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Functional cooperation between HIF-1 α and c-Jun in mediating primary and acquired resistance to gefitinib in NSCLC cells with activating mutation of *EGFR*

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Abstract

Objective—Hypoxia-inducible factor 1 (HIF-1) and activator protein 1 (AP-1) are important transcription factors regulating expression of genes involved in cell survival. HIF-1 α and c-Jun are key components of HIF-1 and AP-1, respectively, and are regulated by epidermal growth factor receptor (EGFR)-mediated cell signaling and tumor microenvironmental cues. The roles of HIF-1 α and c-Jun in development of resistance to EGFR tyrosine kinase inhibitor (TKI) in non-small cell lung cancer (NSCLC) with activating mutation of *EGFR* have not been explored. In this study, we investigated the roles of HIF-1 α and c-Jun in mediating primary and acquired resistance to gefitinib in NSCLC cells with activating mutation of *EGFR*.

Materials and Methods—Changes in HIF-1 α protein and in total and phosphorylated c-Jun levels in relation to changes in total and phosphorylated EGFR levels before and after gefitinib treatment were measured using Western blot analysis in NSCLC cells sensitive or resistant to gefitinib. The impact of overexpression of a constitutively expressed HIF-1 α (HIF-1 α / ODD) or a constitutively active c-Jun upstream regulator (SEK1 S220E/T224D mutant) on cell response to gefitinib was also examined. The effect of pharmacological inhibition of SEK1-JNK-c-Jun pathway on cell response to gefitinib was evaluated.

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Conflict of interest

None declared.

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Results—Downregulation of HIF-1 α and total and phosphorylated c-Jun levels correlated with cell inhibitory response to gefitinib better than decrease in phosphorylated EGFR did in NSCLC cells with intrinsic or acquired resistance to gefitinib. Overexpression of HIF-1 α / ODD or SEK1 S220E/T224D mutant conferred resistance to gefitinib. There exists a positive feed-forward regulation loop between HIF-1 and c-Jun. The JNK inhibitor SP600125 sensitized gefitinib-resistant NSCLC cells to gefitinib.

Conclusions—HIF-1 α and c-Jun functionally cooperate in development of resistance to gefitinib in NSCLC cells. The translational value of inhibiting HIF-1 α /c-Jun cooperation in overcoming resistance to EGFR TKI treatment of NSCLC cells with activating mutation of *EGFR* deserves further investigation.

Keywords

Non-small cell lung cancer; EGFR mutation; TKI; HIF-1 α ; c-Jun

1. Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have been used worldwide to treat patients with advanced non-small cell lung cancer (NSCLC) since the first-generation EGFR TKIs (gefitinib and erlotinib) were approved in early 2000s. In NSCLC patients with tumors harboring *EGFR* activating mutations in exons 19–21 of EGFR kinase domain, treatment with a first-generation EGFR TKI prolonged progression-free survival time by about 9.7 months [1].

However, not all NSCLC patients with the *EGFR* activating mutations in their tumors have such favorable outcomes in response to EGFR TKIs. About 20% to 30% of NSCLC patients with the *EGFR* activating mutations in their tumors have no objective tumor-regressive response to initial treatment with a first-generation EGFR TKI [1–4]. A number of mechanisms can cause intrinsic (primary) resistance of NSCLC to EGFR TKIs, including EGFR downstream pathway redundancy (activated by overlapping signal pathways), pathway reactivation (independent of EGFR due to oncogenic mutations or mutational inactivation of key signaling molecules, such as Ras [5,6] and PTEN [7,8]), and pathway alternation (escape from EGFR signaling regulation via recruiting an alternate signaling pathway) [9]. In addition, tumor microenvironmental cues and tumor heterogeneity can cause intrinsic resistance to EGFR TKI [10].

Moreover, even in patients who initially have a partial or complete response to an EGFR TKI, acquired resistance may ultimately occur. The mechanisms underlying development of acquired resistance of NSCLC to first-generation EGFR TKIs include *EGFR* T790M secondary mutation (present in ~60% cases of acquired resistance), *MET* amplification (5%–10%), *PIK3CA* mutation (~5%), *BRAF* mutation (~1%), and small-cell cancer transformation (~5%) [11]; in another approximately 20% to 25% of cases of acquired resistance, the underlying mechanisms remain unclear.

