ICBKKE Is a Substrate of EGFR and a Therapeutic Target in Non-Small Cell Lung Cancer with Activating Mutations of EGFR

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Abstract

Non–small cell lung cancers (NSCLC) marked by EGFR mutations tend to develop resistance to therapeutic EGFR inhibitors, often due to secondary mutation EGFR{T790M} but also other mechanisms. Here we report support for a rationale to target ICBKE, an iκB kinase family member that activates the AKT and NF-κB pathways, as one strategy to address NSCLC resistant to EGFR inhibitors. While wild-type and mutant EGFR directly interacted with ICBKE, only mutant EGFR phosphorylated ICBKE on residues Y153 and Y179. The unphosphorylatable mutant ICBKE-Y153F/Y179-F that lost kinase activity failed to activate AKT and inhibited EGFR signaling. In clinical specimens of NSCLC with activating mutations of EGFR, we observed elevated levels of phospho-Y153 ICBKE. ICBKE ablation with shRNA or small-molecule inhibitor amlexanox selectively inhibited the viability of NSCLC cells with EGFR mutations in vitro. In parallel, we found that these treatments activated the MAPK pathway due to attenuation of an ICBKE feedback mechanism. In vivo studies revealed that combining amlexanox with MEK inhibitor AZD6244 significantly inhibited the xenograft tumor growth of NSCLC cells harboring activating EGFR mutations, including EGFR{T790M}. Overall, our findings define ICBKE as a direct effector target of EGFR and provide a therapeutic rationale to target ICBKE as a strategy to eradicate EGFR-TKI–resistant NSCLC cells. Cancer Res; 76(15): 4418–29. ©2016 AACR.

Introduction

EGF receptor (EGFR) is the major driver pathway of non–small cell lung cancer (NSCLC), especially lung adenocarcinoma (1, 2). Activating mutations of EGFR have been detected in 15% and 30% of NSCLC in Western and Asian populations, respectively (3). EGFR-mutated NSCLCs depend on EGFR signaling for their survival and growth. While MAPK, PI3K/AKT, and the STAT pathways have been shown to be the major signaling cascades involved in the EGFR oncogenic signaling (3), the molecular mechanism of active mutant EGFR driving NSCLC growth remains elusive.

Activating EGFR mutations dictate responsiveness of NSCLC to reversible EGFR tyrosine kinase inhibitors (TKI). Despite promising initial responses, virtually all patients, ultimately, progress because of the acquisition of resistance (3). Secondary EGFR{T790M} mutation is the most common resistance mechanism and accounts for approximately 60% of patients after disease progression (4, 5). Second-generation irreversible, EGFR-TKIs have been developed and extensively studied. Despite promising preclinical evidences of activity against EGFR-mutated cell lines harboring the T790M mutation (6–8), they, as monotherapy, have failed to overcome T790M-mediated resistance in patients (9–12). In addition to efforts of developing and characterizing “third-generation” EGFR-TKIs that are designed to target T790M, inhibition of EGFR downstream signaling, such as MEK inhibitor and PI3K/Akt/mTOR inhibitors, has been used to overturn the resistance (13–15).

ICBKKE is a noncanonical iκB kinase family member frequently upregulated/activated in NSCLC (16) and its overexpression leads to malignant transformation in various cell types (17). In addition to phosphorylation of IRF3, IRF7, and STAT1 (18–21), ICBKE can activate the NF-κB pathway by inhibiting several key regulators of NF-κB pathway (22–25), which lead to induction of CCL5 and IL6 and subsequent activation of IAK/STAT3 pathway (26, 27). In addition, we and others have demonstrated that ICBKE activates AKT by direct phosphorylation of AKT-Thr308 and Ser473 independent of PI3K and mTORC2 (28, 29). ICBKE is also able to exhibit its oncogenic function by phosphorylation and degradation of FOXO3a (30). In this study, we showed that wild-type and mutant EGFR interact with ICBKE and ICBKE is directly activated by mutant but not wild-type EGFR. The ICBKE inhibitor amlexanox, a small-molecule therapeutic presently used in the clinic to treat aphthous ulcers, asthma, and obesity (31–33), synergized with MEK inhibitor AZD6244, leading to significant reduction of tumor growth in NSCLC cells bearing EGFR mutation.
Materials and Methods

