Stress hormones promote EGFR inhibitor resistance in NSCLC: Implications for combinations with β-blockers

Monique B. Nilsson,1 Huiying Sun,1 Lixia Diao,2 Pan Tong,2 Diane Liu,3 Lerong Li,2 Youhong Fan,1 Alissa Poteete,1 Seung-Oe Lim,4 Kathryn Howells,5 Vincent Haddad,5 Daniel Gomez,6 Hai Tran,1 Guillermo Armaiz Pena,7 Lecia V. Sequist,8 James C. Yang,9 Jing Wang,2 Edward S. Kim,10 Roy Herbst,11 J. Jack Lee,3 Waun Ki Hong,1 Ignacio Wistuba,1,12 Mien-Chie Hung,4 Anil K. Sood,7 John V. Heymach1*

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) resistance mediated by T790M-independent mechanisms remains a major challenge in the treatment of non–small cell lung cancer (NSCLC). We identified a targetable mechanism of EGFR inhibitor resistance whereby stress hormones activate β2-adrenergic receptors (β2-ARs) on NSCLC cells, which cooperatively signal with mutant EGFR, resulting in the inactivation of the tumor suppressor, liver kinase B1 (LKB1), and subsequently induce interleukin-6 (IL-6) expression. We show that stress and β2-AR activation promote tumor growth and EGFR inhibitor resistance, which can be abrogated with β-blockers or IL-6 inhibition. IL-6 was associated with a worse outcome in EGFR TKI–treated NSCLC patients, and β-blocker use was associated with lower IL-6 concentrations and improved benefit from EGFR inhibitors. These findings provide evidence that chronic stress hormones promote EGFR TKI resistance via β2-AR signaling by an LKB1/CREB (cyclic adenosine 3′,5′-monophosphate response element–binding protein)/IL-6–dependent mechanism and suggest that combinations of β-blockers with EGFR TKIs merit further investigation as a strategy to abrogate resistance.

INTRODUCTION

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are effective therapies for non–small cell lung cancer (NSCLC) patients with EGFR-activating mutations. However, resistant disease inevitably emerges (1). The most common mechanism of resistance to EGFR TKIs is the acquisition of secondary EGFR T790M mutations (2, 3). Second- and third-generation EGFR inhibitors have shown activity against T790M-driven resistance (4, 5). However, in nearly 50% of cases, resistance is T790M-independent. Interleukin-6 (IL-6) has been implicated as a mediator of T790M-independent EGFR TKI resistance (6). The identification of targetable mechanisms of T790M-independent resistance is critical for improving outcomes in these patients.

Chronic stress results in increased production of stress hormones such as norepinephrine (NE) and epinephrine (E) from the adrenal medulla and sympathetic neurons. The effects of stress hormones are mediated through binding to β1,2- or β1,2,3-adrenergic receptors (ARs) on target cells. The AR pathway contributes to tumor development and progression in multiple malignancies including NSCLC (7–9). In lung cancer patients, psychological distress is a known predictor of mortality (10), and in preclinical models, β-AR signaling is associated with lung adenocarcinoma development and initiation of proliferative and invasive cellular programs (7, 11, 12). AR signaling has been linked to increased expression of cytokines including IL-6 (13). We previously reported that stress hormones including NE and E activate ARs present on ovarian cancer cells and increase tumor–derived IL-6 (14). Given the reports implicating IL-6 as a mediator of T790M-independent EGFR TKI resistance, we investigated whether stress hormones promote IL-6 expression in NSCLC cells and whether activation of ARs facilitates resistance to EGFR TKIs.

Here, we report that stress hormone activation of β2-AR promotes EGFR TKI resistance in cell lines and in mouse models of EGFR-mutant NSCLC and that this can be blocked by anti–IL-6 antibodies or β-blockers. Using reverse phase protein array (RPPA) to identify signaling pathways activated by β2-AR in NSCLC cells, we found that β2-AR signaling inactivates the tumor suppressor liver kinase B1 (LKB1), the loss of which promotes therapeutic resistance and metastasis in NSCLC (15). Analysis of clinical data sets revealed that high circulating concentrations of IL-6 were associated with a worse progression-free survival (PFS) and overall survival (OS) in patients treated with EGFR inhibitors and that circulating IL-6 concentrations were lower in patients incidentally receiving β-blockers. Furthermore, our analysis of incidental β-blocker use in the LUX-Lung3 study comparing afatinib with chemotherapy in EGFR mutant NSCLC suggests that patients receiving β-blockers may receive greater benefit from EGFR-targeting agents. Collectively, these data suggest the need for clinical testing of EGFR TKIs in combination with β-blockers.