Many NSCLC patients, whose tumor initially responds to a first-generation EGFR TKI but develops resistance due to secondary *EGFR* T790M mutation, benefit from treatment with a

third-generation EGFR TKI, such as osimertinib; however, a significant percentage of patients with acquired resistance due to *EGFR* T790M mutation do not respond to a third-generation EGFR TKI [12,13]. Novel insights into the mechanisms of resistance to EGFR TKIs are critical for developing new therapeutic strategies for improving the outcome of NSCLC patients with advanced disease.

Hypoxia-inducible factor-1 (HIF-1), a master regulator of response to tumor hypoxia, is a heterodimer consisting of an oxygen-sensitive alpha subunit (HIF-1 α) and a constitutively expressed beta subunit (HIF-1 β) [14–18]. The level of HIF-1 α is increased dramatically in hypoxic tumor microenvironments because of decreased ubiquitination and degradation of HIF-1 α protein associated with tumor hypoxia [19,20]. The level of HIF-1 α is also upregulated by aberrant cell signaling through increased expression [21–25]. We previously showed that downregulation of HIF-1 α through inhibiting EGFR downstream cell signaling is required for the antiproliferative effects of the anti-EGFR antibody cetuximab in head and neck cancer, colorectal cancer, and NSCLC models [26–36].

Activator protein-1 (AP-1) is a transcription factor that regulates gene expression in response to a variety of extracellular stimuli, growth factors, cytokines, heat shock, UV irradiation, hypoxia, and so forth [37]. AP-1, like HIF-1, is a dimeric complex; AP-1 is assembled through heterodimerization between members containing a leucine zipper motif, including c-Jun, c-Fos, ATF (activating transcription factor), and MAF (musculoaponeurotic fibrosarcoma) [38,39]. c-Jun is regulated by a combination of enhanced expression and phosphorylation on specific serine residues (S63 and S73) of c-Jun by c-Jun N-terminal kinase (JNK) [40–42], which is also known as stress-activated MAP kinase (SAPK), a member of the mitogen-activated protein (MAP) kinase family [43]. JNK is activated by dual phosphorylation on threonine and tyrosine residues (T183 and T185) by a member of the MAPKK group of protein kinases [44,45], specifically, by SAPK/Erk kinase (SEK) [46].

AP-1 has been shown to functionally cooperate with HIF-1 in hypoxia-induced gene transcription [47]. The response of AP-1/c-Jun to chronic hypoxia was reported to be HIF-1 α -dependent [48]. In this study, we tested the hypotheses that HIF-1 α serves as a biomarker of NSCLC cell response to gefitinib and that HIF-1 α and c-Jun functionally cooperate in mediating resistance to gefitinib in NSCLC cells with activating mutation of *EGFR*.

2. Materials and methods

2.1. Reagents

Gefitinib (ZD1839) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). SP600125 (1,9-pyrazoloanthrones) was purchased from Calbiochem/EMD Chemicals Inc. (Gibbstown, NJ). Antibodies against total and S73-phosphorylated c-Jun, total and T183/Y185-phosphorylated JNK, total and Y1068-phosphorylated EGFR, PARP, and β -actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-HIF-1 α antibody was purchased from BD Biosciences. cDNA constructs containing HIF-1 α / ODD and constitutively active SEK1 S220E/T224D mutant were provided by Dr. L. Eric Huang (University of Utah School of Medicine, Salt Lake City, UT) and Dr. Jonathan Kurie (The

University of Texas MD Anderson Cancer Center, Houston, TX), respectively. Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell lines and cell cultures

Human lung adenocarcinoma cells H3255 (harboring *EGFR* L858R mutation in exon 21), HCC827 (harboring *EGFR* E746-A750 in-frame deletion in exon 19), H1650 (harboring *EGFR* E746-A750 in-frame deletion in exon 19 and *PTEN* mutation), and H1975 (harboring *EGFR* L858R mutation in exon 21 and T790M mutation in exon 20) were maintained in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% FBS, 100U/mL penicillin, and 100 µg/mL streptomycin under the condition of 5% CO₂ at 37°C.