Cell culture, plasmids, and antibodies

NSCLC cells were obtained from Moffitt Cancer Center Lung Cancer SPORE Cell Core and maintained in RPMI1640 containing 10% FBS and 1% penicillin/streptavidin (34). The cell lines were authenticated using STR analysis and were used within six months of resuscitation. Knockdown of IKBKE was performed using shRNAs described previously (16). siRNAs specific for EGFR were obtained from IDT Technologies. The sequences for EGFR siRNAs are: siEGFR1: sense 5’-GCACAUCUCCUGAAAGCCATT-3’, antisense 5’-UAGCCUUGUGUAGACUGA-3’; and siEGFR2: sense 5’-GGAGCACAAGCCACAAUUCA-3’, antisense 5’-GACUUGUGUAGUACUGAG-3’. Aflatinib was purchased from LC Biotech. Amlexanox and AZD6244 were from Sigma. The pCMV-Tag3b Myc-IBKKE plasmid was obtained as described previously (35). Tyrosine-153 and -179 residues were mutated to phenylalanine using the Stratagene Quick Change kit according to the manufacturer protocol. GST-fusion peptides (20 amino acids) spanning the phosphorylation tyrosines and their mutant forms were obtained by cloning oligonucleotides into pGEX-4T1 vector. Transfections of IKBKE were performed by PCR amplification and ligated into pCMV-Tag3b vector.

Antibodies against pIKBKE-Ser172, pAKT-Thr308, pAKT-Ser473, AKT, pEGFR-Y1068, and GAPDH were purchased from Cell Signaling Technology. IKBKE antibody was from Sigma. For the EGFR, Myc, pan-phospho-tyrosine (pTyr), and GST antibodies were purchased from Santa Cruz Biotechnology.

Western blot, immunoprecipitation, and in vitro kinase assays

Western blot analysis was performed as described previously (28). For detection of phosphorylated IKBKE-tyrosine, cell lysates were immunoprecipitated with anti-Myc (for transfected myc-IBKKE) or anti-IBKKE antibodies, followed by Western blot analysis with pTyr antibody. For coimmunoprecipitation, cells expressing myc-IBKKE and EGFR were lysed in a lysis buffer containing 50 mMolar/L Tris-HCl, 300 mMolar/L NaCl, 4 mMolar/L EDTA, 1 mMolar/L DTT, 0.5% CHAPS. IKBKE and EGFR kinase assays were performed as described previously (28, 36). Recombinant IKBKE and EGFR were purchased from Life Technologies and Sigma, respectively.

MTT and clonogenic assays

Cells indicated in the figure legends were plated in 96-well plates at a density of 4,000 cells/well. After culture for 12 hours, growth media were replaced with media containing indicated amount of Amlexanox (IKBKE inhibitor) or AZD6244 (MEK inhibitor) and the combination of these two inhibitors. After 48 hours, MTT assay (Sigma) was performed according to manufacturer’s protocol. Drug synergy was calculated using CompuSyn software (37). For clonogenic assay, cells were plated at a density of 500 cells/well in a 12-well plate and treated with the IKBKE inhibitor or MEK inhibitor and their combination. After culture for 9 days, the cells were fixed with crystal violet stain.

Mass spectrometry

IKBKE from in vitro EGFR kinase assay reactions were separated on SDS-PAGE. The band corresponding to IKBKE was in-gel digested with trypsin and the phosphorylated peptides were identified by MS/MS.

Confocal microscopy and IHC

After transfection of GFP-IBKKE and EGFR, the cells were fixed with 10% formalin containing methanol and the cells were stained with EGFR antibody. Colocalization of IKBKE and EGFR in H1299 cells were analyzed using confocal microscopy (30). IHC was performed as described previously (16). High pH antigen unmasking solution (Vector Laboratories) was used for antigen retrieval.

Cell migration/invasion assay, cell-cycle analysis, and xenograft study

Cell migration and invasion assays were performed as described previously (38). Cell-cycle analysis was performed using PI staining and followed by flow cytometry.

