay (A) and detection of PARP and caspase-3 cleavage (B) in HCC827 cells treated with Gefitinib (5 nM), PP1 (1  $\mu$ M), both, or control (DMSO). Actin was used as a loading control.

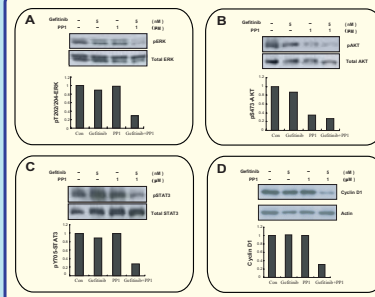


Figure 5. Combined treatment with Gefitinib and PP1 enhances the inhibition of EGFR and SFK downstream mediators. HCC827 cells treated with Gefitinib (5 nM) and PP1 (1  $\mu$ M) alone or in combination were subjected to Western blot analysis to detect expression and phosphorylation of EGFR and SFKs downstream mediators. The intensities of the bands were quantified by densitometry analysis, and normalized by total protein levels (A,B,C) or actin levels (D). Phosphorylation levels were expressed relative to that of control cells treated with DMSO, which were set at 1.

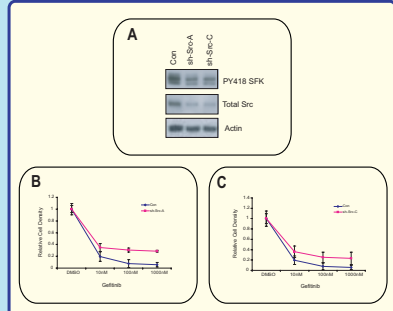


Figure 6. SFKs activation is required for Gefitinib sensitivity in HCC827 cells. A, Total expression of c-Src and the phosphorylation of Tyr418 in HCC827 cells were down-regulated by two specific shRNA sequences for c-Src, sh-Src-A and sh-Src-C. Actin was used as a loading control. B and C, Down-regulation of total Src and phosphorylation of SFKs confer partially resistance to Gefitinib in HCC827 cells.  $P < 0.001$ ; HCC827 control vs. sh-Src-A or sh-Src-C.

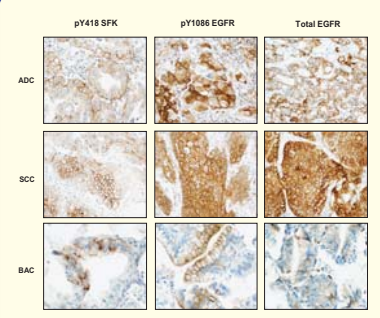


Figure 1. Figure 1. Immunohistochemical analysis of total EGFR, pY1088-EGFR, and pY418-SFK in representative samples of adenocarcinoma (ADC), squamous-cell carcinoma (SCC), and bronchioloalveolar-cell carcinoma (BAC).

## CONCLUSIONS

1. SFKs activation correlated with EGFR activation in tumor tissues from NSCLC patients.
2. The cell lines with high SFKs activation had EGFR mutations.
3. The SFKs inhibitor PP1 induces apoptosis of EGFR-mutant NSCLC Cells.
4. Inhibition of SFKs in EGFR-mutant NSCLC cells induced apoptosis and enhanced the proapoptotic effect of EGFR TKIs.
5. SFKs activation contributes to the sensitivity to Gefitinib in HCC827 cells.

Taken together, SFKs and EGFR cooperate in NSCLCs to maintain cell survival, and therapeutic strategies to inhibit both EGFR and SFKs may be more efficacious than strategies to inhibit EGFR alone.