RESULTS

Acquired resistance to EGFR TKIs is associated with increased IL-6

IL-6 is implicated as a driver of EGFR TKI resistance (6). We cultured HCC827 and HCC4006 (both EGFR mutant) cells with increasing concentrations of erlotinib, an EGFR inhibitor, until resistant variants
emerged. EGFR TKI–resistant (ER) variants HCC827 ER-1, ER-3, and ER-6 and HCC4006 ER-3, ER-5, and ER-6 were T790M-negative, as determined by quantitative polymerase chain reaction (PCR) (fig. S1). T790M-negative erlotinib-resistant cells were also resistant to gefitinib, osimertinib, afatinib, and CO-1686 (fig. S2, A to D) and expressed significantly higher IL-6 compared to parental cells (HCC827 ER-1,3,6 versus HCC827, \( P = 0.005 \); HCC4006 ER-3,5,6 versus HCC4006, \( P = 0.0005 \); Fig. 1A). IL-6 expression was minimal in cells where EGFR TKI resistance was associated with MET amplification (HCC827 ER-2) or T790M (PC9 ER-8, ER-9, and ER-11, and H1975; Fig. 1A).

Next, we analyzed pretreatment plasma concentrations of IL-6 by enzyme-linked immunosorbent assay (ELISA) in 209 patients in the erlotinib arm of the phase 3 ZEST (ZACTIMA Efficacy Study versus Tarceva; NCT00364351) (16) clinical study. All patients had platinum-refractory NSCLC and were not genotype-selected. In this population, high IL-6 (greater than median) was associated with a significantly worse OS compared to patients with lower than median IL-6 concentrations (\( P < 0.0001 \); Fig. 1B and table S1). The median OS for erlotinib-treated patients with high IL-6 was 4.8 months, whereas the median OS was 11.5 months in patients with low IL-6.

High IL-6 was also associated with a significantly worse progression-free survival (\( P = 0.0092 \); table S1 and fig. S2E). Smokers had higher concentrations of IL-6 than nonsmokers (table S2). The findings are consistent with earlier preclinical studies (6) and suggest that higher IL-6 may be associated with EGFR TKI resistance.

Stress hormones induce IL-6 expression in NSCLC cells through activation of \( \beta_2 \)-AR

NE and E are increased under stress conditions and can be substantially higher in tissues compared to circulating concentrations (17, 18). Effects of stress hormones are mediated through binding to \( \alpha \)-AR1,2 or \( \beta \)-AR1,2,3. We examined ADRB1, ADRB2, and ADRB3 gene expression in 159 NSCLC clinical samples and 116 lung cancer cell lines. Tumor samples and cell lines tested positive for ADRB1, ADRB2, and ADRB3 expression (Fig. 1C and fig. S3A). NSCLC cell lines harboring EGFR-activating mutations expressed constitutively phosphorylated
EGFR (fig. S3B). We treated NSCLC cells harboring EGFR-activating mutations (HCC827, HCC4006, and H3255) with 1 or 10 μM NE for 24 hours and measured IL-6 secretion by ELISA. NE induced a marked rise in IL-6 (Fig. 1D). IL-6 mRNA expression was significantly increased after NE (10 μM) stimulation for 3 hours, as determined by quantitative real-time reverse transcription PCR (P < 0.001; Fig. 1E). NE concentrations of 0.1 and 0.01 μM also increased IL-6 (fig. S4, A and B). E and the β-AR agonist isoproterenol (ISO) similarly induced IL-6 in EGFR mutant NSCLC cells; however, the effect on non-malignant human bronchial epithelial cells was minimal (fig. S4, C and D).

Propranolol (PPL; β-AR inhibitor) but not phenolamine hydrochloride (α-AR inhibitor) blocked NE-induced IL-6 (Fig. 1F and fig. S4E). Treatment with a β2-AR agonist (salbutamol) but not a β1-AR agonist (dobutamine) increased IL-6 (Fig. 1G).

β-ARs activate the cyclic adenosine 3′,5′-monophosphate (cAMP) signaling pathway through stimulation of adenylyl cyclase (19). We treated NSCLC cells with an adenylyl cyclase activator, forskolin, and observed an increase in IL-6 similar to that induced by NE (Fig. 1H). β-ARs can also activate Epac (20); however, treatment with 8-CPT-2Me-cAMP, a cAMP analog that specifically targets Epac, did not affect IL-6 secretion (fig. S4F).

To determine whether β-blocker use may be associated with a reduction of IL-6 in patients, we measured circulating concentrations of IL-6 by ELISA in patient samples from the BATTLE trial (21). IL-6 concentrations were significantly lower in patients using pan-β-blockers compared to patients not receiving β-blockers, supporting the contribution of β-ARs in regulating IL-6 concentrations (P = 0.02; Fig. 1I).