Three cell lines H1975, HCC827, and H1650 were injected subcutaneously into 7-week old female nu/nu mice (5 x 10⁶/mouse, Charles River Laboratories). After the tumors reached a volume of 100 mm³, mice were randomized into four groups, that is, vehicle, Amlexanox (50 mg/kg), AZD6244 (25 mg/kg), and combination of Amlexanox and AZD6244. The mice were treated five days a week by oral gavage. Tumor volumes were measured using calipers. The animal experiments were performed according to the IACUC protocol.

Statistical analysis

Student t tests were used to analyze statistical significance. P ≤ 0.05 was considered as statistically significant. The error bars indicate SD.

Results

NSCLC cell lines with activating EGFR mutation are more sensitive to IKBKE inhibition and activating mutations of EGFR activate IKBKE

We have previously shown frequent overexpression of IKBKE in NSCLC (16). To investigate its functional significance in NSCLC, we knocked down IKBKE in a panel of NSCLC cell lines. Interestingly, we observed that except PC9, cells harboring the activating mutations of EGFR, including EGFR-T790M–mutant H1975 and H820, are more sensitive to IKBKE knockdown than NSCLCs with wild-type EGFR (Fig. 1A). Further analysis of IKBKE activation, by measuring pIKBKE-Ser172, revealed that IKBKE was highly activated in the EGFR-mutant cell lines (Fig. 1B). To validate the clinical relevance of these findings, we analyzed a public database containing 720 lung adenocarcinoma patient samples available at KM Plotter (39) and observed that higher expression of IKBKE was significantly correlated with poor overall survival regardless disease stage (Supplementary Fig. S1A). We further examined if activating mutations of EGFR induces IKBKE kinase activity. After transfection of various EGFR mutants and myc-IBKKE, we immunoprecipitated IKBKE and performed in vitro IKBKE kinase assay. Notably, we found that IKBKE kinase activity was significantly induced by active EGFR mutants but not wild-type EGFR and that EGF stimulation failed to activate IKBKE (Fig. 1C, Supplementary Fig. S1B). Collectively, these findings
suggest that IKBKE is regulated by activating mutations of EGFR and could serve as a therapeutic target and a prognostic marker in NSCLC, especially in the tumors with EGFR mutations.

**IKBKE directly interacts with EGFR**

It has been shown that Kaposi sarcoma–associated herpesvirus GPCR (kGPCR) directly interacts with IKBKE, resulting in activation of IKBKE (40). To understand the mechanism by which IKBKE is activated by mutant EGFR, we first investigated whether activating mutation of EGFR is able to form a complex with IKBKE. Following expression of EGFR\(^{L858R/T90M}\) and myc-IKBKE in H1299 cells, coimmunoprecipitation revealed that the mutant EGFR interacted with IKBKE (Fig. 2A). Interestingly, IKBKE also bound to wild-type EGFR and addition of EGF ligand led to dissociation of EGFR-IKBKE complex (Supplementary Fig. S2A). We further found that endogenous IKBKE coimmunoprecipitates with EGFR in H1975 cells, which harbor EGFR\(^{L858R/T90M}\) mutation (Supplementary Fig. S2B). Furthermore, inhibition of EGFR kinase activity has no effect on this interaction in H1975 (Fig. 2B), HCC827 and H292 (Supplementary Fig. S2C and S2D) cells. In addition, confocal microscopy showed that IKBKE was colocalized with EGFR (Supplementary Fig. S2C and S2D). These data imply that EGFR kinase activity is dispensable for its interaction with IKBKE.

To examine whether this interaction is direct, we performed in vitro binding assay using recombinant GST-tagged intracellular domain of EGFR\(^{L858R/T90M}\) and IKBKE proteins. After immunoprecipitation with IKBKE antibody, IKBKE was readily detected in the immunoprecipitates with IKBKE antibody (Fig. 2C). Furthermore, wild-type EGFR or EGFR\(^{L858R/T90M}\) was immunoprecipitated with IKBKE antibody after incubation of recombinant GST-IKBKE and EGFR\(^{WT}\) or EGFR\(^{L858R/T90M}\) proteins in vitro (Supplementary Fig. S2F). In addition, an in vitro GST-pulldown assay by incubating GST-EGFR or GST-vector with lysates from HEK293T cells expressing myc-IKBKE revealed that myc-IKBKE is specifically pulled down by GST-EGFR\(^{L858R/T90M}\) or GST-EGFR\(^{WT}\) (Fig. 2D and Supplementary Fig. S2G). To identify the domains in IKBKE that are critical for its interaction with EGFR, we generated constructs expressing myc-tagged N-terminal/kinase and C-terminal domains of IKBKE. Immunoprecipitation revealed that N-terminal region of IKBKE bound to EGFR (Fig. 2E). Collectively, these results suggest that intracellular domain of EGFR directly interacts with N-terminal region of IKBKE.

