Inhibition of Casein Kinase 1 Alpha Prevents Acquired Drug Resistance to Erlotinib in EGFR-Mutant Non–Small Cell Lung Cancer

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Abstract

Patients with lung tumors harboring activating mutations in the EGFR receptor (EGFR) show good initial treatment responses to the EGFR tyrosine kinase inhibitors (TKI) erlotinib or gefitinib. However, acquired resistance invariably develops. Applying a focused shRNA screening approach to identify genes whose knockdown can prevent and/or overcome acquired resistance to erlotinib in several EGFR-mutant non–small cell lung cancer (NSCLC) cell lines, we identified casein kinase 1 α (CSNK1A1, CK1α). We found that CK1α suppression inhibits the NF-κB prosurvival signaling pathway. Furthermore, downregulation of NF-κB signaling by approaches independent of CK1α knockdown can also attenuate acquired erlotinib resistance, supporting a role for activated NF-κB signaling in conferring acquired drug resistance. Importantly, CK1α suppression prevented erlotinib resistance in an HCC827 xenograft model in vivo. Our findings suggest that patients with EGFR-mutant NSCLC might benefit from a combination of EGFR TKIs and CK1α inhibition to prevent acquired drug resistance and to prolong disease-free survival.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Approximately 85% of lung carcinomas are non–small cell lung carcinoma (NSCLC), with adenocarcinoma representing the most frequently occurring histologic subtype (1). About 10% to 40% of patients with adenocarcinoma harbor activating mutations in the receptor tyrosine kinase (RTK) gene EGF receptor (EGFR) and frequently show good initial responses to treatment with the EGFR tyrosine kinase inhibitors (TKI) erlotinib and gefitinib (1, 2). However, most patients eventually acquire resistance, resulting in a modest overall survival benefit compared with standard chemotherapeutics (1).

Modeling acquired resistance in cancer cell line models and examining relapsed patient tumor samples has identified both genetic and epigenetic mechanisms of resistance to EGFR targeted TKIs (3, 4). Most frequently, acquired resistance is achieved through reactivation of signaling pathways that were originally suppressed by RTK inhibition (3, 4). This can result from secondary mutations in the drug target, such as the T790M mutation in EGFR, observed in 50% of EGFR-mutant lung cancers with acquired resistance (5–7). Alternatively, key downstream signaling pathways can be reactivated through mechanisms independent of the original target, for example, through mutational activation of components of the PI3K/AKT and MEK/ERK pathways or through activation of bypass RTKs, such as MET (8–10), ErbB2 (11), IGF1R (12–14), FGFR1 (15), or AXL (16, 17). Furthermore, activation of alternative pathways, such as upregulation of the prosurvival NF-κB signaling pathway, as well as a switch from epithelial-to-mesenchymal cell morphology (EMT) have been connected to both intrinsic and acquired resistance to EGFR TKIs (16, 18, 19).

Cancer cell lines used to model acquired resistance show initial sensitivity to the drug and undergo apoptosis, similar to the initial treatment response observed in patients with cancer. However, as in patients, the treatment response in cell lines is rarely complete, resulting in a subpopulation of initially quiescent drug-tolerant persisters (DTP), that eventually start proliferating in the presence of the drug and give rise to a drug-resistant cell population (3, 20, 21). In such initially drug-sensitive cancer cell lines, genetic loss-of-function screens have been successfully applied to identify genes whose suppression can confer resistance to the drug (22–25). Loss-of-function screens to identify genes whose suppression enhance drug treatment and thereby potentially prevent drug tolerance or drug resistance are more challenging in these cell line models. This is due to the fact that only a small subpopulation of DTPs survives drug treatment, thus making it more difficult to achieve a high representation of individual shRNAs and a robust signal-to-noise ratio in the screen.

Using loss-of-function genetic screens, we identified that suppression of casein kinase 1 α (CSNK1A1, CK1α) can prevent
and/or overcome erlotinib resistance in several erlotinib-sensitive, EGFR-mutant NSCLC cell lines. Suppression of CK1α inhibits the NF-κB signaling pathway. Moreover, direct downregulation of NF-κB signaling, by approaches independent of CK1α suppression, also attenuates the development of acquired resistance to erlotinib, supporting a role for activated NF-κB signaling in conferring erlotinib resistance. Importantly, suppression of CK1α can also prevent resistance to erlotinib in an HCC827 xenograft model in vivo, making CK1α a potential novel target to attenuate resistance to erlotinib in EGFR-mutant NSCLC.

Materials and Methods

Cell lines and culture conditions

HCC827, HCC4006, HCC2935, H3255, and H5238T cells were obtained and cultured as recommended by the ATCC. PC9 cells were kindly provided by Prof. Nishio (Kinki University of Medicin, Osaka, Japan) and cultured in RPMI-1640 supplemented with 10% FBS (Hyclone). All cell lines were authenticated by SNP fingerprinting and used for experiments within 20 passages.

Generation of lentiviruses

Viral constructs were packaged in HEK293T cells plated at 80% to 90% confluence in a 10-cm culture dish by cotransfection with 2.4 μg of shRNA-encoding plasmid, 0.6 μg of VSVG envelope plasmid, and 2.4 μg of D8.9 ( gag/pol/rev) packaging plasmid, using TransIT-293 reagent (Mirus) for transfection. Growth media were exchanged the following day and lentivirus-containing supernatant was harvested 48 hours later. Target cell lines were transduced at MOI < 0.5.

