**Cancer Therapeutics Insights**

**Src Mediates Cigarette Smoke–Induced Resistance to Tyrosine Kinase Inhibitors in NSCLC Cells**

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**Abstract**

The EGF receptor (EGFR) is a proto-oncogene commonly dysregulated in several cancers including non-small cell lung carcinoma (NSCLC) and, thus, is targeted for treatment using tyrosine kinase inhibitors (TKI) such as erlotinib. However, despite the efficacy observed in patients with NSCLC harboring oncogenic variants of the EGFR, general ineffectiveness of TKIs in patients with NSCLC who are current and former smokers necessitates identification of novel mechanisms to overcome this phenomenon. Previously, we showed that NSCLC cells harboring either wild-type (WT) EGFR or oncogenic mutant (MT) L858R EGFR become resistant to the effects of TKIs when exposed to cigarette smoke, evidenced by their autophosphorylation and prolonged downstream signaling. Here, we present Src as a target mediating cigarette smoke–induced resistance to TKIs in both WT EGFR- and L858R MT EGFR–expressing NSCLC cells. First, we show that cigarette smoke exposure of A549 cells leads to time-dependent activation of Src, which then abnormally binds to the WT EGFR causing TKI resistance, contrasting previous observations of constitutive binding between inactive Src and TKI-sensitive L858R MT EGFR. Next, we show that Src inhibition restores TKI sensitivity in cigarette smoke–exposed NSCLC cells, preventing EGFR autophosphorylation in the presence of erlotinib. Furthermore, we show that overexpression of a dominant-negative Src (Y527F/K295R) restores TKI sensitivity to A549 exposed to cigarette smoke. Importantly, the TKI resistance that emerges even in cigarette smoke–exposed L858R EGFR–expressing NSCLC cells could be eliminated with Src inhibition. Together, these findings offer new rationale for using Src inhibitors for treating TKI-resistant NSCLC commonly observed in smokers. *Mol Cancer Ther;* 12(8); 1579–90. ©2013 AACR.

**Introduction**

Despite extensive preclinical and clinical studies, lung cancer accounts for more than one million deaths per year and its prognosis remains meager, with only a 5% to 15% 5-year survival rate (1). In response, advanced molecular studies identified the EGF receptor (EGFR) as a key target in non–small cell lung carcinoma (NSCLC; refs. 2, 3) that becomes overexpressed in the bronchial epithelium of smokers (4–6). EGFR is a member of the ErbB family of receptor tyrosine kinases (RTK), which includes ErbB2, ErbB3, and ErbB4 that autophosphorylate themselves upon homo- or heterodimerization after ligand binding, acting as mediators of proliferation and survival (7). EGFR overexpression is observed in tumors from more than 60% of patients with metastatic NSCLC and is correlated with poor prognosis (8). Although smoking has been established as the most important cause of lung cancer, it has been recently shown that somatic mutations of the EGFR are responsible for approximately 10% to 25% of all nonsmoking-related lung cancer cases (9, 10). These initial findings triggered the development of tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib that eventually became standard therapies for NSCLC (11).

Many oncologists currently treat patients with NSCLC with promising small-molecule TKIs, such as gefitinib (Iressa) or erlotinib (Tarceva), which reversibly inhibit kinase activity via competitive binding to the ATP-binding site of the EGFR. A high response rate to TKIs was observed in women and in never-smokers with adenocarcinoma, specifically in the Japanese population (12), who were subsequently shown to harbor specific somatic mutations (L858R substitution and Δ746-750 deletion) in the kinase domain of the EGFR, resulting in a constitutively activated EGFR that provides a selective growth advantage to the affected lung cells (13–17). However, it has been recently observed that the same EGFR mutations are not limited to adenocarcinoma from female never-smokers; a large number (40%) of these EGFR mutations are actually found in adenocarcinoma tumor specimens from both men and women who are current and former smokers (18). Yet, patients who are smokers harboring...
TKI-sensitive EGFR mutations were not rigorously screened and tested in any clinical trial to evaluate whether or not they would benefit from TKI treatment (19). It is also remarkable, though still an anecdotal observation, that patients receptive to TKI therapy acquire resistance to TKIs if they began smoking (P. Lara; personal communication).