**EGFR phosphorylates IKBKE at tyrosine-153 and -179 residues**

Having demonstrated EGFR direct interaction with IKBKE, we sought to identify whether IKBKE is phosphorylated by EGFR at tyrosine residue(s). After coexpression of myc-IKBKE with EGFR\(^{L858R/T90M}\) or EGFR\(^{WT}\) in H1299 cells, we immunoprecipitated IKBKE with myc antibody and then immunoblotted the immunoprecipitates with pan-phosphotyrosine (pTyr) antibody. Figure 3A showed that tyrosine phosphorylation of IKBKE was induced by EGFR\(^{L858R/T90M}\) but not by EGFR\(^{WT}\), even when treated with EGF ligand. We further investigated whether inhibition of EGFR will inhibit the tyrosine phosphorylation of IKBKE. Two activating mutation of EGFR cell lines H1975 and HCC827 were treated with afatinib, a second-generation irreversible inhibitor of EGFR (12), and then were subjected to immunoprecipitation with IKBKE antibody and immunoblotting with...
pan-pTyr antibody. The phosphotyrosine level of IKBKE was significantly reduced by afatinib in both cell lines (Fig. 3B and C). However, afatinib had no effect on IKBKE-tyrosine phosphorylation in either H292, in which EGFR is amplified, or H322, in which EGFRWT was ectopically overexpressed (Fig. 3C). In addition, decrease in phosphotyrosine of IKBKE was observed after knockdown of EGFR in EGFR-mutant H1975 cells but not in wild-type EGFR H292 and H322 cells (Fig. 3D). We next examined whether EGFR directly phosphorylates IKBKE. In vitro kinase assay with recombinant EGFR<sup>L858R/T790M</sup> or EGFR<sup>WT</sup> and IKBKE showed that IKBKE was phosphorylated by EGFR<sup>L858R/T790M</sup> but not by EGFR<sup>WT</sup> (Fig. 3E). These data strongly suggest that constitutively active mutant EGFR is a bona fide kinase leading to IKBKE tyrosine phosphorylation.

To identify the residues phosphorylated by EGFR, recombinant IKBKE from in vitro EGFR kinase assay was subjected to mass spectrometric analysis. Five tyrosine residues were identified as potential phosphorylation sites by EGFR (Fig. 4A and Supplementary Fig. S3A). To further define the EGFR phosphorylation residue(s) in IKBKE, in vitro EGFR kinase assay was performed using GST-fused peptides for each of potential phosphorylation tyrosine sites and their mutant (i.e., 20 amino acids of IKBKE with the candidate tyrosine and its phenylalanine substitution in the middle). We noted that EGFR phosphorylated IKBKE-Y153 and -Y179 more significantly than the other tyrosine residues while their tyrosine-to-phenylalanine (Y-F) mutants failed to be phosphorylated by EGFR in vitro (Supplementary Fig. S3B). In support of this finding, we found that the N-terminal region of IKBKE binds to EGFR. HEK293 cells were transfected with truncation mutants of myc-IKBKE (left) and EGFR<sup>L858R/T790M</sup> and then were subjected to immunoprecipitation with EGFR antibody and immunoblot with myc antibody. Un, untreated.
the EGFR-induced tyrosine phosphorylation (Fig. 4B). We also observed the conservation of Y153 and Y179 residues of IKBKE across different species (Fig. 4C). We further generated specific pIKBKE-Y153 antibody. To determine the antibody specificity, we initially incubated GST-fused IKBKE-Y153 peptides with and without recombinant constitutively active EGFR and then immunoblotted with pIKBKE-Y153 antibody. We observed that EGFR phosphorylated IKBKE-Y153 is recognized by pIKBKE-Y153 antibody (Supplementary Fig. S3E). We also found that the pIKBKE-Y153 antibody could detect endogenous level of pIKBKE-Y153 in H1975 cells, which was reduced by either pretreatment of the cell lysate with alkaline phosphatase or IKBKE knockdown (Supplementary Fig. S3F). Moreover, IKBKE-pY153 was elevated in EGFR-activating mutation NSCLC cell lines (Supplementary Fig. S4A). In addition, we examined pIKBKE-Y153 in 68 NSCLC specimens in which 9 have EGFR mutation. Six of the 9 EGFR mutation cases exhibited elevated pIKBKE-Y153 (Supplementary Fig. S4B). These data show that our pIKBKE-Y153 antibody specifically recognizes endogenous p-IKBKE-Y153 and IKBKE is a bona fide substrate of EGFR.