RNAi

shRNA sequences were designed to include EcoRI and AgeI restriction sites to allow subsequent cloning into the pLKO. Tet-On inducible vector system in which the H1-TetO promoter had been replaced with U6-TetO. The CTCGAG stem loop was used for shNTC. For shCK1α#1, shCK1α#2, shCK1α#3, and shCK1α#4, the Collecta stem loop sequence GATTAATTACATGC was used for the top oligonucleotide and GCCATGATTATAAC for the bottom oligonucleotide. The sequences of the oligonucleotides used are as follows from 5′ to 3′:

shCK1α#1-top: CCGGCATCTATTTGGCGATTAACATGTTAATATTCATAGCCTAGCA-
shCK1α#1-bottom: AATTAAAAACATCTATTTGGCGATCAACATGCTATGAATATTAACATGTTGATCGCCAAATAGATGTTTTT; shCK1α#2-top: CCGCCTGCCGTTAATTTTGGTGATTTGAATTATTCATACATGGCTAGCA-
shCK1α#2-bottom: AATTAAAAACATCTATTTGGCGATCAACATGCTATGAATATTAACATGTTGATCGCCAAATAGATGTTTTT; shCK1α#3-top: CCAGCTCTGCTAAATTGCCTAGCTAGATTAATCCATACGCTATACAGCA-
shCK1α#3-bottom: AATTAAAAACATCTATTTGGCGATCAACATGCTATGAATATTAACATGTTGATCGCCAAATAGATGTTTTT; shCK1α#4-top: CGGCAGCTTGTACGAGAGCAGACGATTAATCTCTTACAGCCAGAGCT-
shCK1α#4-bottom: AATTAAAAACATCTATTTGGCGATCAACATGCTATGAATATTAACATGTTGATCGCCAAATAGATGTTTTT.

Ectopic expression of mCK1α and IκBα superrepressor alleles

The kinase-dead mCK1α (D136N) was generated from the murine CK1α-cDNA construct (Life Technologies, clone ID: IOM18458), using the Q5 Mutagenesis Kit (New England Biolabs) with primers 5′-TATACACAGAAACATTTAACCAG-3′ and 5′-AAATCTTTTATGCACTATATTC-3′. Additional silent mutations were added to further increase the resistance of murine CK1α to shRNA#1 and #3 using the primers 5′-AATAAACACTGGGAGAAGTGG-3′ and 5′-ATAAGCGGATGACAGTCCGG-3′, respectively. The stop codon was deleted with primers 5′-TACCCAGCTTTTGATGAC-3′ and 5′-GAAACCTGTTGGGATG-3′, and constructs were cloned into pLent6.3-V5-CMV plasmid (Life Technologies) to generate V5-tagged fusion constructs. Plasmids were packaged into lentiviral particles, and after infection, cells were selected with 25 μg/mL puromycin for 1 week to eliminate nontransduced cells. IkBα superrepressor was cloned into a tetracycline-inducible pLKO-TREX plasmid. Lentivirus-transduced cells were selected with 2 μg/mL puromycin. Expression of the IkBα superrepressor was induced with 100 ng/mL doxycycline (Clontech).

shRNA screen

A custom shRNA library containing 6,500 shRNAs against ~350 potentially cancer-relevant genes (~17 shRNAs per gene) described previously (26) was screened. Cells were transduced at an MOI of 0.5. Selection with 2 μg/mL puromycin was started 24 hours after transduction and continued for 72 hours. Cells were trypsinized and the percentage of positively transduced cells was more than 90% for all cell lines screened as tested by RFP expression. Fifteen million cells, representing the baseline of shRNA representation in DMSO-treated versus baseline condition was calculated to estimate the effects of shRNAs on cell proliferation. A robust treatment versus baseline condition was calculated to estimate the effects of shRNAs on cell proliferation. A robust z-score was
calculated using the median and MAD for the calculated fold changes across the entire shRNA library. For gene-based hit calling, two statistical measures were used, redundant shRNA activity (RSA; ref. 27) and Q1-z-score (26, 28). The RSA score is a statistical score (P value) representing the probability of a gene hit based on the collective activities of multiple shRNAs per gene. It is a measure of how significantly the rank order of shRNAs against a given gene differs from the population of other shRNAs in the library. In this approach, genes with multiple moderately active shRNAs score higher than genes with few but highly active shRNAs (27). The Q1-z-score represents the z-score of the shRNA at the first quartile (Q1), that is, the z-score of the fourth best (in a library that contains an average of 16–17 shRNAs per gene) performing shRNA per gene. By using both RSA-score and Q1-z-score for hit calling, the most robust hits with both consistent and high shRNA activity are identified.