We have shown before that gefitinib and erlotinib treatments cannot inhibit the EGFR activation/autophosphorylation in NSCLC cells exposed to cigarette smoke. Therefore, smoking-related TKI resistance of NSCLC cells exists and is attributed to posttranslational changes in the EGFR conformation and signaling (20). We presented evidence for a novel, active EGFR conformation caused by oxidative stress from cigarette smoke exposure. Unlike the canonical EGF-induced conformation, it does not dimerize, and it interacts strongly with Src, resulting in ligand-independent EGFR activation that is resistant to inhibition by TKIs. Importantly, we established that cigarette smoke exposure renders TKIs ineffective in L858R EGFR-transformed cells, thus sustaining clonal growth of lung tumors even in the presence of TKI drugs (20). Notably, this effect was observed for several TKIs (gefitinib, erlotinib, and AG1478) and with different cell lines (A549, HCC827, and NIH-3T3 overexpressing L858R EGFR).

Src, the first proto-oncogene described, is a key participant downstream of the EGFR family. Phosphorylation of several tyrosine residues within the EGFR has been shown to increase following Src overexpression both in vitro and in vivo, indicating that Src is needed for full biologic response following EGF stimulus (21, 22). Furthermore, using chimeric EGF/ErbB2 receptors (21, 23), it was shown that Src specifically associates with ErbB2 but not with wild-type (WT) EGFR or other ErbB family members. However, mutant EGFRs isolated from lung adenocarcinomas have the capacity to associate with Src and these EGFR mutants require Src kinase activity for transformation (23, 24).

Here, we show that cigarette smoke/oxidative stress leads to robust Src phosphorylation/activation, which becomes aberrantly associated with EGFR in NSCLC cells. We used Src mutants and inhibitors to analyze the role of Src in generating resistance to TKIs in EGFR-overexpressing NSCLC cells exposed to cigarette smoke. Our findings suggest that the association of the EGFR with activated Src is critical for the TKI-resistant phenotype observed in both the WT- and L858R MT EGFR–expressing cells during cigarette smoke exposure, supporting our theory that simultaneous targeting of Src during TKI treatment may overcome smoking/oxidative stress–related resistance to TKIs in NSCLC.

Materials and Methods

Cell culture, treatments, and reagents

A549 adenocarcinoma (American Type Culture Collection), NCI-H3255 (generous gifts from Dr. Philip Mack, University of California, Davis, Davis, CA), NIH-3T3 (generous gifts from Dr. Hamid Band, University of Nebraska Medical Center, Omaha, NE; ref. 24), and Chinese hamster ovarian (CHO) cells have been used in this study. All the cell lines used in this study were previously characterized by others, as reported previously (24–26); in addition, we verified the correct expression of WT and L858R EGFR mutants in all the cell lines [the mRNA of EGFR was sequenced after real-time PCR (RT-PCR) and extraction of the PCR product from agarose gel]. A549 cells were cultured in F12K medium ( Gibco) supplemented with 10% FBS ( Gibco) and 1% penicillin/streptomycin ( Gibco). NCI-H3255 cells were cultured in RPMI medium ( Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. NIH 3T3 cells stably expressing WT EGFR or L858R mutant EGFR, CHO cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin.

Transfections of WT EGFR, kinase dead K721M EGFR, oncogenic L858R EGFR, constitutively active (CA) Y527F Src-GFP, and dominant-negative (DN) Y527F/K295R Src-GFP constructs were conducted using Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer’s protocol. CA-Src-GFP and DN-Src-GFP constructs were kindly provided by Dr. Yoav Henis ( Tel Aviv University, Tel Aviv, Israel). Cells were transfected at approximately 80% confluence in either 35- or 60-mm dishes and treated after approximately 24 hours.

For treatments, DMEM supplemented with 20 mmol/L HEPES, pH 7.4 was used. EGF was added directly into treatment medium at a final concentration of 100 ng/mL. AG1478 (Cell Signaling Technology), erlotinib ( Tarceva) and dasatinib ( Sprycel) were dissolved in dimethyl sulfoxide (DMSO) and then added to the treatment medium at a final concentration of 1 μmol/L. PP1 and PP2 were dissolved in DMSO and then added to the treatment medium at a final concentration of 5 μmol/L. DMSO was added at a final concentration of less than 0.1% to controluntreated (−) cells.

Cells were collected by scraping directly in lysis buffer: 1% NP-40 ( Igepal; Sigma), 50 mmol/L Tris, 10% glycerol, 0.02% NaNO₂, 150 mmol/L NaCl, pH 7.4, containing a cocktail of phosphatase and protease inhibitors (Sigma) as well as 1 mmol/L NaF and Na₃VO₄. Lysates were passed 5 times through a 30-gauge needle before centrifugation and further processing of the samples (either immunoprecipitation or immunoblotting). All the other reagents were from Sigma, unless differently specified.