Phosphorylation of IKBKE by EGFR increases IKBKE kinase activity and IKBKE mediates EGFR signaling and cellular function

We further investigated whether EGFR phosphorylation of IKBKE regulates its kinase activity. After transfection of H1299 cells with myc-IKBKE, myc-IKBKE-Y153F/Y179F, which was no longer phosphorylated by EGFR, together with and without EGFRL858R/T790M, IKBKE was immunoprecipitated with myc antibody and then subjected to in vitro kinase assay using IKB

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**Figure 3.**

EGFR<sup>L858R/T790M</sup> phosphorylates IKBKE at tyrosine residues. **A,** expression of EGFR<sup>L858R/T790M</sup> induces IKBKE-tyrosine phosphorylation. H299 cells were cotransfected with indicated plasmids. After immunoprecipitation (IP) with myc antibody, immunoprecipitated IKBKE was blotted with pan-phospho-tyrosine antibody (pTyr, top). Panels 2 and 4 show the immunoprecipitated IKBKE and expression of transfected EGFR, respectively. **B** and **C,** IKBKE-tyrosine phosphorylation was inhibited by EGFR inhibitor in EGFR-mutant NSCLC but not EGFR<sup>WT</sup> cells. Indicated cells were treated with 200 nmol/L afatinib for 16 hours and the endogenous IKBKE was immunoprecipitated followed by Western blot analysis with indicated antibodies. Panels 3 and 4 are immunoblots with phospho- and total-EGFR antibodies. **D,** knockdown of EGFR leads to reduced IKBKE-tyrosine phosphorylation in EGFR-mutant cells. Indicated cells were transfected with two siRNAs targeting EGFR and a control siRNA. After incubation for 48 hours, the cells were lysed and endogenous IKBKE was immunoprecipitated followed by Western blot analysis with indicated antibodies. Knockdown of EGFR was shown in panel 3. **E,** IKBKE is directly phosphorylated by EGFR<sup>L858R/T790M</sup>. GST-IKBKE was incubated with GST-EGFRL858R/T790M or GST-EGFR<sup>WT</sup> in vitro. After immunoprecipitation with IKBKE antibody, the immunoprecipitates were blotted with indicated antibodies (panels 1 and 2). Bottom panel shows GST-EGFR used for the assay.
as a substrate. We found that expression of EGFRL858R/T790M significantly induced IKBKE kinase activity and that IKBKE-Y153F/Y179F mutant not only lost basal kinase activity but also could not be activated by EGFRL858R/T790M (Fig. 5A).

Moreover, we examined whether EGFR phosphorylation of IKBKE affects downstream signaling of IKBKE. We and others have previously showed that IKBKE is an AKT kinase, that is, IKBKE activates AKT by direct phosphorylation of AKT-T308 and -S473 (28). Thus, we coexpressed myc-IKBKE or myc-IKBKE-Y153F/Y179F with and without EGFRL858R/T790M in H1299 cells. Immunoblot analysis showed that pAKT-T308 and -S473 were induced by expression of IKBKE, which was further enhanced by coexpression of IKBKE and EGFRL858R/T790M. However, expression of IKBKE-Y153F/Y179F failed to activate AKT. Also, expression of constitutively active EGFR had no effect on IKBKE-Y153F/Y179F toward AKT activation (Fig. 5B).