Cell viability and proliferation analysis

For short-term cell viability assays, 2,000 to 4,000 cells were seeded in triplicate in 96 wells 1 day before compound addition and incubated for 5 days with various concentrations of compounds. Cell viability was determined by CellTiter-Glo Luminescence Assay (Promega). Luminescence signal was recorded on an EnVision plate reader (PerkinElmer), and the inhibition of viability relative to DMSO-treated cells was calculated. For long-term colony formation assays, 100,000 to 200,000 cells were plated per 6-well the day before the start of treatment. Cells were retreated with fresh media with or without compound every 3 days until the appropriate confluence as estimated by the control condition was reached. Each condition was done in triplicate. Plates were stained with 0.2% crystal violet/10% formaldehyde, and crystal violet signal was quantified on Odyssey CLx (LI-COR) at 700 nm. To measure proliferation over time, cells were plated at 10,000 cells per well in a 24-well plate in triplicate. Twenty-four hours later, the indicated treatment was started and confluence measurements were taken every 12 hours using an Incucyte Kinetic Imaging System (Essen BioScience).

Luciferase assay

Cell lines stably expressing an NF-kB luciferase reporter plasmid (pLent6-NF-kB-Luc2P) or a WNT-responsive Super-TopFlash (STF) luciferase reporter plasmid (pLent6-STF) were generated by lentiviral infection. Forty thousand cells per 96-well were plated and the luciferase signal was measured after 4 days using Luciferase Assay System (Promega) and normalized to cell viability assessed by CellTiter-Glo Luminescence Assay (Promega). For transient NF-kB luciferase reporter assays, cells were cotransfected with firefly luciferase construct driven by NF-kB–binding sequences (pTranslucent-NFκB-Luc) and Renilla luciferase plasmid (pRL-SV40-Renilla) at a ratio of 25 to 1. Luciferase signal was measured using Dual Luciferase Reporter Assay System (Promega) 48 hours after transfection, and firefly luciferase signal was normalized to Renilla luciferase signal. All conditions were done in triplicate.

Caspase-3/7 apoptosis assay

Ten thousand cells were plated per 96-well in triplicate and treated with 2 μmol/L erlotinib or DMSO the following day. Caspase activity was measured 24 hours posttreatment using the Caspase Glo 3/7 assay (Promega), and normalized to cell viability assessed by CellTiter-Glo Luminescence Assay (Promega).

Quantitative real-time PCR

Total RNA was extracted using RNaseasy Plus Mini Kit (Qiagen). cDNA was generated using iScript cDNA Synthesis Kit (BioRad). The qRT-PCR reactions were performed using TaqMan Universal PCR Master Mix (Life Technologies) and were run on 7900HT Fast Real-Time PCR System (Applied Biosystems). TaqMan probes for Serpine1, TNFα, and β-actin were purchased from Life Technologies. All experiments were performed in triplicate and normalized to β-actin transcript levels using comparative CT method.

Microarray analysis

HCC827 and PC9 cells were treated for 8 or 30 days with DMSO or 2 μmol/L erlotinib. Total RNA was isolated using the RNaseasy Mini Kit (Qiagen). Gene expression profiling was performed using Affymetrix U133Plus2 Arrays and data were analyzed as described previously (29). Differential analysis was performed by computing fold changes of erlotinib treatment relative to DMSO. The data have been deposited at the National Center for Biotechnology information (NCBI Gene Expression Omnibus with the accession number GSE67051). GeneGo pathway enrichment analysis was performed to identify differentially regulated pathways between treated and untreated conditions.

Immunoblotting

Cell pellets were lysed in RIPA buffer, and immunoblot analysis was performed using standard methods. The following antibodies were used: CK1α (Santa Cruz Biotechnology, sc-6477), V5 (Invitrogen, 46-0705), GAPDH (Millipore, MAB374), pERK (T202, Y204; CST, 4377S), ERK (CST, 4695S), pAKT (S473+CST, 4058L), AKT (CST, 4685S), pEGFR (Y1086; Abcam, ab32086), EGFR (CST, 4267S), and cleaved PARP (Asp214; CST, 5625S).

Xenograft studies

Mice were handled in accordance with the Novartis Institutes for BioMedical Research (NIBR) Animal Care and Use Committee protocols and regulations. Ten million cells in 50% Matrigel (BD Biosciences) were implanted subcutaneously into female athymic nude mice (20–25 g; 7–8 weeks old). The cells were free of mycoplasma and viral contamination (IMPACT VIII PCR Profile, IDEXX BioResearch). Treatment with compound (formulation 40% Captisol, 0.1 mol/L tartaric acid) and doxycycline-supplemented food (PharmaServ diet #5515; PharmaServ 5053 with 400 ppm doxycycline) started 10 to 14 days after implant when average tumor volume reached approximately 350 mm³. Animals were administered once daily with vehicle or erlotinib (LC Laboratories; 1, 2.5, 5, or 10 mg/kg, oral) for the duration of the study. Tumor volume was measured using calipers and calculated as (length × width × width)/2. At the end of the study and 1 hour after the last dose, tumor tissues were excised and snap-frozen in liquid nitrogen for biomarker analysis.

