Cigarette Smoke Induces Aberrant EGF Receptor Activation That Mediates Lung Cancer Development and Resistance to Tyrosine Kinase Inhibitors

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Abstract

The EGF receptor (EGFR) and its downstream signaling are implicated in lung cancer development. Therefore, much effort was spent in developing specific tyrosine kinase inhibitors (TKI) that bind to the EGFR ATP-pocket, blocking EGFR phosphorylation/signaling. Clinical use of TKIs is effective in a subset of lung cancers with mutations in the EGFR kinase domain, rendering the receptor highly susceptible to TKIs. However, these benefits are limited, and emergence of additional EGFR mutations usually results in TKI resistance and disease progression. Previously, we showed one mechanism linking cigarette smoke to EGFR-driven lung cancer. Specifically, exposure of lung epithelial cells to cigarette smoke-induced oxidative stress stimulates aberrant EGFR phosphorylation/activation with impaired receptor ubiquitination/degradation. The abnormal stabilization of the activated receptor leads to uncontrolled cell growth and tumorigenesis. Here, we describe for the first time a novel posttranslational mechanism of EGFR resistance to TKIs. Exposure of airway epithelial cells to cigarette smoke causes aberrant phosphorylation/activation of EGFR, resulting in a conformation that is different from that induced by the ligand EGF. Unlike EGF-activated EGFR, cigarette smoke-activated EGFR binds c-Src and caveolin-1 and does not undergo canonical dimerization. Importantly, the cigarette smoke-activated EGFR is not inhibited by TKIs (AG1478; erlotinib; gefitinib); in fact, the cigarette smoke exposure induces TKI-resistance even in the TKI-sensitive EGFR mutants. Our findings show that cigarette smoke exposure stimulates not only aberrant EGFR phosphorylation impairing receptor degradation, but also induces a different EGFR conformation and signaling that are resistant to TKIs. Together, these findings offer new insights into cigarette smoke-induced lung cancer development and TKI resistance.

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Introduction

Smoking-related lung cancer is the leading cause of cancer deaths in both men and women in the United States. However, the molecular mechanisms underlying the induction of lung cancer by cigarette smoke are still poorly understood. It is currently known that overexpression and deregulation of receptor tyrosine kinases (RTK) are tightly connected to tumorigenesis. Of importance is the EGF receptor (EGFR), a member of the ErbB family of RTKs, which also includes ErbB2, ErbB3, and ErbB4 (1). EGFR is implicated in a number of cancers including lung cancer (2–4).

This receptor plays a role in normal cell processes, but the deregulation of its activation and downstream signaling leads to aberrant cell proliferation and cancer development (5–7).

The activation mechanism of EGFR in lung cancer has been a subject of intense studies, and a classical model of EGFR activation has been established wherein ligand binding induces receptor dimerization. This leads to activation of the EGFR intracellular tyrosine kinase domain and subsequent autophosphorylation of specific tyrosines on the C-terminal tail of the receptor, initiating a cascade of downstream signaling.

Over the last decade, specific tyrosine kinase inhibitors (TKI) have been developed to block EGFR activation/signalin. TKIs are small molecules that inhibit TK activity via binding reversibly to the EGFR ATP binding site; several TKIs are approved by the U.S. Food and Drug Administration for the treatment of metastatic lung cancer, including erlotinib (Tarceva), while other TKIs are in preclinical trials (e.g., TKI AG1478; refs. 8–11). Trials with TKIs indicated that tumor responses to the drugs were remarkable and sustainable in specific subsets of non-small cell lung cancer (NSCLC) patients that possess activating EGFR mutations (12–14). Intriguingly, however, it has been noted anecdotally that patients responsive...
to TKI therapy developed TKI resistance if they began smoking (15).

Notably, molecular structure studies of the L858R mutant (MT) EGFR (16) showed that the conformation of this TKI-sensitive mutant differs from that of the wild-type (WT) EGFR at the level of the kinase domain, which carries a constitutively open activating loop. This open loop turns the EGFR to be constitutively active. Moreover, such a conformation allows better access of TKIs to the EGFR pocket of ATP binding.