β-AR signals cooperatively with mutant EGFR

Although NE induced a robust increase in IL-6 in cells with EGFR-activating mutations (HCC827, HCC4006, H3255, PC9, H1975, and H1650), the effect was minimal in EGFR wild-type cells (A549, H441, Calu6, H460, H661, H23, HCC15, and H1993) (Fig. 2A). Similarly, salbutamol (a β2-AR agonist) and forskolin induced IL-6 expression in NSCLC cells with mutant but not wild-type EGFR

**Fig. 2.** β-ARs signal cooperatively with mutant EGFR and inactivate LKB1. (A) NSCLC cells harboring wild-type EGFR or EGFR-activating mutations were stimulated with NE (10 μM), and IL-6 secretion was measured by ELISA. *P ≤ 0.001. Data are means ± SD. (B) Coimmunoprecipitation of endogenous β2-AR and EGFR in HCC827 cells (EGFR mutant) after NE stimulation. (C) HCC827 (EGFR mutant) and A549 (EGFR wild-type) cells were transfected with control vector or a FLAG-tagged β2-AR expression vector. After treatment with NE, cells were immunostained with antibodies directed against EGFR or FLAG-tag and assessed by Duolink proximity ligation assay. Data are means ± SD. (D) Representative images from Duolink assay. Red foci indicate interactions between endogenous EGFR and FLAG-tagged β2-AR. Scale bar, 10 μm. (E) Heatmap depicting mean protein expression after treatment with NE (10 μM; 15 min). (F) Alterations in phosphorylation of LKB1 at the inhibitory site S428, AMPK activation, and mechanistic target of rapamycin (mTOR) activity (p70S6K) after NE stimulation as determined by RPPA. Data are means ± SD. (G) RPPA results were confirmed by Western blotting. (H) Western blot demonstrating the effect of NE (10 μM; 15 min) on the phosphorylation of LKB1 at the inhibitory site S428 and phosphorylation of mTOR and p70S6K in H1975 cells.

(fig. S5, A and B). Dobutamine (a β1-AR agonist) treatment did not affect IL-6 secretion. β2-AR expression was similar across EGFR mutant and wild-type cell lines (fig. S3A). To determine whether EGFR interacts with β2-AR in cells with EGFR-activating mutations, HCC827 cells were treated with or without NE, lysates were immunoprecipitated (IP) with anti-EGFR antibodies, and blots were immunoblotted (IB) with anti-β2-AR antibodies. We observed an interaction between endogenous β2-AR and EGFR in HCC827 cells (EGFR mutant) (Fig. 2B). A similar experiment was performed using HCC827 cells transfected with a FLAG-tagged β2-AR expression vector. Lysates were immunoprecipitated with anti-EGFR antibodies, and blots were immunoblotted (IB) with anti-β2-AR antibodies. We observed an interaction between β2-AR and EGFR in HCC827 cells (EGFR mutant) (fig. S5C). To visualize the in situ interaction between EGFR and β2-AR, we performed a Duolink proximity ligation assay. HCC827 (EGFR mutant) and A549 (EGFR wild-type) cells were transfected with a control vector or a FLAG-tagged β2-AR expression vector and treated with or without NE for 15 min. Confocal microscopy revealed NE-induced interactions between β2-AR and mutant EGFR but not wild-type EGFR (Fig. 2, C and D, red spots).

**Stress hormone signaling inactivates the tumor suppressor LKB1 in a PKC-dependent manner**

We stimulated NSCLC cells with NE and conducted a proteomic analysis by RPPA. NE induced LKB1S428 phosphorylation—a modification that inhibits LKB1 function (Fig. 2, E and F) (22). LKB1 plays a pivotal role in energy sensing, suppressing the mTOR pathway via activation of AMPK and TSC2 (23). NE concurrently decreased AMPK activation and increased mTOR activity, as indicated by p70S6K phosphorylation (Fig. 2, E to G). Total amounts of LKB1, AMPK, and p70S6K were unchanged (fig. S6A). RPPA data were confirmed by Western blotting (Fig. 2G). The effect of NE on LKB1 inactivation was also observed at lower concentrations of NE (fig. S6B). NE had a minimal effect on p-LKB1S428 in two EGFR wild-type cell lines (A549 and H661; fig. S6C). Forskolin induced a similar rise in p-LKB1S428 (fig. S6D). NE-induced inactivation of LKB1 was abrogated by PPL (Fig. 2G). Similarly, in H1975 cells (EGFR mutant), NE stimulation induced a rise in p-LKB1S428 and increased p-mTOR and p-p70S6K (Fig. 2H).