Statistical analysis

Except noted otherwise, all average results are presented as mean ± SD. *Pvalues were calculated using a two-tailed t test; ***, P < 0.05 by t test; ***, P < 0.01 by t test; ***, P < 0.001; n.s., not significant by t test.
Results

shRNA screening identifies CK1α as a synergizer with erlotinib in EGFR-mutant NSCLC cells

To identify novel genes involved in acquired resistance to the EGFR TKI erlotinib, we performed loss-of-function genetic screens in several EGFR-mutant NSCLC cell lines in the presence or absence of the drug. We screened three EGFR-mutant NSCLC cell lines, HCC827, HCC4006, and PC9 cells, all of which are highly sensitive to erlotinib (Fig. 1A, left and Supplementary Fig. S1A and S1B) but acquire resistance upon long-term treatment with the drug. We purposely chose highly drug-sensitive cell lines in which treatment with the clinically relevant dose of 2 μmol/L erlotinib allows only the survival of a small subpopulation of DTPs, as an enhancer screen in

Figure 1.
shRNA screening identifies CK1α (CSNK1A1) as a dropout hit in EGFR-mutant NSCLC cells treated with the EGFR TKI erlotinib. A, schematic outline of the dropout shRNA screens for genes whose inhibition prevents or overcomes resistance to erlotinib in EGFR-mutant NSCLC cells. Cells were infected with lentiviral particles containing an shRNA library composed of 6,500 shRNAs against ~350 genes and then treated with DMSO or 2 μmol/L erlotinib for 10 days or 24 days. shRNA representation from both populations was determined by next-generation sequencing. Strategy 1 (left) outlines screening of erlotinib-sensitive EGFR-mutant NSCLC cells, strategy 2 (right) outlines screening of erlotinib-resistant PC9 cells. B, representation of the abundance of shRNAs at gene level in erlotinib- versus DMSO-treated conditions. For high-confidence hit calling at the gene level, RSA score and Q1-z-score were calculated for the 17 shRNAs for each gene. RSA < −1.5 (P < 0.05) and Q1-z-score < −1 were used as parameters for hit selection (boxed area) and hits shared across several NSCLC cell lines were prioritized. Red dots highlight CSNK1A1, blue dots CSNK1D, and green dots CSNK1E.

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such cell models has the potential to identify genes whose knockdown can prevent the occurrence of DTPs and thereby prevent drug resistance in the long-term. In addition, to identify genes whose knockdown can overcome drug resistance, we screened an erlotinib-resistant PC9 cell line generated by continuous treatment and then maintained with a clinically relevant dose of erlotinib (2 μmol/L; Fig. 1A, right and Supplementary Fig. S1A and S1B). Importantly, as described previously, erlotinib-resistant PC9 cells did not show any additional resistance conferring mutations in the EGFR gene or in other genes when analyzed by RNA sequencing (data not shown; ref. 20). We then screened a focused, deep coverage (17 shRNAs per gene) shRNA library of 6,500 shRNAs against about 350 potentially cancer-relevant genes (Supplementary Table S1; ref. 26). The deep-coverage shRNA library of 17 shRNAs per gene enables high-confidence hit calling at the gene level rather than analysis of individual shRNAs in the dataset. HCC827, HCC4006, PC9 cells, and erlotinib-resistant PC9 cells were infected with shRNA library containing lentiviral particles and library-infected cells were cultured for 10 or 24 days in the absence or presence of 2 μmol/L erlotinib (Fig. 1A), corresponding to approximately 50-fold of the IC50 in the cell lines used (Supplementary Fig. S1A and S1B). To avoid the follow-up of cell line specific as opposed to more generalizable phenotypes, we focused on shRNA dropout hits that were shared across several NSCLC cell lines. We prioritized those hits that came up in the 10-day treatment condition, as long-term culture can increase the noise of shRNA data.

Using these criteria for hit selection, CK1α (CSNK1A1) was identified as a novel dropout hit that decreased cell survival in the presence of erlotinib in parental HCC827, HCC4006, and PC9 cells, as well as in erlotinib-resistant PC9 cells (Fig. 1B and Supplementary Fig. S3). In the presence of DMSO, CK1α shRNAs dropped out only in PC9 cells (Supplementary Fig. S2), explaining the lower scores of CK1α in PC9 in the comparison of erlotinib versus DMSO treatment (Fig. 1B). Other isoforms of the CK1 family represented in the shRNA library, namely CSNK1D (CK1δ) and CSNK1E (CK1ε), did not exhibit significant dropout in the presence of erlotinib (Fig. 1B and Supplementary Fig. S3), suggesting a specific role of CK1α in resistance to erlotinib.

Suppression of CK1α increases the maximum dose response and enhances the apoptotic response to erlotinib

To further confirm the role of CK1α in cell survival in the presence of erlotinib, we generated stable cell lines expressing three independent doxycycline-inducible shRNAs against CK1α or a nontargeting control (NTC) shRNA. Cells were initially treated with or without doxycycline for 72 hours to induce knockdown of CK1α. Subsequently, cells were plated in the presence or absence of doxycycline and treated with different concentrations of erlotinib for 5 days. Knockdown of CK1α increased the maximum dose response (Amax) to erlotinib in HCC827 and HCC4006 cells and sensitized erlotinib-resistant PC9 cells to the drug in this short-term assay (Fig. 2A). No separation of the dose–response curves was detectable upon knockdown of CK1α in the presence of doxorubicin or cisplatin, suggesting that CK1α knockdown specifically increases the response to erlotinib (Supplementary Fig. S4).

Because knockdown of CK1α specifically increased the Amax rather than shift the IC50 in combination with erlotinib, the effect of CK1α knockdown on the apoptotic response to erlotinib was examined. Indeed, knockdown of CK1α enhanced apoptosis in the presence of erlotinib in HCC827, HCC4006, and erlotinib-resistant PC9 cells, as shown by increased PARP cleavage (Fig. 2B) and increased caspase-3/7 activity (Fig. 2C). Collectively, these findings indicate that knockdown of CK1α increases the maximum dose response, at least in part, by increasing the extent of the apoptotic response to erlotinib in EGFR-mutant NSCLC cells.