In this study, we investigated whether smoking-related TKI resistance may be explained mechanistically by alterations in the EGFR conformation and signaling. Recently, we showed that EGFR activation induced by cigarette smoke was in fact independent of ligand binding (5). Specifically, one of the major reactive oxidants in the gas phase of cigarette smoke, hydrogen peroxide (H$_2$O$_2$), caused aberrant phosphorylation and activation of the EGFR (5) in human airway epithelial cells. The abnormal phosphorylation of the receptor in cells exposed to H$_2$O$_2$-induced oxidative stress also acquired an aberrant activated conformation that impairs canonical dimerization of EGFR (17). This activated EGFR was neither ubiquitinated nor subsequently degraded due to its inability to bind the E3-lygase, c-Cbl. This allowed EGFR to remain active for a longer period at the plasma membrane, thereby causing prolonged survival signals that contributed to uncontrolled cell growth (5–6, 18).

Here, we present 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 c-Src, resulting in ligand-independent EGFR activation that is resistant to inhibition by TKIs.

In fact, both WT and TKI-sensitive EGFR MTs develop TKI resistance through this mechanism after cigarette smoke exposure. These findings help to elucidate how cigarette smoke induces aberrant EGFR signaling that promotes lung cancer and therapy resistance.

Materials and Methods

Cell culture, treatments, and reagents

A549 adenocarcinoma (American Type Culture Collection), NCI-HCC827 (generous gifts from Dr. Philip Mack, University of California at Davis, Davis, California), and NIH-3T3 cells (generous gifts from Dr. Hamid Band, University of Nebraska Medical Center; ref. 19) have been used in this study. All the cell lines used in this study were previously characterized by others, as reported (19–21); no authentication was done by the authors. Details regarding the various media and source of reagents are provided in the online supplement.

Cigarette smoke exposure

Serum-starved cells were exposed to cigarette smoke as described before (5). Details about the exposure chamber are in the online supplement.

Immunoprecipitation

Total protein extracts (200–400 µg) were incubated for 3 hours with 2 to 4 µg of antibodies: anti (α) 528 (against EGFR) or α4-2 [active-EGFR, kindly provided by Dr. K. Omi, Fujirebio Inc., Tokyo, Japan (22)]. A total of 50 µL of 50% protein A-agarose bead complexes (Repligen) was 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 immunoprecipitations (IP) in the loading dye for SDS-PAGE, as described before (5, 17).

SDS-PAGE and immunoblotting

Acrylamide gels (5%, 6%, 8%, 10%, or 12%) were prepared and used in a 2 Cell system (BioRad) for 1 to 4 hours at 100 V at room temperature. Total protein extracts (20–100 µg) or the IP samples were loaded into each well of the SDS-PAGE in the presence of a SDS/DTT reducing loading dye. After SDS-PAGE separation, the proteins were transferred to a nitrocellulose membrane and blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 for 120 minutes or overnight, as described (17). Full details about the antibodies used are provided in the online supplement.

Anchorage-independent growth assay (soft agar/agarose assay)

A total of 5,000 single-suspended NIH-3T3 cells overexpressing the L858R EGFR MT were mixed in culture medium (38°C) containing 0.275% low melting point agarose (Fermentas) and then seeded in 6-well plate dishes (Falcon) on top of 0.6% agarose and then seeded in 6-well plate dishes (Falcon) on top of 0.6% agarose (BioTech Sources LLC) layer, as previously described by others (19). Growing colonies (composed of at least 3 cells) were randomly counted by optical microscopy at ×100 magnification.

Statistical analysis

Each treatment and experiment [immunoblotting (IB), IP/IB or soft agar/agarose assay] was repeated at least 3 times. The plotted data are reported as mean ± SD. Statistical significance was determined by Student t test, and P < 0.05 was considered statistically significant.