The LKB1 S428 residue can be phosphorylated by protein kinase C (PKC) (24) and p90RSK (22), and activation of β-AR triggers activation of PKC (25) and the mitogen-activated protein kinase (MAPK)/p90RSK pathways (fig. S6E) (26). Ro31-8220, an inhibitor of PKC and p90RSK, reduced NE-induced phosphorylation of PKC and LKB1S428 (Fig. 3A and fig. S6F) and abrogated NE-induced IL-6 (Fig. 3B and fig. S6G). To distinguish between the potential contributions of the PKC and MAPK/p90RSK pathways, we treated HCC827 cells with a control medium, a negative control peptide, or a PKC inhibitor peptide (27) with or without NE. PKC inhibition blocked NE-induced p-LKB1S428 (Fig. 3C), whereas p90RSK knockdown failed to inhibit NE-induced increases in p-LKB1S428 or IL-6 (fig. S6, H and I). β-AR signaling can also activate cAMP-dependent protein kinase (PKA) (28, 29). The PKA inhibitor H89 did not block NE-induced p-LKB1S428 (fig. S6J). To investigate the contribution of LKB1 inactivation in β-AR–mediated IL-6, we overexpressed LKB1 in HCC827 cells (fig. S6K). NE increased IL-6 in green fluorescent protein (GFP) control cells but not in cells overexpressing LKB1 (Fig. 3D).
Stress hormones induce IL-6 expression in a CREB-dependent manner

Transcription of the IL6 gene is complex, involving multiple transcription factors including nuclear factor κB (NF-κB) and cAMP response element–binding protein (CREB). In HCC827 cells, NE treatment increased p-CREBS133 (fig. S7, A and B), and inhibition of β-AR but not α-ARs abrogated NE-induced p-CREBS133 (fig. S7C). The NE-induced rise in p-CREBS133 in EGFR mutant cells was not observed in EGFR wild-type cells (Fig. 3E). Activated CREB binds CREB-binding proteins (CREBBPs), allowing transcription of target genes. SGC-CBP30 (CREBBP inhibitor) abrogated β2-AR–induced IL-6 (Fig. 3F). EGFR activation was not necessary for β-AR–mediated IL-6 expression, because erlotinib (1 μM) failed to inhibit NE-induced p-CREB and p-LKB1S428 (fig. S7D) and failed to block NE-induced IL-6 expression (fig. S7E). NE had minimal effects on NF-κB nuclear localization; NF-κB inhibition failed to block NE-induced IL-6 (fig. S8). Inhibition of PKC by RO31-8220 or a PKC inhibitor peptide diminished NE-induced rises in p-CREB (Fig. 3, A and C). In contrast, p90RSK knockdown failed to inhibit NE-induced p-CREB (fig. S6H). NE increased p-CREBS133 in GFP control cells but not in cells overexpressing LKB1 (fig. S6L), suggesting that CREB activation occurs downstream of LKB1 suppression.

Stress hormones promote EGFR TKI resistance and growth of NSCLC xenografts

We treated EGFR mutant NSCLC cell lines with NE 24 hours before the addition of erlotinib. NE promoted erlotinib resistance in vitro (Fig. 4A). The addition of the β-AR inhibitor PPL or IL-6 neutralizing antibodies blocked the effect on EGFR TKI resistance (Fig. 4, B and C).

Next, we injected HCC827 and HCC4006 cells into the flanks of nude mice. Chronic stress was initiated using an established restraint model (30). Tumor volumes were more than doubled in stressed mice relative to controls (Fig. 4D; P ≤ 0.04 for both). Immunohistochemistry revealed increased IL-6 expression in tumors from stressed mice compared to controls (P = 0.002; fig. S9, A and B). Tumor cell proliferation was not significantly different in stressed mice, but they had reduced microvessel density (MVD; fig. S9C).

We established HCC827 xenografts in female nude mice. Once tumors reached 400 mm³, animals were randomized to receive erlotinib (100 mg/kg) alone or with daily ISO. Erlotinib treatment resulted in near-complete tumor regression (Fig. 4F). After prolonged erlotinib treatment,
resistant disease emerged in mice treated with erlotinib plus ISO compared to mice receiving erlotinib alone ($P = 0.018$; Fig. 4G). The addition of PPL ($P = 0.035$), or the IL-6 antibody siltuximab ($P = 0.009$), inhibited the effect of ISO on EGFR TKI resistance, indicating a role for IL-6 in $\beta$-AR–mediated resistance.