2.3. Western blot analysis

After desired treatments, cells were washed twice with cold PBS and lysed in a lysis buffer (50mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 50mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 25µg/ml aprotinin, and 25 µg/ml leupeptin) for 15minutes on ice [36,49]. Insoluble cell debris was removed by centrifugation. Equal amounts of protein lysates, quantified by Pierce Coomassie Plus, were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with various primary antibodies. The signals were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ), and the bands of interest were quantified using Image J.

2.4. MTT proliferation assay

Cells were seeded in 48-well plates with 0.2 mL/well of medium containing 0.5% FBS at 37°C. Following the indicated treatment, cells were incubated with 20 µL/well of 10mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 2hours and then lysed with a lysis buffer (200 µL/well) containing 20% SDS in dimethyl formamide/H₂O (1:1, v/v; pH 4) at 37°C overnight. The relative number of surviving cells in each group was determined by measuring the optical density (OD) of the cell lysates at an absorbance wavelength of 570 nm. The OD value of each treatment group was expressed as a percentage of the OD value of the untreated control cells [32,50]. IC₅₀ value was determined using Excel FORECAST.

2.5. cDNA transfection

Cells were transfected with cDNA plasmids using Lipofectamine 2000. Briefly, cells were seeded in 60-mm plates at 80% to 90% confluency overnight. cDNA and Lipofectamine 2000 were mixed in 100 µL of minimal essential medium (Opti-MEM, Life Technologies, Carlsbad, CA) for 15 minutes, and then this mixture was added into the culture medium. Six hours later, the medium was replaced with regular medium, and the cells were cultured for an additional 48 hours before detection of expression of cDNA constructs by Western blotting.

2.6. Statistical analysis

Experimental data are presented as mean $\pm$ standard deviation. Student's t-test was used in statistical analyses using SPSS 19.0 software. P value <0.01 was considered statistically significant.

3. Results

3.1. Downregulation of HIF-1 α and total and phosphorylated c-Jun protein correlates with cell inhibitory response to gefitinib in NSCLC cells

As shown in Fig. 1, four human NSCLC cell lines harboring *EGFR* mutation in exon 19 or 21, which is generally considered a predictor of response to the first-generation EGFR TKIs [51–53], responded differently to treatment with gefitinib. HCC827 and H3255 cells were sensitive to gefitinib, with IC₅₀ values of 8.26 nM and 22.75 nM, respectively (Fig. 1A, left). In contrast, H1650 and H1975 cells were resistant to gefitinib, with IC₅₀ values greater than 2000 nM (Fig. 1A, right). As shown in Fig. 1B, after overnight exposure to gefitinib at various concentrations, HCC827 and H3255 cells but not H1650 and H1975 cells exhibited apoptosis as indicated by appearance of PARP cleavage.

Of note, the tyrosine kinase activity of EGFR was inhibited in an EGFR TKI dose-dependent manner in all four cell lines, although there were differences in sensitivity: HCC827 (gefitinib-sensitive) was the most sensitive, H3255 (gefitinib-sensitive) and H1650 (gefitinib-resistant) had intermediate sensitivity, and H1975 (gefitinib-resistant) was relatively less sensitive. In contrast, levels of HIF-1 α and c-Jun seemed to be more closely associated with the response to gefitinib treatment. Specifically, both HIF-1 α and c-Jun showed remarkable decrease in protein level detected by Western blotting after gefitinib treatment in the gefitinib-sensitive cell lines (HCC827 and H3255) but not in the gefitinib-resistant cell lines (H1650 and H1975) at doses as high as 500 nM gefitinib. This interesting finding suggests that HIF-1 α and c-Jun are important signaling molecules mediating NSCLC cell response to EGFR TKI.

3.2. Levels of HIF-1 α and c-Jun are increased in NSCLC cells with acquired resistance to gefitinib

To further investigate whether decreased levels of HIF-1 α and c-Jun correlate with inhibitory response to EGFR TKI treatment, we exposed H3255 and HCC827 cells (both gefitinib-sensitive) to gefitinib at increasing concentrations for an extended period of time. Through this process, we generated a subline of H3255 cells, H3255RR, that was relatively resistant to gefitinib compared to the parental H3255 cells, and a subline of HCC827 cells, HCC827R, that exhibited remarkable resistance to gefitinib. As shown in Fig. 2A, the IC₅₀ value for H3255RR was 92 nM, approximately 11 times the IC₅₀ value for the parental H3255 cells (8.26 nM, Fig. 1A), and the IC₅₀ value for HCC827R was over 500 nM, dramatically higher than the IC₅₀ value for the parental HCC827 cells (22.75 nM, Fig. 1A).