Results

Cigarette smoke-induced phosphorylation of EGFR is not inhibited by TKIs, AG1478, erlotinib, and gefitinib

Small-molecule TKI, that is, AG1478, erlotinib, and gefitinib are used to target the EGFR, suppressing its kinase activity by reversibly blocking the ATP-binding site of the receptor. Thus, we tested the efficacy of these TKIs on cigarette smoke-induced phosphorylation of EGFR.

Serum-starved A549 cells were incubated (or not) with 1 µmol/L AG1478 or erlotinib or gefitinib for 30 minutes and then treated (or not) with 100 ng/mL EGF or smoke from 1 cigarette for an additional 30 minutes. Cells were lysed, EGFR was immunoprecipitated and immunoblotted for total receptor, total tyrosine (Tyr) phosphorylation, and active-EGFR, kindly provided by Dr. K. Omi, Fujirebio Inc., Tokyo, Japan (22). A total of 50 µL of 50% protein A-agarose bead complexes (Repligen) was 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 immunoprecipitations (IP) in the loading dye for SDS-PAGE, as described before (5, 17).
phosphorylation, and specific Tyr (Y) phosphorylation sites, as indicated in Fig. 1. Cells treated with EGF showed an increase of phosphorylation at the EGFR autophosphorylation sites Y1068 and Y1173 compared with untreated cells. Of the cells exposed to cigarette smoke, activation at the auto-phosphorylation sites highly increased along with the c-Src-dependent transphosphorylation site (Y845) compared with control (we previously showed that c-Src is highly activated in human airway epithelial cells exposed to cigarette smoke (5). With incubation of TKIs before treatment, AG1478 (Fig. 1A), erlotinib (Fig. 1B), and gefitinib (Fig. 1C) EGF-induced phosphorylation was inhibited as expected, highly decreasing the phosphorylation at sites Y1068 and Y1173. However, with cigarette smoke treatment, the TKIs failed to suppress EGFR phosphorylation at all sites (Fig. 1A for AG1478, Fig. 1B for erlotinib, and Fig. 1C for gefitinib).

**Cigarette smoke exposure leads to downstream survival and proliferation signaling that is not inhibited by TKIs**

Extracellular signal-regulated kinases (ERK) 1/2 and Akt (also known as protein kinase B) are 2 well-known mediators of cell proliferation and survival and are known to be involved in cell transformation when persistently activated (23–24). We have shown previously (5) that exposure to smoke from 1 cigarette for 45 minutes activates downstream ERK 1/2 and Akt signaling. Furthermore, both EGFR and ERK1/2 phosphorylation persisted for up to 2 hours after the removal of the treatment medium exposed to cigarette smoke, whereas removal of EGF returns phosphorylation to near baseline levels at these time points. This further showed that these signals may be prolonged due to the inability of the EGFR to be degraded under cigarette smoke-induced oxidative stress.

Importantly, here we show, in the Supplementary Fig. S1, that under cigarette smoke exposure not only are the survival signals prolonged but also the TKI treatment cannot quench the cigarette smoke-dependent phosphorylation/activation of ERK 1/2 and Akt/protein kinase B.

**c-Src binds EGFR under cigarette smoke exposure**

Next, we investigated whether cigarette smoke exposure could induce the association of EGFR with c-Src. Serum-starved A549 cells were treated (or not) with 100 ng/mL EGF for 15 minutes or exposed to smoke from 1 cigarette for 30 minutes. Cells were lysed, EGFR was immunoprecipitated and immunoblotted for total EGFR and active (p-Y416) c-Src. As shown in Fig. 2, after cigarette smoke exposure, c-Src (active c-Src) strongly associated with EGFR; whereas, upon EGF stimulation no differences in the interaction between c-Src and EGFR were observed in comparison with control [not treated (NT)] conditions (Fig. 2). Because it has been shown that conformational changes in the intracellular domain of EGFR may lead to direct interaction between EGFR and c-Src (7, 19), such an observed interaction under cigarette smoke exposure provides an indication for a novel conformation