β-Blocker use is associated with improved benefit from afatinib in the LUX-Lung3 study

Finally, we evaluated the influence of incidental β-blocker use on treatment effect in the randomized phase 3 LUX-Lung3 study (31) comparing the EGFR TKI afatinib with chemotherapy in EGFR mutation–positive NSCLC patients. In patients not receiving β-blockers, afatinib improved PFS time, with a median PFS of 11.1 and 6.9 months for afatinib and chemotherapy, respectively (log-rank test $P = 0.001$; Fig. 5, left), and a hazard ratio (HR) of 0.60, corresponding to a 40% reduction in the likelihood of progression for afatinib. In patients receiving β-blockers, afatinib was associated with a greater relative PFS benefit, with a median PFS of 13.6 and 2.5 months for afatinib and chemotherapy, respectively (HR = 0.25, log-rank test $P = 0.001$; Fig. 5, left), and a hazard ratio (HR) of 0.60, corresponding to a 40% reduction in the likelihood of progression for afatinib. In patients receiving β-blockers, afatinib was associated with a greater relative PFS benefit, with a median PFS of 13.6 and 2.5 months for afatinib and chemotherapy, respectively (HR = 0.25, log-rank test $P = 0.001$; Fig. 5, right), corresponding to a 75% reduction in the likelihood of progression. Although the analysis of these randomized trials was exploratory and limited by the modest number of patients receiving β-blockers, the findings are consistent with the preclinical data that β-blockade could delay the emergence of EGFR TKI resistance.

DISCUSSION

Acquired resistance to EGFR-targeted therapies is a major challenge in the treatment of NSCLC patients. The development of second- and third-generation EGFR inhibitors with activity against T790M-positive EGFR mutants is a clear advancement for targeting resistance. However, effective strategies are needed to delay and target resistant disease that emerges through T790M-independent mechanisms. This study revealed that stress hormones act on $\beta_1$-AR on NSCLC tumor cells and promote EGFR inhibitor resistance through the up-regulation of IL-6 (fig. S10). By using a high-throughput proteomic analysis, we identified a mechanism of LKB1- and CREB-dependent induction of IL-6 that could be inhibited by β-blockers. Our preclinical findings were supported by clinical data indicating that high plasma concentrations of IL-6 are associated with a worse response to EGFR inhibitors, and β-blocker use is associated with lower circulating concentrations of IL-6. Finally, our analysis of the phase 3 LUX-Lung3 study suggests that β-blocker use may improve response to EGFR inhibitors in NSCLC patients with EGFR-activating mutations.

The $\beta$-AR pathway contributes to tumor progression in a variety of malignancies, enhancing tumor cell proliferation and invasion (7, 32–36), inducing vascular and lymphatic remodeling (37), and enhancing lymph node metastasis (37). In lung cancer patients, psychological distress is a predictor of mortality (10). Retrospective clinical analyses have indicated that β-blocker use is associated with a reduced risk of cancer development (38) and decreased breast cancer–specific (39) and prostate cancer–specific mortality (40), but whether β-blockers improve clinical outcome remains controversial. Whereas first-generation β-blockers (PPL, nadolol, and timolol) are nonselective and thus block $\beta_1$- and $\beta_2$-ARs, second-generation cardioselective β-blockers (metoprolol, acebutolol, and busoprolol) have activity against $\beta_1$-ARs but not $\beta_2$-ARs. In the treatment of patients with cardiovascular conditions, cardioselective β-blockers are more frequently used in effort to minimize adverse effects. Here, we show that $\beta_2$-ARs are highly expressed on NSCLC tumor cells and that stress hormones activate signaling pathways that contribute to therapeutic resistance in NSCLC cell lines though $\beta_2$-AR but not $\beta_1$-AR. These findings indicate that pan-β-blockers but not cardioselective β-blockers may improve the clinical outcome of this patient population. Moreover, there were differential effects between cells with or without EGFR-activating mutations. It is feasible that the conflicting clinical data regarding the effect of β-blocker use on clinical outcome are related to the specificity of the β-blocker used and the mutational context of the tumors. Note that the tobacco-specific nitrosamine NKK is a high-affinity agonist for $\beta_1$-ARs and $\beta_2$-ARs (12, 41). Therefore, it is feasible that the established association between smoking status and IL-6 expression occurs in part through activation of the adrenergic pathway as described here.

Findings from this study and others (6) indicate that IL-6 is a key driver of T790M-independent EGFR TKI resistance. The present report highlights the regulation of IL-6 by stress hormones, but smoking status and comorbidities including obesity and diabetes are also associated with chronic inflammation and enhanced expression of IL-6 (42–44) and thus may affect the emergence of therapeutic resistance. Moreover, the deleterious effects of chronic stress on the clinical outcome of EGFR mutant NSCLC patients may extend beyond the induction of IL-6. Stress hormone–mediated activation of $\beta_1$-ARs can further activate the β-arrestin pathway, triggering degradation of p53 and the accumulation of DNA damage (45). In addition, β-arrestins are thought to facilitate GPCR (heterotrimeric GTP-binding protein–coupled receptor)–mediated transactivation of EGFR in cancer cells (46).