In addition, we found that knockdown of endogenous IKBKE in EGFRL858R/T790M transfected H322 and in EGFR mutation bearing H1975 cells significantly decreased phosphorylation of AKT and RelA/p65 (Fig. 5C and D). Moreover, depletion of IKBKE in 4 cell lines with activating mutations of EGFR inhibited cell survival, growth, and invasion (Fig. 5E–G and Supplementary Fig. S5A–S5C). We further investigated the significance of EGFR phosphorylation of IKBKE-Y153/Y179 in EGFR cellular function. We expressed wild-type IKBKE or nonphosphorylatable IKBKE-Y153F/Y179F individually and in combination in H1299 cells. Boyden chamber assays showed that while expression of IKBKE alone had no significant effects on cell migration and invasion, coexpression of IKBKE and constitutively active EGFR significantly induces these phenotypes. Notably, constitutively active EGFR-promoted cell migration and invasion were inhibited by expression of IKBKE-Y153F/Y179F mutant (Supplementary Fig. S5D and S5E). Collectively, these data...
Indicate that constitutively active EGFR induces IKBKE kinase activity through phosphorylation of Y153 and Y179 and that IKBKE is an important downstream target of EGFR to mediate EGFR signaling and function.

Therapeutic targeting of IKBKE for NSCLC cells with EGFR mutation and TKI resistance

A recent study identified an anti-inflammatory drug, amlexanox, as a selective inhibitor of IKBKE/TBK1 (33). Because IKBKE is directly activated by activating mutation of EGFR and amlexanox is an approved small-molecule therapeutic presently used in the clinic to treat aphthous ulcers (31), and in clinical trial for obesity (32), we assumed that amlexanox could preferentially inhibit cell survival in NSCLCs with activating EGFR mutation including acquired EGFRT790M. We treated a panel of NSCLC cell lines with increasing concentrations of amlexanox and found that the EGFR-mutant cell lines, including acquired-resistant lines H1975 and H1650, are more sensitive to amlexanox (GI50 20 and 30 μmol/L) compared to the NSCLCs with wild-type EGFR (GI50 > 80 μmol/L; Fig. 6A). We also performed cell-cycle analysis in H1975 cells and observed that amlexanox induced G1-S arrest and predominate enrichment of G1 phase cells by 24 hours of treatment (Supplementary Fig. S6A). Interestingly, amlexanox treatment led to a moderate increase in pERK1/2, while decrease in pAKT, in EGFR mutant H1975, H1650, and HCC827 cells (Fig. 6B and C and Supplementary Fig. S6B), suggesting a feedback activation of MAPK pathway upon IKBKE inhibition.

In addition to IKBKE and AKT, EGFR mutations also significantly activate MAPK in NSCLC (3). Moreover, our data show that inhibition of IKBKE further induces pERK1/2 in EGFR-mutant NSCLC cells. These findings prompted us to examine the synergistic antitumor activity of combination of IKBKE inhibitor amlexanox with MEK inhibitor AZD6244, a potent ATP-competitive inhibitor of MEK1/2 and currently in phase II/III clinical trial (41). We treated H1975 (Fig. 6D-F) and HCC827

Figure 5. IKBKE-Y153/Y179 phosphorylation by activating mutant EGFR regulates its signaling. A, EGFR L858R/T790M phosphorylation of IKBKE modulates IKBKE kinase activity. In vitro IKBKE kinase assay was performed using IKBKE immunoprecipitated from H1299 cells transfected with indicated plasmids and GST-IκB as a substrate (panels 1 and 2). Expression of transfected plasmids is shown in panels 3 and 4. B, IKBKE-Y153F/Y179F lost ability to activate AKT and inhibited EGFR-induced AKT activation. H1299 cells were transfected with indicated plasmids and then immunoblotted with indicated antibodies. C and D, IKBKE mediates downstream signaling of activating mutant EGFR. Indicated plasmids were introduced to H322 (C) and H1975 (D) cells. After incubation for 48 hours, immunoblot was performed with indicated antibodies. E–G, depletion of IKBKE reduces active EGFR cellular function. After knockdown of IKBKE in H1975, cell viability (E), colony growth (F), and cell invasion (G) were measured. Each experiment was repeated three times. *P < 0.05; **P < 0.005.
Figure 6. 
IKBKE inhibitor amlexanox preferentially inhibits cell viability in NSCLC cells with activating mutations in EGFR and synergizes with MEK inhibitor AZD6244. 
A, amlexanox (Amx) was more potent in cell lines with activating EGFR mutations. A panel of NSCLC cell lines were treated with increasing concentrations of amlexanox for 72 hours and then subjected to MTT assay. B and C, amlexanox inhibits IKBKE signaling but induces pERK in EGFR-mutant cells. H1975 (B) and H650 (C) cells were treated with indicated compounds for 24 hours and then were immunoblotted with indicated antibodies. D–F, amlexanox synergizes with AZD6244. H1975 cells were treated with amlexanox and AZD6244 individually and the combination. Cell viability (D) and colony growth (F) were assayed. 