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**Figure 1.** Cigarette smoke-induced EGFR phosphorylation is not inhibited by TKIs AG1478, erlotinib, and gefitinib. Serum-starved A549 cells were incubated (or not) with 1 μmol/L AG1478 (A), 1 μmol/L erlotinib (B), or 1 μmol/L gefitinib (C) for 30 minutes. Cells were treated for 30 minutes with 100 ng/mL EGF or exposed to smoke from 1 cigarette for 30 minutes. EGFR was immunoprecipitated from the total cell lysates, separated by SDS-PAGE and immunoblotted for total receptor, Tyr phosphorylation level (p-EGFR), and specific Tyr phosphorylation (Y845, Y1068, and Y1173). Each treatment and experiment/IP-IB was repeated 3 times. Sequential IBs of the immunoprecipitates were done by stripping the antibodies off the membranes in between each IB. The images shown are representative of 3 independent experiments. CS, cigarette smoke; NT, not treated.
of the EGFR. This interaction also explains the strong level of phosphorylation observed at Y845 (Fig. 1).

Activated EGFR does not dimerize under cigarette smoke

We have shown before that H$_2$O$_2$-generated oxidative stress did not induce formation of EGFR dimers in the presence of the cross-linker EDAC both in airway epithelial cells and in NIH-3T3 cells overexpressing EGFR (17). Here, we investigated the mechanism of EGFR dimerization under cigarette smoke. Serum-starved A549 cells were initially exposed (or not) for 15 minutes to 100 ng/mL EGF or to smoke from half a cigarette. Then, a cross-linking agent, EDAC (1 mmol/L), was added (or not) for additional 15 minutes (in the presence of EGF or cigarette smoke, respectively). Cells were lysed, and an equal amount of proteins (50 μg) were separated by SDS-PAGE and immunoblotted for total and Tyr-phosphorylated EGFR (Fig. 3). As expected, treating with EGF and the cross-linking agent resulted in the formation of a dimer with a band at approximately 340 to 360 kDa. On the contrary, cigarette smoke induced the activation of EGFR, but no dimer was detected in the presence of EDAC (Fig. 3). This shows that cigarette smoke-induced activation of EGFR does not result in the classical dimerization of EGFR.

Cigarette smoke induces a novel active conformation of the EGFR intracellular domain

To substantiate our hypothesis that the EGFR acquires a new active conformation under cigarette smoke exposure, we used a novel conformational change-sensitive EGFR monoclonal antibody (mAb), anti-(α4-2 antibody (22). This antibody recognizes epitopes (amino acids 956–998) of EGFR in nondenaturing conditions. These epitopes are in the intracellular domain of EGFR and were found to be exposed only in the canonically ligand-activated WT EGFR. In addition, the 4-2 mAb binds constitutively to the L858R EGFR MT, because the same epitopes are constitutively exposed in that mutant (22).

Serum-starved A549 cells were treated (or not) with EGF or cigarette smoke as before. EGFR was immunoprecipitated from 300 μg of total cell lysate with either α4-2 mAb or α528 mAb and then immunoblotted for total EGFR (α2232; Fig. 4A and B). The α528 mAb pulled down EGFR with the same affinity for all of the treatments, as expected (Fig. 4A). The α4-2 mAb bound significantly higher to the EGF-treated EGFR than to the untreated receptor. However, under cigarette smoke exposure, although EGFR is activated (Fig. 1) as verified by strong blotting of its autophosphorylation sites (using αp–Y1173 antibody), its affinity to that special antibody, which marks a canonical activated conformation, did not change (Fig. 4A). The α4-2 mAb binds EGFR under EGF stimulation with an affinity approximately 700% greater than control conditions, but this does not happen under cigarette smoke activation (Fig. 4B), indicating that the intracellular active conformation of EGFR under cigarette smoke does not resemble that of the EGF-activated receptor.