Knockdown of CK1α attenuates resistance to erlotinib in EGFR-mutant NSCLC cells

To determine the effects of CK1α knockdown on the development of resistance to erlotinib in long-term colony formation assays, cell lines generated to express doxycycline-inducible CK1κ- or NTC-shRNAs were cultured in erlotinib in the presence or absence of doxycycline until colonies formed (generally 21–35 days). Strikingly, in this long-term assay, CK1α knockdown attenuated the development of resistance to erlotinib in HCC827, HCC4006, and PC9 cells. The effect of CK1α knockdown on cell proliferation in the absence of erlotinib was less pronounced, with no significant effect in HCC827 cells, but moderate and significant effects in HCC4006 and PC9 cells (Fig. 3A and Supplementary Fig. S5A). Interestingly, knockdown of CK1α was also effective when induced in DTPs, that is, after initial erlotinib treatment for 8 days, showing that CK1α knockdown can also attenuate the outgrowth of DTPs to resistant clones (Supplementary Fig. S6A, right), in addition to preventing the emergence of DTPs (Fig. 3A and Supplementary Fig. S6A, left). Furthermore, knockdown of CK1α inhibited the proliferation of resistant PC9 cells, and this effect was more pronounced in the presence than in the absence of erlotinib (Supplementary Fig. S6B). In addition, the CK1α inhibitor D4476 (30) in combination with erlotinib attenuated resistance to erlotinib in HCC827, HCC4006, and PC9 cells, albeit with modest effects on cell proliferation when D4476 was used as a single agent (Supplementary Fig. S7). We extended the finding that CK1α knockdown attenuates acquired resistance to erlotinib to two additional EGFR-mutant and erlotinib-sensitive NSCLC cell lines, HCC2935 and H3255, that were not part of the shRNA screen (Fig. 3B and Supplementary Figs. S1A, S1B, and S5B). Taken together, these data suggest that inhibition of CK1α can attenuate resistance to erlotinib across a panel of EGFR-mutant NSCLC cell lines and that the synergy between CK1α inhibition and erlotinib represents a general mechanism.

We next tested whether the effect of CK1α knockdown on preventing resistance to erlotinib could be reversed by ectopic expression of CK1α. To this end, we generated cell lines that stably express V5-tagged shRNA-resistant murine wild-type or kinase-dead mCK1α cDNA (31) and doxycycline-inducible CK1α- or NTC-shRNAs. As shown in Fig. 3D, shRNA-resistant mCK1α-V5 was refractory to knockdown by the human specific CK1α-shRNAs, and wild-type and kinase-dead mCK1α-V5 were expressed at similar levels. Importantly, expression of wild-type mCK1α-V5, but not kinase-dead mCK1α(D136N)-V5, partially rescued the attenuation of resistance to erlotinib in the presence of human CK1α knockdown (Fig. 3C). These results support the importance of CK1α and its kinase activity in acquired resistance to erlotinib in EGFR-mutant NSCLC.
Suppression of CK1α increases the maximum dose response ($A_{\text{max}}$) and enhances the apoptotic response to erlotinib. A, knockdown of CK1α increases the maximum dose response ($A_{\text{max}}$) to erlotinib. Lentiviral infection was used to introduce three independent doxycycline-inducible CK1α- or NTC-shRNAs into HCC827, HCC4006, or erlotinib-resistant PC9 cells. Cells pretreated for 72 hours with doxycycline were plated in triplicate, and treatment with various doses of erlotinib was started the following day. Cell viability was assessed by CellTiter Glo after 5 days, and inhibition of viability relative to DMSO-treated cells was calculated. Data represent one of three independent experiments. **p-value of the difference between the maximum dose response of plus versus minus doxycycline condition at the highest concentration of erlotinib (5 μmol/L). B, knockdown of CK1α enhances the apoptotic response to erlotinib as measured by cleaved PARP. HCC827 and HCC4006 cells expressing doxycycline-inducible CK1α- or NTC-shRNAs were treated for 72 hours with doxycycline followed by ± 2 μmol/L erlotinib treatment for 24 hours. Erlotinib-resistant PC9 cells cultured in the presence of 2 μmol/L erlotinib were treated ± doxycycline and ± 2 μmol/L erlotinib for 96 hours. Immunoblot analysis of total cell lysates was performed. C, knockdown of CK1α enhances the apoptotic response to erlotinib as measured by caspase-3/7 activity. Treatment of cells was performed as in B. Caspase activity was normalized to cell viability measured by CellTiter Glo. Data are represented as fold change relative to −dox/+erlotinib condition ± SD. Data represent one of three independent experiments. The subtle apoptotic signal caused by the NTC-shRNA may result from cell line-specific small seed homology off-target effects or from general cell perturbations due to the presence of exogenous vectors and/or RNA species (43).
Suppression of CK1α attenuates acquired resistance to erlotinib in EGFR-mutant NSCLC cells. A, HCC827, HCC4006, and PC9 cells expressing doxycycline-inducible CK1α- or NTC-shRNAs were treated with doxycycline and 2 μmol/L erlotinib. Cells were fixed, stained, and photographed at the indicated time points. Each condition was done in triplicate and a representative image is shown. Data represent one of three independent experiments.