Cigarette smoke exposure

Serum-starved cells were exposed to cigarette smoke gas phase as described before (27). Cells were placed in a vacuum oven with a chamber volume of 0.45 ft³ at a constant temperature of 37°C. A negative pressure was generated by vacuum (~10 Hg) and smoke from one cigarette (University of Kentucky, Lexington, KY; 2R4F) was drawn into the chamber by equilibrating the pressure.
inside the chamber with atmospheric pressure through a valve. For some experiments (requiring prolonged exposure to cigarette smoke–induced oxidative stress) medium preconditioning with cigarette smoke was used: 6 mL of medium were put in a 50 mL syringe, which was further used to aspirate approximately 60 cm$^3$ of cigarette smoke puff through a valve-equipped tube. The cigarette smoke–conditioning of the medium was allowed (inside the syringe) for 30 minutes at 37°C; then the cigarette smoke–conditioned medium was diluted (or not) with fresh medium, before being used in the experiments.

**Immunoprecipitation**  
Two hundred to 400 μg of total protein extracts were incubated for 3 hours with 2 to 4 μg of antibodies: anti-(α) 528 (against EGFR) or αSrc (Santa Cruz Biotechnology). Fifty microliter of 50% protein A-agarose bead complexes (Repligen) were added to the samples and incubated for 90 minutes. Four washes (by sequential centrifugation and resuspension) with the NP-40-lysis buffer were done before resuspending the immunoprecipitates in the loading dye for SDS-PAGE, as described before (27, 28).

**SDS-PAGE and immunoblotting**  
Of note, 6% to 12% acrylamide gels were used in a 2-cell system (Bio-Rad) for 1 to 4 hours at 100 V. Twenty to 100 μg total protein extracts or the immunoprecipitation samples were loaded into each well of the SDS-PAGE in the presence of dithiothreitol (DTT) reducing loading dye. After SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane and "blocked" with 5% skimmed milk in TBS with 0.05% Tween-20 (TBS-T) for 120 minutes or overnight, as described previously (28). Primary antibodies were incubated in 5% milk–TBS-T for 2 hours at room temperature. Secondary antibodies, either goat α-mouse or goat α-rabbit horseradish peroxidase–conjugated (Jackson ImmunoResearch), were incubated for 90 minutes at room temperature at 1:1,000 dilution in 5% milk–TBS-T. Bands were imaged using enhanced chemiluminescence (Supersignal West Pico Luminol Enhancer; Thermo Scientific). Extensive washes with TBS-T were done in between each step. When conducting sequential immunoblottings to assess the specific sites of EGFR phosphorylation, antibodies were stripped off the membranes using stripping buffer (Restore PLUS; Thermo Scientific) in between each immunoblotting. Primary antibodies used in this study for immunoblottings were: α2232 (αEGFR; Cell Signaling Technology; 1:1,000), αSrc (Santa Cruz Biotechnology; 1:1,000), αphospho-Y416 Src (Cell Signaling Technology; 1:1,000), αp-Y20 (Santa Cruz Biotechnology; 1:3,000), αp-Y1173 EGFR (Santa Cruz Biotechnology; 1:1,000), and αp-Y1086 EGFR, αp-Y1068 EGFR, and αp-Y845 EGFR (Cell Signaling Technology; 1:1,000).

**Cell viability assay**  
NSCLC cell viability was measured by the standard/common MTT method. Briefly, the cells were cultured and treated in phenol red–free medium, which was supplemented with 1 mg/mL MTT. The conversion of the yellow tetrazole (MTT) into purple formazan was carried out for 45 minutes in the cell incubator. The reaction was terminated and the formazan was solubilized by the addition of isopropanol containing 4 mmol/L HCl and 0.1% NP-40 (in a volume equal to that of the reaction medium). Absorbance of the solution was measured by spectrophotometry at 540 nm. The negative control consisted of a sample with no cells/no conversion of tetrazole.

**Statistical analysis**  
Each experiment (immunoblotting, immunoprecipitation/immunoblotting) was repeated at least 3 times and the shown images are representative. The plotted data are reported as mean ± SD. Statistical significance was determined by Student t test and P value less than 0.05 was considered statistically significant.