Earlier studies in other settings have suggested that interactions between $\beta$-AR and mutant EGFR could occur.
Many GPCRs use EGFR as an intermediate signaling protein (47). β-AR transactivates EGFR, resulting in incomplete downstream signaling. However, when EGFR and β-AR are simultaneously activated, broader signal transduction pathways are initiated (48). In animal models of carcinogen-induced lung adenocarcinoma, activation of both β-AR and EGFR is observed, and data suggest receptor cross-talk (41). Our data indicate that the effects of stress hormones on lung cancer cells may be heightened in cells with EGFR-activating mutations.

Here, we uncovered a mechanism of LKB1 inactivation in NSCLC cells, where intact LKB1 is inactivated by stress hormone signaling. Although the LKB1/STK11 tumor suppressor is frequently lost at the genomic level, or mutated, in NSCLC, the mechanism reported here may be particularly important in tumors with EGFR mutations because they nearly always have intact LKB1 (49). Consistent with our proposed model, in cardiac myocytes, β-AR stimulation inactivates the LKB1 target AMPK (50) as part of metabolic changes induced by catecholamines. Our data provide a detailed mechanistic understanding of how this could occur in nonmalignant cells and indicate that NSCLC cells may usurp this physiological process. LKB loss or inactivation has been linked to an enhanced CREB gene expression signature (51) and an immunosuppressive tumor microenvironment characterized by increased infiltration of myeloid-derived tumor suppressor cells and increased IL-6 (52). Studies have found an association between reduced LKB1/AMPK pathway signaling and increased distant metastasis (15, 53). In an analysis of 722 NSCLC patients receiving definitive radiotherapy, β-blocker use was strongly associated with reduced distant metastasis (8). Studies are warranted to determine whether stress hormones promote lung cancer metastasis through inactivation of LKB1.

Our analysis also demonstrated that β-AR activation stimulates the MAPK pathway in EGFR mutant NSCLC cells. The effects of β-AR signaling on MAPK are notable because reactivation of the MAPK pathway has been established as a mechanism of EGFR TKI resistance (54, 55).

Our prediliction findings indicate that stress hormones act on β2-AR on NSCLC cells, inducing increases in IL-6 and, in turn, resistance to EGFR TKIs. Our in vitro observations were supported by an animal model of NSCLC, where β-AR activation promoted the emergence of erlotinib-resistant disease, which was blocked by IL-6 neutralizing antibodies or β-blockers. To provide clinical validation, we analyzed the effect of incidental β-blocker use on benefit from afatinib versus chemotherapy in NSCLC patients with EGFR mutations in the LUX-Lung3 study. In patients receiving β-blockers, afatinib appeared to produce a larger improvement in PFS compared to patients not receiving β-blockers. However, interpreting this effect is difficult because patients receiving β-blockers also had a worse clinical response to chemotherapy (Fig. 5). Although our analysis of the LUX-Lung3 study supports our hypothesis that β-blocker use may improve clinical response to EGFR inhibitors, definitive clinical proof of this hypothesis would require direct clinical testing of EGFR TKIs alone and in combination with β-blockers. Given the potential benefits highlighted here, as well as the favorable tolerability profile and low cost of these drugs, we feel that such clinical testing merits consideration.

In summary, this study reveals a mechanism of EGFR inhibitor resistance and advances the understanding of how chronic stress may influence disease progression. These findings have immediate clinical implications because many well-tolerated and inexpensive β-blockers are available. These data support the future clinical testing of β-blockers in combination with EGFR-targeting agents.

**MATERIALS AND METHODS**

**Study design**

The objectives of this study were to (i) determine whether IL-6 expression was associated with T790M-negative resistance to EGFR TKIs and (ii) determine whether stress hormones could signal on NSCLC tumor cells and promote EGFR TKI resistance through the up-regulation of IL-6. To investigate the effects of β-AR signaling on NSCLC cells and the effects of β-AR activation on EGFR TKI resistance, we used quantitative real-time PCR, in vitro assays, Western blotting, RPPA, ELISAs, immunohistochemistry, and xenograft models of EGFR mutant NSCLC. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center in accordance with National Institutes of Health guidelines. Animals were randomly assigned to control or treatment groups. No statistical method was used to predetermine the sample size. Investigators were not blinded for the preclinical experiments.