The level of knockdown of CK1α protein induced by each shRNA was analyzed by immunoblotting. B, in A, but for two additional EGFR-mutant NSCLC cell lines, HCC2935 and H3255. H3255 cells were treated with 75 nmol/L erlotinib. C, ectopic expression of V5-tagged shRNA-resistant wild-type mCK1α cDNA, but not of kinase-dead mCK1α(D136N) cDNA, rescues CK1α-knockdown cells from erlotinib-induced cell death. HCC827 cells expressing doxycycline-inducible CK1α- or NTC-shRNAs and wild-type or kinase-dead mCK1α-cDNA constructs were generated by lentiviral infection. Cells were treated with doxycycline and 2 μmol/L erlotinib. Cells were fixed, stained, and photographed at the indicated time points. Each condition was done in triplicate and a representative image is shown. Levels of ectopically expressed mCK1α protein were analyzed by immunoblotting. Human hCK1α was detected by anti-CK1α antibody; murine mCK1α-V5 was detected by anti-V5 antibody.

Figure 3.

CK1α Inhibition Prevents Erlotinib Resistance.
Knockdown of CK1α does not affect the MEK/ERK or PI3K/AKT survival pathways, neither does it inhibit Wnt signaling

Resistance to erlotinib often results from reactivation of key downstream signaling pathways, such as the PI3K/AKT and MEK/ERK pathways. This is particularly true in the case of erlotinib-resistant PC9 cells, which downregulate pEGFR but maintain robust activation of pERK and pAKT even in the presence of erlotinib (Supplementary Fig. S8, bottom right). To rule out a direct effect of CK1α on ERK/AKT signaling, we investigated pERK and pAKT levels upon CK1α knockdown in the erlotinib-resistant PC9 cells. pERK and pAKT levels were not significantly affected by CK1α knockdown either in the presence or absence of erlotinib, suggesting an ERK/AKT pathway-independent mechanism for the synergy between CK1α knockdown and erlotinib treatment (Supplementary Fig. S8, bottom right). Consistent with these findings, CK1α knockdown also did not affect pERK, pAKT, or pEGFR levels in HCC827, HCC4006, and PC9 cells under acute treatment in the presence or absence of erlotinib (Supplementary Fig. S8).

Previous studies have shown that CK1α, by virtue of being part of the β-catenin destruction complex, plays a role in WNT pathway regulation, with CK1α knockdown activating WNT signaling in the gut (32–34). However, CK1α knockdown did not activate WNT signaling in HCC827 and PC9 cells as measured by a WNT-responsive Super-TopFlash (STF) luciferase reporter assay (Supplementary Fig. S9), thereby excluding WNT pathway deregulation as the mechanism for preventing resistance to erlotinib in EGFR-mutant NSCLC cells. Consistently, GeneGo pathway enrichment analysis of erlotinib- versus DMSO-treated HCC827 and PC9 cells did not suggest altered WNT signaling upon erlotinib treatment (Supplementary Fig. S10A and S10B).

Knockdown of CK1α decreases NF-κB signaling and inhibition of NF-κB signaling attenuates resistance to erlotinib

CK1α has been implicated in several cellular signaling pathways (35). To better understand the effect of erlotinib on EGFR-mutant NSCLC cells, we generated microarray data from NSCLC cells treated for 8 days (DTP) or 30 days (resistant cells) with 2 μmol/L erlotinib. GeneGO pathway enrichment analysis of erlotinib- versus DMSO-treated cells revealed a prominent upregulation of NF-κB signaling in DTPs and in erlotinib-resistant cells (Supplementary Fig. S10A and S10B). On the basis of these findings, we hypothesized that upregulation of NF-κB signaling contributes to tolerance/resistance to erlotinib and that knockdown of CK1α attenuates resistance to erlotinib through downregulation of NF-κB signaling. To test this hypothesis, we generated HCC827 and PC9 cell lines stably expressing both an NF-κB luciferase reporter and doxycycline-inducible CK1α- or NTC-shRNAs. Knockdown of CK1α significantly decreased the NF-κB luciferase reporter signal (Fig. 4A). Consistent with these findings, CK1α knockdown reduced the expression of the NF-κB target genes TNFα and Serpine1 in the presence of erlotinib (Supplementary Fig. S10C).

On the basis of these results, we next investigated whether inhibition of NF-κB signaling by independent approaches would prevent resistance to erlotinib. To this end, we generated cell lines expressing a doxycycline-inducible dominant-negative version of IκBα, the IκBα superrepressor. Two point mutations at serine 32 and serine 36 to alanine in the IκBα superrepressor prohibit phosphorylation and thus degradation of IκBα, thereby inhibiting NF-κB signaling (36). Indeed, induction of the IκBα superrepressor by doxycycline decreased NF-κB signaling, as measured by NF-κB luciferase reporter assay (Fig. 4B). Importantly, expression of the IκBα superrepressor attenuated resistance to erlotinib in HCC827 and PC9 cells, whereas it only modestly affected cell proliferation in the absence of erlotinib (Fig. 4B and Supplementary Fig. S11A and S11B). These findings recapitulated the observations made for CK1α knockdown, which also downregulated NF-κB signaling and attenuated resistance to erlotinib.