We found that, compared with their respective parental cells, both H3255RR and HCC827R cells exhibited much higher basal protein levels of HIF-1 α and total and phosphorylated c-Jun (Fig. 2B). In the H3255RR cells, even though the resistance to gefitinib relative to

parental H3255 cells was not as great as the resistance of HCC827R cells relative to parental HCC827 cells, the increase in baseline HIF-1 α and c-Jun levels between parental and resistant cells was remarkable, as shown by the fold ratios (Fig. 2B). The levels of HIF-1 α and total and phosphorylated c-Jun were not decreased by gefitinib in HCC827R cells. In H3255RR cells, although the levels of HIF-1 α and total and phosphorylated c-Jun were decreased by gefitinib, the magnitude of the decrease was less than the magnitude of the decrease in the parental H3255 cells, as shown by the fold ratios (Fig. 2B). The decrease likely reflected the difficulty of developing H3255 sublines resistant to gefitinib. As shown in Fig. 2A, H3255RR cells retained considerable sensitivity to gefitinib even though they were relatively resistant compared to the parental H3255 cells.

3.3. c-Jun expression is induced under hypoxia and upregulated by HIF-1, and HIF-1 α and c-Jun contribute to resistance to gefitinib in NSCLC cells

We next investigated the functional roles of HIF-1 α and c-Jun and their interactions in mediating resistance to gefitinib. Tumor hypoxia is common in solid tumors, including NSCLC. HIF-1 α rapidly accumulates in cells under hypoxia, and c-Jun is known to be activated by HIF-1 under hypoxia in certain experimental conditions. We compared HIF-1 α and c-Jun levels between NSCLC cells cultured under normoxia and hypoxia. As shown in Fig. 3A, overnight incubation of the cells in a hypoxic chamber with 1% oxygen led to an increase in HIF-1 α level in all four NSCLC cell lines, which had various degrees of sensitivity to gefitinib. The low-oxygen condition also led to increases in the levels of both total and phosphorylated c-Jun protein and increases in the levels of both total and phosphorylated JNK1 protein, with the degrees of increase differing by cell type. Since hypoxia is one of the characteristic features of solid tumors, these data suggested that the hypoxic microenvironment might play a role in development of resistance to EGFR TKI in NSCLC.

To test our hypothesis that hypoxia contributes to development of resistance to gefitinib in part through upregulating c-Jun, we introduced a constitutively expressed HIF-1 α construct containing a HIF-1 α oxygen-dependent degradation domain deletion mutant (HIF-1 α /ODD) in HCC827 and H3255 cells. We previously reported that HIF-1 α /ODD mutant retains the majority of the transcriptional activity of full-length HIF-1 α and can be stably overexpressed in a normoxic environment [35]. Compared to the control vector-transfected cells, HCC827 and H3255 cells expressing HIF-1 α /ODD exhibited higher levels of total and phosphorylated c-Jun and JNK (Fig. 3B). This result was consistent with our observation of increase in total and phosphorylated c-Jun and JNK under hypoxia (Fig. 3A). To examine whether HIF-1 α has a causal role in mediating resistance to gefitinib, we examined the induction of apoptosis after gefitinib treatment in NSCLC cells with and without overexpression of HIF-1 α /ODD. As shown in Fig. 3C, there was a noticeable decrease in the level of PARP cleavage in HCC827 HIF-1 α /ODD cells and H3255 HIF-1 α /ODD cells compared to the results in the respective control vector-transfected cells. This finding indicated that HIF-1 α overexpression plays a critical role in mediating resistance to EGFR TKI treatment in NSCLC.