Furthermore, we investigated the ability of the α4-2 mAb to bind the L858R EGFR MT. This was done by using NIH-3T3 cells stably overexpressing this mutant. Cells were treated with EGF or cigarette smoke, as before, and EGFR was immunoprecipitated from total cell lysates with either α4-2 or α528 mAbs, and immunoblotted for total EGFR.
(α2232). Figures 4C and D confirm that the α4-2 mAb binds to the L858R EGFR MT with high affinity (Fig. 4C), as previously reported (22). However, its affinity is reduced upon cigarette smoke exposure, even though the phosphorylation of the MT EGFR under EGF and cigarette smoke exposure is comparable (data not shown). Indeed, there is approximately 40% reduction in the amount of MT EGFR pulled down by α4-2 mAb after cigarette smoke exposure (Fig. 4C). This suggests that cigarette smoke changes the conformation of both WT and MT L858R EGFR at the level of the intracellular domain, most likely at the level of the kinase domain. Therefore, this novel active conformation could be no longer inhibited by the TKIs, as described above.

**The TKI-sensitive L858R somatic MT EGFR acquires resistance to TKIs under cigarette smoke**

Because we observed that the TKIs were ineffective in inhibiting EGFR activation under cigarette smoke, we tested their efficacy against the L858R EGFR MT upon cigarette smoke exposure.

NIH-3T3 cells stably overexpressing the L858R EGFR MT were serum starved and incubated (or not) with 1 μmol/L AG1478 or erlotinib for 30 minutes and then treated (or not) with 100 ng/mL EGF or smoke from 1 cigarette for an additional 30 minutes. Cells were lysed, EGFR was immunoprecipitated and immunoblotted for total receptor, Tyr phosphorylation, and specific tyrosine phosphorylation sites, as above.

As shown in Fig. 5, the L858R EGFR MT is constitutively active [as expected (7, 13, 25)], while its phosphorylation is slightly increased under both EGF and cigarette smoke. The constitutive activation of the EGFR MT as well as the EGF-induced activation were inhibited by both TKIs, AG1478 (Fig. 5A), and erlotinib (Fig. 5B), at the auto-phosphorylation sites Y1068 and Y1173 compared with untreated cells. However, this TKI-sensitive EGFR MT
becomes resistant to TKI treatments upon exposure to cigarette smoke (Fig. 5A relative to AG1478 and Fig. 5B relative to erlotinib). Both the autophosphorylation sites Y1068 and Y1173 and the transphosphorylation site Y845 remained active under cigarette smoke exposure even with the preincubation of AG1478 or erlotinib. To further validate this response, another drug-sensitizing MT EGFR with an in-frame deletion in exon 19 (EGFR D746–750; which encodes part of the kinase domain) was subject to the same TKI treatments. As with the L858R EGFR MT, this deletion mutant acquired resistance to both AG1478 and erlotinib under cigarette smoke (data not shown).

Finally, we also repeated the cigarette smoke/TKI treatments in the NSCLC cells, HCC827, which are harboring the TKI-sensitive EGFR MT: Δ746–750 MT (26). Supplementary Figure S2 shows that exposure to cigarette smoke of these TKI-sensitive NSCLC cells also causes EGFR resistance to TKIs.

Overall, our data suggest that cigarette smoke induces a novel active conformation of EGFR that differs from the EGF-induced one and also is different from that of the L858R EGFR MT and other TKI-sensitive mutants. Such a novel acquired conformation could be the reason for the receptor’s acquired resistance to TKIs.

Collectively, all the above new data suggest that under cigarette smoke exposure the conformational change of EGFR may no longer keep the kinase domain of the receptor fully open, so that the TKIs’ (AG1478, erlotinib, and gefitinib) accessibility to the EGFR binding pocket and thus their ability to effectively inhibit EGFR phosphorylation/activation is reduced. This allows the EGFR and its downstream targets, ERK 1/2 and Akt, to remain active.