Underscoring the importance of the NF-κB signaling pathway in acquired resistance, combination treatment with erlotinib and AFN700, an inhibitor of IκB kinase (IKKβ; refs. 37, 38), the primary kinase promoting IκBα phosphorylation and NF-κB activation, attenuated resistance to erlotinib in HCC827, HCC4006, and PC9 cells at concentrations where AFN700 alone did not affect cell proliferation (Fig. 4C and Supplementary Fig. S11C and S11D). Furthermore, treatment with AFN700 inhibited proliferation of resistant PC9 cells and this effect was more pronounced in the presence than in the absence of erlotinib (Supplementary Fig. S11E), suggesting that resistant PC9 cells depend on activated NF-κB pathway for survival and that erlotinib modulates this dependency.

In summary, our data show that the development of drug tolerance to erlotinib is associated with activated NF-κB signaling in EGFR-mutant NSCLC cell lines and that downregulation of NF-κB signaling by CK1α knockdown, as well as by other approaches can attenuate resistance to erlotinib.

Knockdown of CK1α attenuates resistance to erlotinib in an HCC827 xenograft model

Because knockdown of CK1α attenuated acquired resistance in vitro, we next investigated whether CK1α knockdown could affect resistance to erlotinib in vivo. In an initial experiment, we determined the minimum dose of erlotinib that was sufficient to achieve maximum HCC827 tumor regression in vivo to be 5 mg/kg (Fig. 5A). Treatment of mice with 5 mg/kg erlotinib reduced pEGFR levels, demonstrating the effectiveness of 5 mg/kg erlotinib on target modulation in vivo (Fig. 5B). In addition, two CK1α-shRNAs, but not the NTC-shRNA, induced robust knockdown of CK1α in vivo (Fig. 5B). Mice harboring HCC827-derived tumors expressing doxycycline-inducible CK1α- or NTC-shRNAs were kept on a diet supplemented with or without doxycycline and dosed daily with vehicle or 5 mg/kg erlotinib. As shown in Fig. 5C, CK1α knockdown alone did not affect tumor growth in the vehicle treatment group. Treatment of mice with 5 mg/kg erlotinib strongly inhibited tumor growth (relative to vehicle treatment), with resistance to erlotinib starting to emerge after approximately 70 days of erlotinib treatment. Importantly, expression of two independent CK1α-shRNAs but not of the NTC-shRNA prevented the emergence of resistance to erlotinib in the HCC827 xenograft model (Fig. 5C).

These in vivo data further support a role for CK1α as a potential target to prevent resistance to erlotinib in EGFR-mutant NSCLC.

Discussion

We used a loss-of-function screening approach to investigate mechanisms of acquired resistance to erlotinib across a panel of EGFR-mutant NSCLC cells. CK1α was identified as a novel hit whose knockdown could both prevent and overcome acquired...
Figure 4.
Downregulation of NF-κB signaling by CK1α knockdown attenuates acquired resistance to erlotinib in NSCLC cells. A, knockdown of CK1α inhibits NF-κB signaling as measured by NF-κB luciferase reporter assay. HCC827 and PC9 cells expressing doxycycline-inducible CK1α or NTC-shRNAs were transduced with NF-κB luciferase reporter plasmid. Cells were treated for 72 hours ± doxycycline followed by ± 2 μmol/L erlotinib treatment for 24 hours. Luciferase signal was normalized to cell viability assessed by CellTiter Glo. Data are represented as fold change relative to −dox condition ± SD. Data represent one of three independent experiments. B, inhibition of NF-κB signaling by expression of IκBα superrepressor attenuates acquired resistance to erlotinib. HCC827 and PC9 cells expressing doxycycline-inducible IκBα superrepressor were cultured ± doxycycline and ± 2 μmol/L erlotinib. Cells were fixed, stained, and photographed at the indicated time points. Each condition was done in triplicate and a representative image is shown. Data represent one of two independent experiments. Inhibition of NF-κB signaling upon induction of IκBα superrepressor was assessed by NF-κB luciferase reporter. Cells treated for 48 hours ± doxycycline were cotransfected with NF-κB firefly luciferase plasmid and Renilla luciferase plasmid. Forty-eight hours after transfection, the signal of firefly and Renilla luciferase was measured using Luciferase Dual Glo assay. Firefly luciferase signal was normalized to Renilla luciferase signal. Data are represented as fold change relative to −dox condition ± SD. Data represent one of two independent experiments. C, cotreatment of HCC827, HCC4006, and PC9 cells with the IKKβ inhibitor AFN700 and 2 μmol/L erlotinib attenuates acquired resistance. Cells were cultured in the presence of the indicated concentrations of AFN700 and ± 2 μmol/L erlotinib. At the indicated time points, cells were fixed, stained, and photographed. Data represent one of two independent experiments.
resistance to erlotinib. Suppression of CK1α downregulated the prosurvival NF-κB signaling pathway and NF-κB signaling was upregulated in erlotinib-treated drug-tolerant NSCLC cells and in resistant PC9 cells. In addition, inhibition of NF-κB signaling by approaches independent of CK1α suppression similarly attenuated resistance to erlotinib, supporting a causal link between increased NF-κB signaling and erlotinib resistance. Importantly, suppression of CK1α in vivo also prevented resistance to erlotinib in an HCC827 xenograft model. Together, our data suggest CK1α inhibition as a novel combination treatment strategy with EGFR TKIs to attenuate acquired resistance in patients with lung cancer harboring activating EGFR mutations.