**Results**

**EGFR resistance to TKIs in NSCLC exposed to cigarette smoke: Src activation and binding to EGFR**  
To address why cigarette smoke exposure renders the EGFR resistant to TKI treatment in NSCLC cells (20), we tested the role of Src in this phenomenon. Previously, we showed that Src is consistently phosphorylated/activated under oxidative stress (28). Figure 1 presents the kinetics of Src activation, shown by the phosphorylation of Tyr416 (pY416-Src), in NSCLC cells exposed to tobacco smoke from 1 cigarette compared with that of EGF stimulation (100 ng/mL) for 30 minutes. As shown in Fig. 1, Src is robustly activated by cigarette smoke exposure but not by EGF. Furthermore, the cigarette smoke–induced activation of Src was not limited to A549 but was also observed in several cell types used for our current study including NIH-3T3 stably expressing L858R EGFR (Supplementary Fig. S1A and S1B) and CHO cells (Supplementary Fig. S1C).

We first tested whether these 2 activated tyrosine kinases, Src and EGFR, interact under cigarette smoke. Serum-starved A549 cells were incubated with 1 μmol/L erlotinib for 30 minutes and subsequently exposed to either EGF (100 ng/mL; 15 minutes) or cigarette smoke...
Serum-starved A549 cells were incubated (or not) with 100 ng/mL EGF or smoke from 1 cigarette for 30 minutes and exposed to 100 ng/mL EGF or smoke from 1 cigarette for an additional 30 minutes. EGFR was immunoprecipitated (IP) from the total cell lysates and the immunoprecipitates were immunoblotted (IB) for p-Y416 (active) Src (A), total EGFR and p-Y1173 EGFR (B).

We next tested whether the cigarette smoke–induced interaction between EGFR and Src occurs under cigarette smoke exposure, as shown in Fig. 2, the cigarette smoke–activated EGFR is not only unaffected by erlotinib but also abnormally binds the activated Src under these conditions. Furthermore, erlotinib did not prevent Src activation nor its binding to the EGFR during stimulation by cigarette smoke (Fig. 2A). However, minimal-to-no binding of Src to EGFR could be observed under EGFR stimulation, thus indicating that Src may have some role in imparting the TKI-resistant phenotype to EGFR-overexpressing cells (Fig. 2B).

Cigarette smoke–induced TKI resistance is eliminated by inhibition of Src activity

Following the observation that Src binds to the aberrantly activated EGFR during cigarette smoke exposure, we next tested whether Src activity plays a role in the WT EGFR’s resistance to TKIs under cigarette smoke stimuli. Serum-starved A549 cells were incubated (or not) with 1 μmol/L erlotinib for 30 minutes and then treated with 100 ng/mL EGF or cigarette smoke for an additional 30 minutes. EGFR was immunoprecipitated (IP) from the total cell lysates and the immunoprecipitates were immunoblotted (IB) for p-Y416 (active) Src (A), total EGFR and p-Y1173 EGFR (B).

Our findings strongly suggest that the activation of Src controls the EGFR resistance to TKI, which emerges during cigarette smoke exposure in lung epithelial cells (20).

TKI-resistant phenotype emerges when Src is bound to the EGFR in its active, "open" state

We next tested whether the cigarette smoke–induced interaction between EGFR and Src (20) is dependent on Src activity. Serum-starved A549 cells were incubated with 10 μmol/L PP1–PP2 for 30 minutes and then treated with smoke, as before. Cells were lysed, Src was immunoprecipitated, and the immunoprecipitates were immunoblotted for total Src and total EGFR. As shown in Supplementary Fig. S2, Src was pulled down together with Src in the presence of Src inhibitors, showing that Src kinase activity is not suppressed during cigarette smoke exposure. Similar results were observed in NSCLC cells treated with erlotinib and dasatinib, a U.S. Food and Drug Administration–approved Src inhibitor (not shown). Our findings strongly suggest that the activation of Src controls the EGFR resistance to TKI, which emerges during cigarette smoke exposure in lung epithelial cells (20).
To further confirm the requirement of Src in the emergence of EGFR resistance to TKIs, GFP-fused dominant-negative Src (DN-Src; K295R/Y527F) or a constitutively active form of Src (CA-Src; Y527F) were transiently expressed in A549 cells. Binding of these 2 Src constructs to EGFR was tested in the presence of EGF or cigarette smoke exposure. Intriguingly, our coimmunoprecipitation studies showed that both GFP-tagged Src constructs (CA- and DN-Src) bind to EGFR in the presence and absence of cigarette smoke exposure (Fig. 4), reiterating our previous observations that Src remained bound to the aberrantly activated WT EGFR under cigarette smoke stimulation despite Src inhibition. Moreover, it reaffirmed that an “open” conformation of Src is sufficient for its interaction/binding to the WT EGFR.