**Cell lines and reagents**

Cell lines (table S3) were maintained in 10% fetal bovine serum (FBS; Sigma) RPMI medium. NE, E, ISO, phenolamine hydrochloride, salbutamol, dobutamine, and forskolin were obtained from Sigma-Aldrich. 8-pCPT-2-Me-cAMP was obtained from Thermo Fisher Scientific. QNZ (EVP4593), SGC-CBP30, and RO31-8220 were obtained from Selleck Chemicals. H89 was obtained from Tocris Chemicals. IL-6 neutralizing antibodies were obtained from R&D Systems. Erlotinib and siltuximab were obtained from the institutional pharmacy at the University of Texas MD Anderson Cancer Center. LKB1 expression vectors were obtained from Addgene. The PKC inhibitor peptide 19–36 and control peptides (EMD Millipore) were used at a concentration of 100 μM in ice-cold permeabilization buffer for 5 min. Cells recovered in serum-free medium for 2 hours before NE treatment. The following antibodies were used for Western blotting: IKB-α (4814; Cell Signaling), p-CREB (9198; Cell Signaling), pEGFR (3777; Cell Signaling), EGFR (1005; Santa Cruz Biotechnology Inc.), p-LKB1 S428 (3482 and 3051; Cell Signaling), p-p90RSK (9344; Cell Signaling), p-AMPK (2535; Cell Signaling), p-p70S6K (9205; Cell Signaling), p-ERK (9101; Cell Signaling), vinculin (v9131; 1:10,000; Sigma-Aldrich), and β-actin (A5441; 1:10,000; Sigma-Aldrich). For immunohistochemical studies, the following antibodies were used: anti-mouse CD31 antibody (1:400; PharMingen), Ki67 (Dako), and Alexa 594–conjugated secondary antibody (Molecular Probes).

**Detection of IL-6**

NSCLC cells (200,000 cells per well in six-well plates) were treated with 1 ml of serum-free medium with or without NE, E, or ISO for 24 hours, and medium was collected. IL-6 ELISA (R&D Systems) was performed according to the manufacturer’s instructions. For IL-6 staining, antibodies (1:50; Millipore) were used on frozen tumor sections. For inhibitor studies, cells were pretreated with inhibitors for 1 hour before NE stimulation. The doses of catecholamines used for our studies were selected to reflect physiologic conditions of these hormones at the level of the tumor. Whereas circulating plasma concentrations of NE range from 10 to 1000 pM in a normal individual and may reach as high as 100 nM in conditions of stress (56), catecholamine concentrations in...
some tissues are at least 100 times higher and may reach concentrations as high as 10 μM (17, 18).

**ADRB gene expression profiling**

The MD Anderson cohort was obtained from the Profiling of Resistance patterns and Oncogenic Signaling Pathways in Evaluation of Cancers of the Thorax (PROSPECT) study. Surgically resected tumors, collected between 2006 and 2010, from 189 patients were included in PROSPECT; and of these, 152 were lung adenocarcinomas. Gene expression analysis and sequencing of select genes were conducted as reported elsewhere (57–59).

**Immunoprecipitation**

Cells were stably transfected with FLAG-B2ADR or control vector using lentivirus. Cells were serum-starved for 1 hour and then treated with NE (10 μM) for 10 min. Cells were washed with phosphate-buffered saline (PBS) and incubated in dithiobis succinimidyl propionate (DSP; Thermo Scientific; 1 mM in PBS) cross-linker solution and incubated on ice. After 2 hours, tris-HCl stop solution was added at a final concentration of 10 mM and incubated for 15 min. Cells were lysed in protein lysis buffer. Communiprecipitation was performed using Pierce cross-link immunoprecipitation kit according to the manufacturer's instructions (cat. #26147). After elution, DSP cross-linking was quenched using 20 mM DTT (3′-deoxythymidine 3′-triphosphate) loading buffer in 1% SDS, 60 mM tris-HCl, and 10% glycerol, boiling at 100°C for 10 min.

**Duolink II fluorescence assay**

Duolink II fluorescence assay was performed as previously described (60). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 and then blocked in 5% BSA in PBS and incubated with mouse anti-EGFR (1:2000 dilution; Thermo Scientific) and rabbit anti-Flag (1:400 dilution; Cell Signaling) antibodies for 1 hour. The Duolink II PLA probe (Sigma) was used to detect the signals.

**Reverse phase protein array**

NSCLC cells were treated with 10 μM NE for 15 min, and protein lysates were collected. RPPA slides were printed from lysates, and RPPA studies were performed and analyzed as previously described (53, 61–63).