Functional shRNA screens have been successfully used in the past to identify novel players in drug resistance. Several factors determine the quality of shRNA screen data and the ability for high-confidence hit calling. First, the use of deep-coverage shRNA libraries, in our case 17 shRNAs per gene, reduces the likelihood of false-positive hit calling (39). Second, the recovery of sufficiently high cell numbers at the end of the screen is necessary to ensure a high representation of each shRNA in the library and to reduce noise in the screen (39). And third, screening a panel of cell lines of the same lineage helps to avoid the follow-up of cell line specific as opposed to more generalizable phenotypes. In drug-sensitive cell lines, drug treatment often kills the bulk

Figure 5.
Knockdown of CK1α attenuates acquired resistance to erlotinib in HCC827 xenograft model. A, determination of minimum erlotinib dose to achieve maximum tumor regression. Mice bearing HCC827 xenografts expressing doxycycline-inducible NTC-shRNAs were dosed with vehicle or 1, 2.5, 5, or 10 mg/kg erlotinib, respectively, for the indicated time frame. Data are represented as mean tumor volume (mm³) ± SEM. Each group included 6 mice. B, inducible CK1α-shRNAs downregulate CK1α protein, and erlotinib modulates pEGFR in the HCC827 xenograft model. Immunoblot analysis of tumor pharmacodynamic markers from three animals per treatment group. Mice bearing HCC827 xenografts expressing doxycycline-inducible CK1α- or NTC-shRNAs were kept on a diet supplemented with doxycycline and were dosed once daily with 5 mg/kg erlotinib or vehicle for 7 days. C, suppression of CK1α attenuates acquired resistance to erlotinib in HCC827 xenograft model. Mice bearing HCC827 xenografts expressing doxycycline-inducible CK1α- or NTC-shRNAs were kept on a diet supplemented with doxycycline and dosed once daily with 5 mg/kg erlotinib or vehicle for the indicated time frame. Data are represented as mean tumor volume (mm³) ± SEM. Vehicle treatment groups included 6 mice; erlotinib treatment groups included 10 mice.
population (20), hampering the recovery of sufficient shRNAs per gene. Therefore, most shRNA screens to date have been set up to identify genes whose knockdown confers acquired resistance, for which gene hits can also be identified at lower shRNA representation (22–25). Alternatively, shRNAs screens with the aim to identify drop-out hits have mostly been applied in cell line models of intrinsic resistance that lack drug sensitivity (18, 40, 41). In contrast, we successfully performed drop-out shRNA screens in a panel of drug-sensitive EGFR-mutant NSCLC cells of acquired resistance in the presence of clinically relevant erlotinib concentrations. One challenge of this approach is that it requires the handling of initially large cell numbers to account for the treatment-induced cell loss throughout the screen and is therefore more applicable for screening of focused as opposed to genome-wide shRNA libraries. Nonetheless, this screening approach is highly valuable, as it can inform new combination treatment strategies. In our study, it identified CK1α as a potential novel target to prevent resistance to erlotinib in EGFR-mutant NSCLC.

CK1α has previously been shown to play a role in NF-κB signaling pathway in diffuse large B-cell lymphoma (DLBCL; ref. 31). Suppression of CK1α was selectively lethal for activated B-cell–like DLBCL cells that rely on constitutive NF-κB activation for proliferation and survival, but not for NF-κB pathway independent germinal center B–cell–like DLBCL cells (31, 42). We demonstrate that suppression of CK1α can downregulate NF-κB pathway activity in EGFR-mutant NSCLC cells and attenuate resistance to erlotinib. Furthermore, we show that DTPs and resistant PC9 cells show NF-κB activation and that downregulation of NF-κB signaling, through expression of the IκBα super-repressor or through treatment with an IκBκ inhibitor, can attenuate resistance to erlotinib. Together, these findings suggest a causal role for activated NF-κB signaling in resistance to erlotinib and provide evidence that inhibition of NF-κB signaling can attenuate resistance to erlotinib in EGFR-mutant NSCLC cells. Accordingly, it has been described previously that the extent of NF-κB activity may determine the response to EGFR TKI treatment in patients with EGFR-mutant lung cancer, with low IκBκ expression, an indicator of high NF-κB activation, being predictive of worse progression-free survival and decreased overall survival of patients with EGFR-mutant lung cancer treated with erlotinib (18). Furthermore, adaptive NF-κB activation has recently been shown to enable survival of NSCLCs cells upon initial EGFR-targeted therapy, thereby allowing tumor cell persistence manifesting as an incomplete tumor response that may ultimately promote acquired resistance (19).

The present finding that CK1α knockdown is able to prevent as well as overcome acquired resistance to erlotinib opens up some interesting possibilities. Previous studies have shown that a variety of resistance mechanisms operate to drive acquired resistance to erlotinib and that these can frequently co-occur in patients, suggesting that completely overcoming all resistance mechanisms in a patient may be extremely difficult (4). Thus, preventing acquired resistance has a better chance of being curative. In this regard, the preventative potential of targeting CK1α in EGFR-mutant NSCLC may be well worth exploring further.

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

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