Next, we further tested whether Src activity is required for the EGFR’s resistance to erlotinib/TKIs under cigarette smoke exposure by transiently overexpressing either DN-Src or CA-Src in A549 for 24 hours then subsequently incubating the cells with 1 μmol/L erlotinib for 30 minutes and exposing them to either EGF or cigarette smoke, as before. Figure 4 shows that the DN-Src could sensibly improve the efficacy of erlotinib in inhibiting EGFR autophosphorylation during cigarette smoke exposure, compared with the unobstructed cigarette smoke–induced EGFR Tyr phosphorylation in cells expressing the CA-Src treated with erlotinib (Fig. 4).

Overall, these data show that cigarette smoke–induced Src activation controls TKI sensitivity of EGFR. In WT EGFR–overexpressing NSCLC cells exposed to cigarette smoke, resistance to TKIs is being developed posttranslationally not only because WT EGFR is aberrantly activated, but primarily because Src is also abnormally activated and binds the WT EGFR under cigarette smoke exposure. Indeed, this acquired TKI-resistant phenotype reliably disappears upon Src inhibition (for example by PP1-2 or dasatinib treatment or overexpression of DN-Src) despite still being bound to the cigarette smoke–activated EGFR.
Phosphorylation of Tyr845-EGFR does not control its cigarette smoke–induced TKI resistance

Having shown that Src inhibition restored TKI sensitivity to WT EGFR-expressing NSCLC cells, we investigated whether phosphorylation of Y845-EGFR, a Tyr phosphorylation site canonically linked to Src activity (24, 29), plays a role in generating the TKI-resistant phenotype observed during cigarette smoke exposure. To test this, NIH-3T3 cells stably overexpressing either WT or Y845F MT EGFR were incubated with erlotinib and exposed to either EGF or cigarette smoke, as before. Intriguingly, we observed no differences in TKI sensitivity/resistance between WT and Y845F MT EGFR, presented in Supplementary Fig. S4, indicating that trans-phosphorylation on Y845 does not control the cigarette smoke–generated TKI-resistant phenotype of EGFR. Given the potential for trans-phosphorylation of EGFR by other ErbB family members or by endogenous EGFR in NIH-3T3 cells, we further confirmed this finding in CHO cells that do not express endogenous EGFR or any other ErbB member. Again, no differences between WT and Y845F MT EGFR were observed in terms of cigarette smoke–induced EGFR TKI resistance, whereas cigarette smoke–activated Src remained bound to EGFR regardless of the presence of intact Y845 (not shown).

Cigarette smoke–induced TKI resistance in NSCLC cells expressing TKI-sensitive L858R MT EGFR is reversed by Src inhibition

The discovery that somatic-mutant variants of the EGFR (L858R substitution and D746-750 deletion) were uniquely sensitized to TKIs drives further TKI drug development and investigations into conformation states of the EGFR. Previous studies have shown that interactions between WT EGFR and Src was generally not stable (30) but the interactions between Src and mutant EGFRs, which harbor unique structural differences in the activation loop of the kinase domain (31), were more stable. Furthermore, we previously showed that the TKI-sensitive L858R MT EGFR, which is constitutively active, became resistant to TKIs upon cigarette smoke exposure (20).

Subsequently, we tested whether the IC50 of the TKI erlotinib against L858R EGFR was affected by cigarette smoke exposure. For this we used NIH-3T3 cells stably overexpressing the human L858R EGFR MT; the cells were incubated for 30' with increasing concentrations of erlotinib (from 10−12 to 10−4 mol/L) and then exposed (or not) for additional 30' to cigarette smoke; after the treatments, the level of receptor autophosphorylation on Y1173 was measured (by immunoblotting). Figure 5 shows that cigarette smoke exposure not only increases the IC50 of erlotinib against the L858R EGFR MT (~24 times), but further reduces erlotinib maximal inhibition of the receptor (~5 times). As mentioned before (20), the stability/integrity of the drug itself was not affected by cigarette smoke exposure.