One-way ANOVA was used to calculate statistical significance for cell viability and Student t test was used for the colony growth assay. Combination indices for the combination treatment was computed using CompuSyn (E). * P < 0.05; ** P < 0.005.

(Supplementary Fig. S7A) with amlexanox and AZD6244 individually and as combination for 48 hours. Cell viability analysis showed that while inhibition of either IKBKE or MEK reduced cell survival, inhibition of both pathways exhibited significant synergy with combination index values lower than 1.0 (Fig. 6E and Supplementary Fig. S7B). We also observed that amlexanox and AZD6244 synergistically reduced colony growth in both cell lines (Fig. 6F and Supplementary Fig. S7C).

Next, we asked whether the combination of amlexanox with AZD6244 could inhibit EGFR-mutant-driven tumor growth and surmount EGFR-TKI resistance in vitro. Xenograft experiments were carried out with 3 EGFR-mutant cell lines. We noted that amlexanox alone only moderately repressed the tumor growth in TKI-resistant H1975 and H1650 cells (no statistical significance). However, the combination of amlexanox and AZD6244 resulted in significant reduction in tumor growth and tumor weight (Fig. 7A–D). In contrast to the H1975 and H1650, HCC827 xenografts, which carry mutant EGFR but are sensitive to EGFR inhibitor (42), showed better sensitivity to amlexanox or/and AZD6244 (Supplementary Fig. S8A and S8B). Immunoblot analysis of the xenograft lysates revealed that the tumors treated with amlexanox alone expressed elevated pERK1/2, which is consistent with our in vitro findings (Fig. 7E and F and Supplementary Fig. S8C). Significantly, the tumors treated with both amlexanox and AZD6244 displayed low levels of pAKT and pERK1/2 (Fig. 7E and F and Supplementary Fig. S8C). Moreover, immunohistochemical staining of the tumor sections with Ki67 showed that combination treatment with amlexanox and AZD6244 more significantly inhibited cell proliferation compared with the tumors treated with either one alone (Fig. 7G and H). There was no notable toxicity revealed by the body weights when the mice were treated with combined IKBKE and MEK inhibitors (Supplementary Fig. S8D). Together, these data suggest that IKBKE is an...
important therapeutic target in NSCLC with activating EGFR mutations and that combination of inhibitors of IKBKE and MEK could be a promising therapeutics for EGFR-TKI resistance.

Discussion

Accumulating evidence indicates that EGFR mutations are key drivers for NSCLC by initiating several signal transduction cascades, principally the MAPK, AKT, and JNK pathways. Here, we demonstrated that IKBKE is a direct downstream target of activating mutations of EGFR. This is supported by three key findings: First, IKBKE directly binds to EGFR. Second, constitutively active EGFR phosphorylates IKBKE-Y153/Y179 in vitro and in vivo. Third, IKBKE, but not IKBKE-Y153F/Y179F, is activated by activating mutation of EGFR.