**Cell viability assays**

NSCLC cells (2000 cells per well in 96-well plates) were treated with increasing concentrations of EGFR TKIs. After 5 days, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assays were performed. To evaluate the effect of NE on EGFR TKI resistance, we seeded cells in 24-well plates (40,000 cells per well) and treated them with NE for 24 hours, and then, erlotinib was added to the culture medium. After 5 days, cell viability was measured by MTS assay. PPL (1 μM) or IL-6 antibodies (1 μg/ml) were added 1 hour before the addition of NE.

**Mice**

Cancer cells (1 × 10⁶) were injected subcutaneously into female nude mice. Chronic stress was induced using a restraint-stress procedure (64). To test the effect of ISO on IL-6, once tumors reached a volume of 400 mm³, animals received daily ISO treatment (10 mg/kg, intraperitoneally) for 3 days. Tumors were processed for RNA collection and quantitative real-time PCR. For erlotinib resistance studies, 1 × 10⁶ HCC827 cells were injected subcutaneously into nude mice. Once tumors reached 400 mm³, animals (n = 5 mice per group) received vehicle, ISO (10 mg/kg, daily, intraperitoneally), PPL (10 mg/kg, daily, intraperitoneally), or siltuximab (20 mg/kg, twice weekly, intraperitoneally). Erlotinib was given at a dose of 100 mg/kg (oral gavage) on a 4-week-on/2-week-off schedule.

**Clinical analysis**

Biospecimens were obtained after patients gave informed consent, under protocols approved by institutional review boards at all participating institutions. Studies were conducted in accordance with the Declaration of Helsinki. The PROSPECT data set has been detailed previously (65) and includes patients who underwent surgical resection of NSCLC with curative intent between 1996 and 2008 at the MD Anderson Cancer Center. In the PROSPECT study, most of the cases were lung adenocarcinomas. Clinical outcomes from BATTLE (NCT00409968, NCT00411671, NCT00411632, NCT00410059, NCT00410189), the LUX-Lung3 study (NCT00949650) (31), and ZEST (NCT00364351) (16) have been reported. We analyzed baseline plasma samples collected during the BATTLE clinical trial (21) and the phase 3 ZEST clinical study and were blinded to clinical outcome. Each sample was analyzed in duplicate. For the analysis of circulating concentrations of IL-6 in the BATTLE trial, 185 patients received no β-blockers and 3 patients received pan β-blockers. The groups were compared using a two-tailed Student's t test.

**Statistics**

For the in vitro studies, statistical analysis was performed using Student’s t test (two-tailed) or one-way ANOVA. P ≤ 0.05 was considered statistically significant. For the RPPA data, ANOVA was used on a protein-by-protein basis. The resulting P values, computed from F statistic, were modeled using the β-uniform mixture model and used to determine a false discovery rate cutoff to identify significantly differentially expressed proteins. For in vivo studies, we fitted a linear model (with treatment and time effects) using the generalized least squares method to account for repeated-measurements correlation. Dunnett’s test was used to adjust for multiple comparisons in comparing many treatments with a single control.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/9/415/eaao4307/DC1

**Fig. S1.** Frequency of T790M EGFR secondary mutations in erlotinib-resistant cells.

**Fig. S2.** Resistance of HCC827 ER and HCC4006 ER cells to EGFR TKIs.

**Fig. S3.** ADRB expression and EGFR status in NSCLC cell lines.

**Fig. S4.** Induction of IL-6 after β-AR activation.

**Fig. S5.** Differential effects of stress hormone induced p-LKB1, p-CREB, and IL-6.

**Fig. S6.** Effect of chronic stress on IL-6 expression and MVD in NSCLC xenografts.

**Fig. S7.** Effect of IL-6 expression on NSCLC xenografts.

**Fig. S8.** Effect of β-AR signaling on NF-κB activity.

**Table S1.** PFS and OS of NSCLC patients with high or low IL-6 treated with erlotinib.

**Table S2.** Smoking status of patients with high or low circulating IL-6 in the ZEST trial.

**Table S3.** Comparison of circulating IL-6 concentrations by smoking status in NSCLC patients treated with erlotinib.

**REFERENCES AND NOTES**


Destressing cancer with β-blockers

Common wisdom holds that stress is not good for cancer patients, but it can be difficult to avoid considering that both the diagnosis of cancer and the associated treatments are quite stressful for the mind and body. Nilsson et al. investigated this phenomenon in non-small cell lung cancer, providing insights into the underlying mechanism and a potential intervention. The authors found that stress hormones activate β2-adrenergic receptors on cancer cells, activating a signaling cascade that promotes tumor resistance to EGFR inhibitors, a key therapy for this disease. Conversely, β-blockers—a common class of drugs used in humans—blocked this mechanism of resistance and may become a useful adjunct to lung cancer therapy regimens.