We next tested whether Src controlled the TKI-resistant phenotype also observed for the TKI-sensitive L858R MT EGFR. Initially, NIH-3T3 + L858R MT EGFR cells were incubated with 1 µmol/L AG1478 (TKI) for 30 minutes, then exposed to EGF or cigarette smoke (or not), as before. Cell lysates were collected and immunoprecipitated for either Src or EGFR and immunoblotted for pY1173-EGFR, total EGFR, and total Src. Besides

Figure 4. Cigarette smoke (CS)–dependent TKI resistance of WT EGFR–harboring NSCLC cells is caused by Src activation. A549 cells were transiently transfected with WT EGFR and either GFP–tagged DN-Src or GFP–tagged CA-Src; 24 hours posttransfection the cells were serum starved and incubated for 30 minutes with 1 µmol/L erlotinib and then exposed to either EGF or cigarette smoke, as before. A, EGFR was immunoprecipitated (IP) from the total cell lysates and immunoblotted (IB) for total Src (GFP) and total receptor. B, EGFR was immunoprecipitated from the total cell lysates and immunoblotted for total receptor, total tyrosine (Y) phosphorylation level (p-YEGFR), and specific Y site phosphorylation levels (p-Y1173, p-Y1068, and p-Y845).
previous reports that Src binds to the L858R MT EGFR (24, 32), the cigarette smoke–activated L858R MT EGFR showed greater Src binding than that under EGF or no stimulation, shown in Supplementary Fig. S5A. As expected, the constitutively active L858R MT EGFR remained sensitive to the TKI under control conditions and EGF stimulation, shown by suppressed phosphorylation of several Tyr residues, but became resistant to the TKI under cigarette smoke exposure (Supplementary Fig. S5B).

Subsequently, we treated H3255 NSCLC cells (harboring L858R EGFR MT) with both erlotinib and PP1/PP2 under cigarette smoke exposure to further test our theory. Serum-starved H3255 cells were incubated with erlotinib, PP1–PP2, or a combination of them for 30 minutes and subsequently treated with EGF or cigarette smoke, as before. EGFR was immunoprecipitated from the cell lysates and immunoblotted for total EGFR and receptor autophosphorylation on Y1173 and Y1068. Figure 6A shows that the L858R MT EGFR of H3255 cells is constitutively phosphorylated on Y1173 and Y1068, as expected (20), which is further increased by both EGF and cigarette smoke treatments, and that erlotinib treatment could abolish the receptor autophosphorylation on Y1173 and Y1068 in control and EGF stimulation conditions. However, when these cells were exposed to cigarette smoke, erlotinib had reduced efficacy, as expected. Importantly, the combination of TKI with Src inhibitors could block the activity and autophosphorylation of the EGFR in the cigarette smoke–exposed cells much better than either drug alone (Fig. 6A). This further indicates a critical role of Src activity in controlling TKI sensitivity of NSCLC cells in a setting of cigarette smoke exposure and potentially other sources of oxidative stress for both WT EGFR and constitutively active/TKI-sensitive L858R EGFR (see model in Fig. 6B).

Notably, we have previously shown by the soft/agar assay that cigarette smoke exposure abolishes the TKI-dependent inhibition of anchorage-independent growth of EGFR-transformed cells (20). To further assess the biologic relevance of our biochemical findings, we tested the effects of TKI and cigarette smoke exposure treatments on H3255 NSCLC cells proliferation/viability; such cells are dependent on their L858R EGFR mutation for proliferation/viability, compared with control condition/growth, such cigarette smoke exposure also reduced H3255 cell viability, compared with control (33). As shown now, in Fig. 7, incubation of H3255 cells with TKI (1 μmol/L erlotinib) caused a marked decrease in cell proliferation/viability (compared with control). Importantly, while the cigarette smoke exposure also reduced H3255 cell viability, compared with control condition/growth, such cigarette smoke exposure entirely canceled the inhibitory effect of TKI (Fig. 7). Moreover, dasatinib treatment reduced cell viability of cigarette smoke–treated cells, implicating Src activity as survival/proliferative mechanism during cigarette smoke/oxidative stress exposure. Notably, dasatinib treatment was additive to TKI in reducing cell proliferation/viability in the presence and absence of cigarette smoke exposure, providing rationale for combinatorial therapy using both EGFR and Src inhibitors in treating NSCLC both in smokers and nonsmokers.
Discussion