3.4. c-Jun phosphorylation upregulates HIF-1 α expression and renders NSCLC cells resistant to gefitinib

To further understand the interaction between HIF-1 α and c-Jun in mediating NSCLC resistance to gefitinib, we used both genetic and small-molecule-inhibitor approaches to investigate the role of c-Jun in the scenario. SAPK/Erk kinase (SEK1), also known as MKK4 or JNK kinase, can activate the MAP kinase (SAPK and JNK) and subsequently activates c-Jun by phosphorylating c-Jun on serine 63 and serine 73 within its transcriptional activation domain [40,41]. We transfected HCC827 and H3255 cells with a construct containing a constitutively active SEK1 S220E/T224 mutant. As shown in Fig. 4A, transfection with SEK1 S220E/T224D mutant successfully upregulated the levels of phosphorylated SAPK/JNK and phosphorylated c-Jun in HCC827 and H3255 cells. Overexpression of SEK1 S220E/T224D mutant reduced the induction of PARP cleavage by gefitinib treatment in both cell lines (Fig. 4B), suggesting that c-Jun activation can inhibit gefitinib-induced apoptosis in NSCLC cells. We also found that overexpression of SEK1 S220E/T224D mutant upregulated the level of HIF-1 α (Fig. 4A). These data suggested that there is reciprocal regulation between HIF-1 α and c-Jun that forms a positive feed-forward loop that facilitates development of cellular resistance to gefitinib in NSCLC cells.

3.5. SP600125 inhibits c-Jun phosphorylation, downregulates HIF-1 α level, and promotes induction of apoptosis by gefitinib in gefitinib-resistant NSCLC cells

To examine whether pharmacological inhibition of JNK may overcome resistance to gefitinib in NSCLC cells, we subjected gefitinib-resistant H1650 and H1975 cells to overnight treatment with gefitinib with and without SP600125, a potent JNK and c-Jun inhibitor [54,55]. As shown in Fig. 5A, the levels of phosphorylated SAPK/JNK and phosphorylated c-Jun were remarkably decreased after SP600125 treatment. Furthermore, HIF-1 α protein level was downregulated in both cell lines. This result was consistent with our finding shown in Fig. 4A that phosphorylation of c-Jun led to upregulation of HIF-1 α .

Fig. 5B shows that the combination of SP600125 and gefitinib sensitized H1650 and H1975 cells to treatment with gefitinib through induction of apoptosis, whereas gefitinib alone did not increase PARP cleavage beyond the level in untreated cells. Fig. 5C shows that gefitinib alone inhibited cell growth by only 2.25% in H1650 cells and 4.36% in H1975 cells. SP600125 treatment alone inhibited cell growth only moderately (by 12.47% in H1650 cells and 28.69% in H1975 cells). However, combination treatment with gefitinib and SP600125 inhibited cell growth by 45.33% in H1650 cells and 69.1% in H1975 cells.

Together, our findings indicate that inhibition of the c-Jun and HIF-1 α regulatory loop is a novel approach to overcoming resistance to gefitinib in NSCLC.

4. Discussion

In the present study, we explored whether HIF-1 α and c-Jun functionally cooperate in mediating resistance of NSCLC cells with activating mutation of *EGFR* to gefitinib, a prototype first-generation EGFR TKI. We found that downregulation of HIF-1 α and c-Jun was more closely correlated with cell inhibitory response to the EGFR TKI gefitinib than

was decrease in phosphorylated EGFR in NSCLC cells. NSCLC cells with intrinsic or acquired resistance to gefitinib exhibited higher basal levels of HIF-1 α and total and phosphorylated c-Jun. Experimental overexpression of a constitutively expressed HIF-1 α (HIF-1 α / ODD) construct or a constitutively active SEK1 S220E/T224D mutant in NSCLC cells upregulated total and phosphorylated levels of c-Jun and conferred resistance to gefitinib. Pharmacological inhibition of JNK-c-Jun pathway sensitized gefitinib-resistant NSCLC cells to gefitinib treatment.

Despite being predicted to respond to first-line treatment with an EGFR TKI, 20% to 30% of NSCLC patients with activating *EGFR* mutations do not benefit from the treatment [12,13]. Even osimertinib, a potent and irreversible third-generation EGFR TKI, produced an overall objective response rate of only about 67% in the 80-mg cohort when the drug was used as first-line treatment for advanced NSCLC with activating mutation of *EGFR* [56,57]. Novel mechanisms contributing to resistance of advanced NSCLC to EGFR TKI thus need to be identified. Identification of novel molecular biomarkers is clinically important to further improve patient responses to EGFR TKI.