Cigarette smoke exposure abolishes the TKI-dependent inhibition of anchorage-independent growth of EGFR-transformed cells

Next, we assessed the effect of cigarette smoke exposure on the anchorage-independent growth of EGFR-transformed cells in the presence or absence of TKI. We used the NIH-3T3 cells stably overexpressing L858R EGFR, which were previously shown to be very suitable for testing such transforming potential in soft agar/agarose colony assay (19). Single-suspended cells were seeded inside a layer of 0.275% agarose (in culture medium), and on top of a 0.6% agar gel layer, as described in Materials and Methods. Subsequently, the cells were fed daily with culture medium (DMEM + 10% FBS) that was supplemented (or not) with 1 μmol/L gefitinib; the medium was also pre-exposed (or not) for 30’ to smoke from 1 cigarette. Supplementary Figure S3 shows that incubation with 1 μmol/L gefitinib could effectively inhibit the colony formation of the EGFR-transformed cells [in comparison with control-untreated (NT) cells]. However, gefitinib treatment became ineffective upon cigarette smoke exposure of those cells. This confirmed that cigarette smoke exposure can overcome the TKI sensitivity of EGFR-transformed cells, thus sustaining clonal growth of lung cancer even in the presence of TKI drugs.

Discussion

We define here the initial posttranslational changes that occur in EGFR with cigarette smoke exposure of airway epithelial cells. These alterations in EGFR lead to aberrant signaling and cell proliferation, and ultimately constitute a molecular basis for cigarette smoke-induced lung cancer initiation and promotion (5).

Given its frequent deregulation in NSCLC, EGFR became a logical target for therapy. The first targeted
strategies used a mAb antagonist to the ligand-binding domain (cetuximab) (27). However, more dramatic responses were observed with small-molecule reversible inhibitors of the tyrosine kinase domain (TKIs, gefitinib, or erlotinib). Though highly gratifying, these remarkable clinical responses were restricted to a subset of cases, typically adenocarcinoma occurring in female never-smokers of Japanese origin (27). Subsequently, these responders were found to possess mutations in the EGFR TK domain, which were thought to confer a selective growth advantage that could be neutralized by the TKIs (13, 25, 28–30). Most common, or classic, among these somatic mutations are the exon 19 deletion mutation (Δ746–750) and the exon 21 single-point substitution mutation (L858R) (31–32).

However, it has been recently observed that the same EGFR mutations are not limited to adenocarcinoma from female never-smokers. Indeed, a large number (43%) of EGFR mutations are actually found in adenocarcinoma tumor specimens from men and people who are smokers or former smokers (33). Yet, these smoking patient groups (harboring EGFR mutations, which are sensitive to TKIs) do not seem to benefit from the TKI treatment. This is most notably evidenced by the clinical findings that the sensitivity to TKIs is mostly observed in adenocarcinoma of nonsmokers, whereas cigarette smoke exposure is mostly associated with squamous cell carcinoma and adenocarcinoma that are not sensitive to TKIs. Perhaps for this reason, nonsmoking adenocarcinoma patients who initially respond to TKIs develop resistance when they begin to smoke whereas smoking patients (whose cancer developed in the setting of cigarette smoke exposure) are resistant to TKI in the first place.

Therefore, here we propose that the aberrant mechanism of EGFR ligand-independent (5) activation in human airway epithelial cells exposed to cigarette smoke is due to a novel and uncharacterized conformation of the intracellular domain of the receptor that leads to an active, yet stabilized, EGFR that is also resistant to TKI drugs. Therefore, cigarette smoke-induced EGFR changes may contribute to both the initial disease pathogenesis in smokers and to emergence of TKI resistance in nonsmokers who first are sensitive to TKI.

To provide direct evidence for the conformational change of EGFR under cigarette smoke, we used a novel conformational change-sensitive EGFR antibody (α4-2 mAb; ref. 22), which we used before (17). This antibody was shown to bind epitopes of EGFR that are exposed only subsequent to EGFR canonical activation by its ligand, EGF, which induces a conformational change of the kinase domain (16). This antibody also binds constitutively to the L858R EGFR MT because the same epitopes are constitutively exposed in this mutant due to its open activating loop of the kinase domain (16, 22).