Our recently published observations showed that both WT and L858R MT EGFR become resistant to TKIs (a number of TKIs were tested: AG1478, erlotinib, and gefitinib; Supplementary Fig. S6) in NSCLC cells exposed to cigarette smoke–induced oxidative stress (20). We provided evidence that (i) the cigarette smoke–activated EGFR is resistant to TKIs by posttranslational mechanism(s) and not by random somatic mutations, (ii) such cigarette smoke–dependent TKI-resistant phenotype is also observed in terms of activation of Akt/protein kinase B and extracellular signal–regulated kinase (Erk), as well as TKI-unresponsive clonal growth of transformed cells in soft agar, (iii) cigarette smoke did not chemically “damage” and reduce the effectiveness of the TKIs used (20), and then exposed to either EGF or cigarette smoke for additional 30 minutes, as before. EGFR was immunoprecipitated (IP) from the total cell lysates and immunoblotted (IB) for total receptor and specific p-Y1173 and p-Y1068. Notably, however, a constitutively active form of Src alone is not sufficient for causing EGFR resistance to TKI until exposed to cigarette smoke, suggesting that both EGFR and Src should be

![Figure 6](image_url)
aberrantly activated by cigarette smoke to cause TKI resistance. Nevertheless, given the prevalence of smoking and both WT and “activating” MT EGFR expression in NSCLC, the aforementioned studies provide stronger scientific rationale for a novel combinatory therapy using both EGFR and Src inhibitors for treating NSCLC, especially in smokers.

Furthermore, we strongly believe in the high clinical value of these studies. Defining the impact of oxidative stress generated by tobacco smoking on TKI treatments, and the respective mechanism involved, will serve as a message to patients who continue smoking despite undergoing TKI therapy and also guide physicians for decision relating to drug regimens and dosage recommendations. This is of great importance whenever a patient continues smoking or is subjected to conditions of high lung oxidative stress. Notably, lung cancer incidence is significantly elevated in patients with chronic obstructive pulmonary diseases (COPD), who present chronic oxidative stress (39) and aberrantly activated EGFR (40, 41) in their lungs.

As recently described by Mitchell and colleagues (19), we still do not know whether patients with lung cancer carrying the “TKI-sensitive” EGFR mutations and being former or current smokers would benefit from a TKI therapy (in comparison with those that are never-smokers). However, in this article, we present evidence indicating that tobacco smoking/oxidative stress could affect the efficacy of TKIs’ inhibition of both EGFR phosphorylation and cell viability of NSCLC. Moreover, this occurs even if the cells carry a TKI-sensitive EGFR mutation, such as L858R. Importantly, we have previously reported that cigarette smoke exposure affects EGFR activation/phosphorylation specifically via generation of H$_2$O$_2$-induced oxidative stress (27). Consistently, we confirmed that direct exposure of NSCLC cells to H$_2$O$_2$ affects EGFR-active conformation rendering the receptor resistant to TKIs (28).
Src and other members of its family (Src family kinases; SFK) have been thoroughly studied in several cancers, including NSCLC (42–44). Recently, studies by Chung and colleagues established novel cooperative roles for Src in supporting oncogenic mutant EGFR-dependent transformation of airway epithelial cells via the endocytotic recycling pathway and constitutive phosphorylation of Tyr845 in MT EGFRs (24, 29, 45). Importantly, several Src/SFKs are significantly activated in the airways of patients with NSCLC actively smoking compared with those who were never or former smokers (44). Masaki and colleagues had similar observations that both Src activity and expression are elevated in malignant lung tissue samples, particularly in adenocarcinomas, compared with surrounding normal lung parenchyma from the same patients (46).

Previous molecular studies show that Src interacts with EGFR in 2 unique ways that somehow involve the activation loop of the EGFR kinase. Src is known to phosphorylate Tyr845 (Y845) of both WT and MT EGFRs, a tyrosine residue found on the activation loop of the EGFR kinase domain (24, 47). However, past mutagenic studies showed that the phosphorylation of Y845 was not required for activation of the EGFR kinase, though its phosphorylation by Src assists in maximal biologic activity by EGF stimulation (47, 48). Recently, Shan and colleagues presented convincing data showing that phosphorylation of Y845 aids in stabilizing a critical salt-bridge interaction between Lys5721 and Glu738 required for catalytic activity of the EGFR (49). Following these studies, we tested whether Y845 phosphorylation may be important for TKI resistance under cigarette smoke. However, this seemed not to be the case (Supplementary Fig. S4) as the Y845F MT EGFR continued to be activated by cigarette smoke (pY1173), both in the presence and absence of TKIs. Given that Src activity is involved in generating EGFR TKI resistance (Fig. 3), it is possible that aberrant activation, and conformation of the EGFR under cigarette smoke exposure may be caused by Src-dependent phosphorylation of other EGFR tyrosines, which is currently being investigated in our laboratory.