An important finding of our current study was that resistance to gefitinib in NSCLC cells with either intrinsic or acquired resistance did not entirely correlate with the level of EGFR tyrosine kinase inhibition shown by decrease in the level of EGFR activation-specific phosphorylation on Y1068. In other words, EGFR tyrosine kinase activity was noticeably inhibited by gefitinib in the gefitinib-resistant cell lines, although the level of EGFR tyrosine kinase inhibition was not as great as the level of inhibition seen in the gefitinib-sensitive cells. Current efforts to develop newer-generation EGFR TKIs remain focused on effective inhibition of EGFR tyrosine kinase when EGFR TKI induces a secondary T790M mutation in the tyrosine kinase domains, but our findings suggest that aberrations in cell signaling downstream of EGFR is an area that deserves more attention.

We recently reported that cell signaling downstream of EGFR plays important roles in mediating cancer cell response to the EGFR-blocking antibody cetuximab [27,31,34,35]. In particular, we found that downregulation of HIF-1 α appears to correlate better than EGFR tyrosine kinase inhibition with cellular response to cetuximab treatment in head and neck cancer cells [35]. We also found that the role of HIF-1 α in mediating resistance to cetuximab is linked to the key role of HIF-1 in regulating cancer metabolism [32,35].

In the present study, we found that basal levels of HIF-1 α and total and phosphorylated c-Jun protein were higher in NSCLC cells that acquired resistance to EGFR TKI through long-term adaptation. These data indicated that the elevation of HIF-1 α and c-Jun might play a role in development of EGFR TKI resistance in NSCLC. Results of our experiments involving overexpression of a constitutively expressed HIF-1 α / ODD and a constitutively active SEK1 S220E/T224D mutant led us to propose the model depicted in Fig. 6. In this model, intrinsic genetic aberration, in other words, *EGFR* activating mutation, and extrinsic environmental cues, such as tumor hypoxia and stress, lead to upregulation of HIF-1 α and c-Jun phosphorylation by upstream respective signaling and subsequent activation of an array of targeted genes through HIF-1 and AP-1. It was reported that a positive feed-forward loop exists between AP-1 and c-Jun, in which AP-1 could activate c-Jun promoter, thus

enhancing c-Jun expression, which can further enhance the activity of AP-1 [58–60]. Our current data suggest that a positive feed-forward loop also exists between HIF-1 α and c-Jun, in which constitutive activation of the SEK1-JNK-c-Jun pathway can upregulate HIF-1 α protein level. Our data indicate that pharmacological interruption of these positive feed-forward loops using a small molecule inhibitor may offer a new approach for sensitizing EGFR TKI-resistant NSCLC cells to EGFR TKI. In vivo studies and more delicate mechanistic studies are needed to confirm our findings and further elucidate the important roles of HIF-1 α and c-Jun in resistance to EGFR TKI. c-Jun and HIF-1 α can bind to the transcriptional co-activators CBP/p300 and SRC-1 to enhance transcriptional activity [47,61]. c-Jun upregulation under hypoxia masks the ODD domain of HIF-1 α , thus preventing HIF-1 α from being ubiquitinated and degraded [62]. Whether these mechanisms play a role in the development of NSCLC cell resistance to EGFR TKI treatment needs to be further investigated.

In summary, our data reveal novel insights into potential roles of HIF-1 α and c-Jun and their functional cooperation in mediating development of resistance to EGFR TKI in NSCLC cells with activating mutation of *EGFR*. Our findings expand current knowledge on the mechanisms of resistance to EGFR TKI. Further study is warranted of co-targeting HIF-1 α and c-Jun as a novel approach to overcoming NSCLC resistance to EGFR TKI.

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Highlights

- HIF-1 α and c-Jun downregulation correlates with NSCLC cell response to gefitinib
- Gefitinib-resistant NSCLC cells exhibit high basal levels of HIF-1 α and c-Jun
- Constitutively expressed HIF-1 α upregulates c-Jun and makes NSCLC gefitinib resistant
- SEK-JNK-c-Jun inhibition downregulates HIF-1 α and sensitizes NSCLC to gefitinib

Fig. 1. Downregulation of HIF-1α and c-Jun protein correlates with cell inhibitory response to gefitinib treatment in NSCLC cells

A. HCC827 and H3255 cells (left panel) and H1650 and H1975 cells (right panel) were treated with the indicated concentrations of gefitinib for 5 days. Cell survival was measured by MTT proliferation assay. **B.** HCC827, H3255, H1650, and H1975 cells were treated with the indicated concentrations of gefitinib for 24 hours. Cells were then harvested, and equal amounts of protein lysates were subjected to Western blot analysis with the indicated antibodies. The level of β-actin was used as a reference of lysate protein loading control of each cell line.