EGFR mutations are frequently detected in NSCLC patients, (43). Transgenic murine models have shown that expression of mutant EGFR in the lung induces lung adenocarcinoma (44). More importantly, NSCLCs with EGFR mutations exhibit sensitivity to EGFR-TKIs such as erlotinib and gefitinib; however, acquired resistance develops after a median of 9–14 months (45). The most common mechanism of TKI resistance is a second-site mutation (T790M) in the EGFR kinase domain. EGFRT790M allele also has been detected in a minority of tumors with primary resistance to these drugs (10). Our study showed that IKBKE is directly activated by activating mutations of EGFR including L858R, del19, and T790M. Furthermore, we demonstrated that knockdown of IKBKE largely inhibits EGFR-driven NSCLC cell survival. In addition, amlexanox, an IKBKE inhibitor currently in clinical trials for obesity and diabetes (32), preferentially inhibits cell viability in EGFR-mutant NSCLC cell lines. While third-generation EGFR inhibitors, such as AZD9291, CO-1686, and WZ4002, are capable of inhibiting mutant EGFR with T790M, recent studies have shown that patients develop acquired resistance to these inhibitors due to new activating EGFR-C797S mutation (46, 47). The role of IKBKE activation and therapeutic potential of IKBKE inhibitors in the cells bearing this mutation are yet to be studied.
However, our data showed that IKBKE was activated by various mutants of EGFR, and thus inhibition of IKBKE should reduce EGFR-C797S mutation driven cell growth and survival. Interestingly, inhibition of IKBKE moderately induces ERK activation even though the MAPK pathway is already activated in EGFR-mutant NSCLC (Figs. 6B and C, and 7E and F). In agreement with these findings, combination of amlexanox with MEK inhibitor AZD6244 synergistically inhibited cell survival and xenograft tumor growth in EGFR-mutant NSCLC cells including EGFR T790M mutation. Of note, it was previously shown that AZD6244 feedback activation of AKT leads to resistance to MEK inhibition (48). Indeed, analysis of xenograft lysates from AZD6244-treated mice showed elevated level of AKT activation, whereas the combination of amlexanox and AZD6244 abrogated both feedback pathways (Fig. 7E and F). Taken together, these findings suggest that IKBKE is a critical mediator of cellular function of activating mutations of EGFR that and that combination inhibition of IKBKE and MAPK could be an effective therapeutic strategy for NSCLC with EGFR mutation and EGFR-TKI resistance, especially those driven by secondary EGFR mutation.

Extensive studies have focused on downstream targets of IKBKE, however, the upstream kinases responsible for activation of IKBKE are not yet known. In this study, we showed a novel mechanism of regulation of IKBKE, that is, activating mutations of EGFR, but not EGFR-stimulated wild-type EGFR directly phosphorylate and activate IKBKE. This is the first study to identify tyrosine phosphorylation of IKBKE, thus underlining the importance of IKBKE in tumor cells. Interestingly, the EGFR phosphorylation residue, IKBKE-Y153, is conserved across several species and also in its close homology kinase TBK1 but not in IKKα and IKKβ. Hence, active EGFR mutant may also phosphorylate TBK1. Indeed, a recent study showed that in the absence of ligand, wild-type EGFR constitutively interacts with TBK1 and IRF3, leading to activation of TBK1, while underlying mechanism is currently unknown, subsequently TBK1 phosphorylates IRF3 that results in transcription of target genes. When EGFR is added, EGFR/TBK1/IRF3 complex is disrupted and EGFR now activates downstream signaling pathways such as ERK and AKT (49). In agreement with these findings, our data also show the interaction between wild-type EGFR and IKBKE, which is reduced by EGFR stimulation (Supplementary Fig. S2A). Further investigation is needed to understand the mechanism and physiopathologic significance of IKBKE interaction with wild-type EGFR.

Classically, activated EGFR triggers downstream signaling through recruiting various cytoplasmic proteins that transduce and regulate the EGFR function. The proteins recruited to active EGFR include many Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain–containing proteins, which bind to the tyrosine phosphorylated residues in EGFR. However, recent studies showed that activated EGFR could form a complex with the proteins that do not have SH2 or/and PTB domain, such as RNA helicase A, TBK1, and IRF3 (49, 50). In this study, we showed that N-terminal region of IKBKE, in which there is no sequence homology to SH2 and PTB motifs, interacts with EGFR. Thus, we predict IKBKE/EGFR interaction via an unconventional manner, which needs further investigation. Nevertheless, our study suggests that IKBKE is a direct downstream effector of activating mutant EGFR, including T790M mutation, responsible for half of acquired TKI resistance in NSCLC and that inhibition of IKBKE and its feedback activation of MAPK could be an effective therapeutic strategy for secondary EGFR mutation–associated TKI-resistant NSCLC.

Disclosure of Potential Conflicts of Interest

E.B. Haura reports receiving a commercial research grant from Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

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