Interestingly, even though EGFR is highly activated by cigarette smoke, the α4-2 mAb binds to the cigarette smoke-stimulated EGFR with a much lower affinity than to that activated by EGF (Fig. 4A and B). Furthermore, we showed that the high affinity of the α4-2 mAb for the L858R EGFR MT also dropped approximately 40% upon cigarette smoke exposure. This was not the case upon EGF stimulation (Fig. 4C and D), indicating that cigarette smoke exposure induces an active state of the EGFR that differs from that of the conventional/EGF-stimulated EGFR.

Another indication for a unique conformational change of EGFR under cigarette smoke-induced oxidative stress was supported by the finding that EGFR was strongly associated with c-Src only upon cigarette smoke (and not EGF) exposure of human airway epithelial cells (Fig. 2). We reported previously that highly phosphorylated Cav-1 is strongly bound to EGFR under cigarette smoke-induced oxidative stress (5, 18). Others reported that c-Src stably interacts with ErbB2, but not with WT EGFR, because of the difference in their kinase domains (38). This c-Src binding was shown to confer elevated transformation ability (39). Furthermore, the L858R EGFR MT could also bind c-Src (19).

Collectively, these findings suggest that cigarette smoke exposure may induce TKI resistance solely through post-translational molecular changes without additional somatic mutations. These molecular alterations consist of an EGFR aberrant phosphorylation pattern caused by cigarette smoke exposure (5) accompanied by an aberrant conformational change. A possible hypothesis is that this conformational change limits TKI accessibility to the ATP

Cigarette Smoke, EGFR, TKI Resistance, Lung Cancer
binding site of the receptor, thereby preventing the inhibitory effect of the drug and allowing continuous EGFR signaling and cell proliferation (see model in Fig. 6). Accordingly, we also provide evidence that cigarette smoke exposure can abolish the TKI-dependent inhibition of ERK 1/2 and Akt activation (Supplementary Fig. S1) and of the anchorage-independent growth of EGFR-transformed cells in soft agar/agarose assay (Supplementary Fig. S3).

Of note is that even an irreversible EGFR inhibitor, such as CL387785, turned to be only 50% efficient in inhibiting EGFR activation (phosphorylation) if the cells were simultaneously exposed to the inhibitor and to cigarette smoke (data not shown). Importantly, we have also carried out control experiments addressing whether the TK inhibitors may be damaged under cigarette smoke exposure. We found that it was not the case. The effectiveness of the cigarette smoke-exposed TKIs was evaluated in EGF-stimulated cells and was shown to be as potent as before the TKIs were exposed to cigarette smoke (data not shown).

Taken together, we show herein that EGF-stimulated WT EGFR, L858R, and Δ746–750 EGFR MTs (with or without EGF stimulation) have dramatic inhibition of activation/phosphorylation in the presence of the TKIs AG1478, erlotinib, or gefitinib (Figs. 1 and 5; Supplementary Fig. S2). In contrast, upon cigarette smoke exposure, both the WT and those EGFR MTs become resistant to TKIs, showing a strong phosphorylation at the autophosphorylation sites Y1173 and Y1068. The receptors'...
acquired TKI resistance supports once again a cigarette smoke-induced conformational change in the intracellular kinase domain of EGFR, which could reduce TKIs' accessibility to their binding site. These findings may explain the clinical observation, reported anecdotally, that lung cancer patients who resume smoking while receiving TKI treatment rapidly develop resistance and disease progression (15).

Therefore, we conclude that cigarette smoke-induced posttranslational changes in EGFR could provide an important mechanism of disease pathogenesis underlying TKI resistance in smokers. Additional studies are required to elucidate the exact conformation of EGFR under cigarette smoke-induced oxidative stress; a direction that may lead to the development of more effective TKIs capable of inhibiting EGFR signaling even in the presence of cigarette smoke or other forms of oxidative stress.

Cigarette Smoke, EGFR, TKI Resistance, Lung Cancer

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

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