Fig. 2. The levels of HIF-1α and c-Jun are increased in NSCLC cells with acquired resistance to gefitinib

A. HCC827R and H3255RR cells were treated with the indicated concentrations of gefitinib for 5 days. Cell survival was measured by MTT proliferation assay. **B.** HCC827, HCC827R, H3255 and H3255RR cells were treated with 20 nM gefitinib or DMSO vehicle control for 24 hours. Cells were then harvested, and equal amounts of protein lysates were subjected to Western blot analysis with the indicated antibodies. The level of β-actin was used as a reference of lysate protein loading control of each cell line.

Fig. 3. c-Jun expression is induced under hypoxia and upregulated by HIF-1, and c-Jun and HIF-1α contribute to resistance to gefitinib in NSCLC cells

A. HCC827, H3255, H1650, and H1975 cells were cultured in normoxia or in a hypoxia chamber (1% O₂) for 24 hours. Cells were then harvested immediately, and equal amounts of protein lysates were subjected to Western blot analysis with the indicated antibodies. **B.** HCC827 and H3255 cells were transfected with a construct containing HIF-1α/ ODD mutant or pcDNA3.1 control vector as described in Materials and Methods. Cell lysates were prepared for Western blotting with the indicated antibodies. **C.** HCC827 and H3255 cells expressing control vector or HIF-1α/ ODD mutant were treated with 20 nM gefitinib or vehicle control for 24 hours. Cell lysates were prepared for Western blotting with the indicated antibodies. The level of β-actin was used as a reference of lysate protein loading control of each cell line.

Fig. 4. c-Jun phosphorylation upregulates HIF-1α expression and renders NSCLC cells resistant to gefitinib

A. HCC827 and H3255 cells were transfected with a construct containing SEK1 S220E/T224D or pcDNA3.1 control vector as described in Materials and Methods. Cell lysates were prepared for Western blotting with the indicated antibodies. **B.** HCC827 and H3255 cells expressing SEK1 S220E/T224D or the control vector were treated with 20 nM gefitinib or vehicle control for 24 hours. Cell lysates were prepared for Western blotting with the indicated antibodies. The level of β-actin was used as a reference of lysate protein loading control of each cell line.

Fig. 5. SP600125 inhibits c-Jun phosphorylation, downregulates HIF-1α level, and promotes induction of apoptosis by gefitinib in gefitinib-resistant NSCLC cells

A. H1650 and H1975 cells were treated with 10 μM SP600125 or vehicle control for 24 hours. Cell lysates were then prepared and subjected to Western blot analysis with the indicated antibodies. **B.** H1650 and H1975 cells were treated with vehicle control, 500 nM gefitinib, 10 μM SP600125, or 500 nM gefitinib and 10 μM SP600125. Cell lysates were then prepared for Western blotting with the indicated antibodies. **C.** H1650 and H1975 cells were treated with 500 nM gefitinib, 10 μM SP600125, or 500 nM gefitinib and 10 μM SP600125. Cell inhibition was measured by MTT proliferation assay. The data are presented as a percentage of cells treated with vehicle control (n=3; **P <0.005).

Fig. 6. Proposed model of functional cooperation between HIF-1 α and c-Jun in regulating primary and acquired resistance to gefitinib in NSCLC cells with activating mutation of *EGFR* HIF-1 α and c-Jun are upregulated or phosphorylated by cell signal pathways downstream of EGFR and by environmental cues. Both HIF-1 and AP-1 activate transcription of target genes. AP-1 enhances c-Jun expression through a positive feed-forward loop. c-Jun upregulation prevents HIF-1 α ubiquitination and degradation. HIF-1 may upregulate c-Jun expression through another positive feed-forward loop. *EGFR activating mutation.