Canonically, only ErbB2/Her2, and not WT EGFR, directly interacts with Src despite ErbB2 and EGFR sharing more than 95% homology in amino acid sequence of the kinase domain. Previous studies by Kim and colleagues clearly showed that interactions between Src and ErbB2 are conformation-dependent, requiring the structure of the catalytic kinase domain of ErbB2 for binding (30). Similarly, Chung and colleagues showed that Tyr845 of EGFR was required for binding and oncogenic cooperativity of Src to both L858R substitution mutant and Δ746-750 deletion mutant EGFRs (24). However, interactions between WT EGFR and Src under normal conditions (unstimulated or EGF-stimulated) seem to be transient and/or nonexistent, which intrigued us when we observed that Src strongly binds to the WT EGFR only during exposure to cigarette smoke–induced oxidative stress (Fig. 2; refs. 20, 28). Our results show that this aberrant interaction between Src and EGFR continues to occur during cigarette smoke exposure despite Src inhibition (Supplementary Fig. S3A) or a kinase-dead EGFR (Supplementary Fig. S3B), suggesting that neither Src nor EGFR kinase activity is solely responsible for their interaction, but rather, their conformations that may be initiated and/or sustained by cigarette smoke–induced oxidative stress (See “Model” in Fig. 6B).

Although structural studies on Src are ongoing, the active conformation(s) of Src triggered by oxidative stress are incompletely resolved. The phosphorylation of Y527-Src (pY527) is important for downregulation of Src activity, because it renders Src inactive due to a "closed" conformation involving Src’s SH2 phospho-Tyr–binding domain (50, 51). Substituting Tyr527 for a phenylalanine (F) forfeits this negative-regulatory function, thereby leaving the Src in an "open" conformation (23, 52). Marceotto and colleagues showed that an "open" Src conformation induced by the Y527F mutation was sufficient for Src binding to ErbB2 (23). Intriguingly, our current study mirrored that finding, shown in Fig. 4A, that Y527F-Src (CA-Src) and Y527F/K295R (DN-Src), which are both in the "open" state, constitutively bind to the EGFR regardless of stimuli (EGF; cigarette smoke). Ultimately, understanding the mechanism of how cigarette smoke aberrantly evokes an active conformation in both Src and EGFR will provide clues for designing new inhibitors specifically for treatment of NSCLC in smokers.

Functionally, we have shown before by the soft/agar assay that cigarette smoke exposure eradicates the TKI-dependent inhibition of anchorage-independent growth of EGFR-transformed cells (20). In addition, others have shown previously that TKIs induce apoptosis in H3255 cells (33). Our data showed that erlotinib (TKI) induces a drastic reduction in cell viability (as expected). This was shown further to be reduced by dasatinib cotreatment. However, the TKI effect was significantly counteracted by cigarette smoke exposure (Fig. 7). It is interesting that at the same time, dasatinib treatment alone reduced the cell viability of cigarette smoke–treated cells, implicating Src activity as a survival/proliferative mechanism during cigarette smoke/oxidative stress exposure. Dasatinib treatment was additive with TKI in reducing cell viability in the presence and absence of cigarette smoke exposure, providing the rationale for a combinatory therapy using both EGFR and Src inhibitors to treat NSCLC both in smokers and nonsmokers.

In summary, we provide insight into the initial structure/function alterations of EGFR and Src following exposure to cigarette smoke. We present for the first time a posttranslational mechanism accounting for drug (TKI) resistance development in NSCLC cells, offering not only a paradigm shift in the way we think of cancer therapy resistance, but also a specific target, Src, in the context of tobacco smoke/oxidative stress-related lung cancer. Specifically, the results of our investigation into Src as a molecular target for acquired drug resistance to TKIs in NSCLC provide strong support for continued evaluation of combinatory therapies for treatment of NSCLC.
particularly in patients who are exposed to lung oxidative stress (such as in smokers) and thus harbor aberrantly activated EGFR and Src. However, it should be noted that expression of KRAS mutants or other oncoproteins may de novo overcome the need of EGFR and Src signaling for cell survival and proliferation limits the patient population where such a combined therapy targeting both EGFR and Src should be considered. Further studies should elucidate the dynamic interaction/cooperation between EGFR and Src during cigarette smoke-induced oxidative stress, which may lead to novel opportunities for therapeutic intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: S. Filosto, T. Goldkorn
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Filosto, D.S. Baston
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Filosto
Study supervision: S. Filosto, T. Goldkorn

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