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Altered regulation of HIF-1α in naïve- and drug-resistant EGFR mutant NSCLC: implications for a VEGF-dependent phenotype

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Abstract

Introduction: Treatment of patients with *EGFR* mutant NSCLC with vascular endothelial growth factor (VEGF) inhibitors in combination with EGFR inhibitors provides greater benefit than EGFR inhibition alone, suggesting that *EGFR* mutation status may define a patient subgroup with greater benefit from VEGF blockade. The mechanisms driving this potentially enhanced VEGF dependence are unknown.

Methods: We analyzed the effect of EGFR inhibition on VEGF and HIF-1α in NSCLC models *in vitro* and *in vivo*. We determined the efficacy of VEGF inhibition in xenografts and analyzed the impact of acquired EGFR inhibitor resistance on VEGF and HIF-1α.

Results: NSCLC cells with *EGFR* activating mutations exhibited altered regulation of VEGF compared to *EGFR* wild-type cells. In *EGFR* mutant cells, EGFR, not hypoxia, was the dominant regulator or HIF-1α and VEGF. NSCLC tumor models bearing classical or exon 20 *EGFR* mutations were more sensitive to VEGF inhibition than *EGFR* wild-type tumors, and combination

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of VEGF and EGFR inhibition delayed tumor progression. In models of acquired EGFR inhibitor resistance, while VEGF remained overexpressed, the hypoxia-independent expression of HIF-1α was delinked from EGFR signaling, and EGFR inhibition no longer diminished HIF-1α or VEGF expression.

Conclusions: In *EGFR* mutant NSCLC, EGFR signaling is the dominant regulator of HIF-1α and VEGF in a hypoxia-independent manner, hijacking an important cellular response regulating tumor aggressiveness. Cells with acquired EGFR inhibitor resistance retained elevated expression of HIF-1α/VEGF, and the pathways was no longer EGFR-regulated. This supports VEGF targeting in *EGFR* mutant tumors in the EGFR inhibitor naïve and refractory settings.

Keywords

Non-small cell lung cancer; epidermal growth factor receptor; hypoxia-inducible factor; vascular endothelial growth factor

Introduction

Hypoxia-inducible factor-1 (HIF-1) is a master regulator of the hypoxic transcriptional response and is composed of α and β-subunits. The β-subunit is constitutively expressed, while the α-subunit is regulated primarily by oxygen-dependent proteasome degradation, which is hindered under hypoxic conditions[1]. Several oxygen-independent mechanisms have also been shown to regulate HIF expression[1]. For instance, HIF-1α is transcriptionally upregulated in a cell type–specific manner by various growth factors and receptor tyrosine kinases (RTKs), including EGF, VEGFR and RET[1, 2]. EGFR has been implicated as a hypoxia-independent driver of HIF expression as inhibition of EGFR activation has been shown to reduce HIF protein translation in some cells[3]. We previously reported that expression of *EGFR* mutations is associated with increased HIF-1α levels in NSCLC and NIH-3T3 cells even under normoxic conditions, which implies that cells harboring *EGFR* activating mutations may have distinct regulation of HIF-1α expression[4].

HIF-1α drives the transcription of genes involved in glycolysis and angiogenesis. *VEGF*, a HIF-1α target gene, is modulated by activation of several RTKs, and EGFR inhibition has been shown to decrease VEGF expression in many tumor types[5–7]. The molecular mechanisms underlying the link between EGFR signaling and VEGF expression include both HIF-1–dependent and –independent mechanisms[3, 8]. The effects of *EGFR*-activating mutations leading to altered regulation of HIF may play a critical role in the cross-talk between EGFR downstream signaling and effects on angiogenesis and tumor growth. Therefore, we hypothesized that regulation of VEGF and HIF-1α may be distinct in *EGFR* mutant NSCLC tumor cells, and VEGF blockade may be particularly effective in *EGFR* mutant tumors.

We examined the relationship between *EGFR* activating mutations and HIF-1α in NSCLC cells. Our data revealed that *EGFR* mutant tumors are highly dependent on VEGF, and in tumor cells with *EGFR* mutations, EGFR is the predominant regulator of HIF expression and generates a hypoxic gene signature in normoxia. Moreover, in *EGFR* mutant NSCLC cells with acquired resistance to EGFR TKIs, HIF-1α expression becomes disassociated

from EGFR signaling. These findings offer insight into the mechanism by which *EGFR* activating mutations promote tumor angiogenesis and aggressiveness in NSCLC and provide a mechanism for the clinical observations indicating that VEGF blockade may enhance the efficacy of EGFR TKIs.

Materials and Methods

Cell lines and reagents.

H3255, H1975, H1993, and HCC827 cells were obtained from Drs. Minna and Gazdar (UT Southwestern Medical School, Dallas, TX, USA). A549, H1299, H1650, H23, and Calu-6 cells were obtained from ATCC. Ba/F3 cells were obtained from Creative-Biogene. YUL-0019 cells were obtained from Dr. Politi (Yale Medical School)[9]. VEGF and HIF-1α ELISAs were obtained from R&D systems. Plasmids used for for promoter assays included pGL2-VEGF-luciferase, pRL-TK (Promega), empty vector, a WT EGFR, or EGFR E746_A750del (a gift from Dr. Kurie, MD Anderson Cancer Center)[10]. Detailed methods for generation of resistant cell lines, real-time PCR, measurement of HIF-1α half-life,Western blotting, immunohistochemistry (IHC), promoter assays and ELISA assays are listed in Supplementary Material and Methods.

Microarray analysis of NSCLC cell lines.

Two microarray gene expression datasets were used.We analyzed gene expression microarray data from 53 NSCLC cell lines using the gene chip HG-U133A (Affymetrix, Santa Clara, CA) as previously described[11]. CEL type data files were downloaded from the publicly available NCBI-GEO datasets (www.ncbi.nlm.nih.gov/gds, GSE 4824). The dCHIP software package was used to analyze HIF-1α gene expression levels in cell lines expressing wild-type (n = 45) or mutant EGFR (n = 8). We also analyzed illumina microarray gene expression data from NSCLC cell lines (n=110) expressing wild-type EGFR or classical EGFR activating mutations (Illumina WG v2 and v3 platform) generated and processed by Dr. Minna at the University of Texas Southwestern Medical Center (Dallas, TX).

Gene set enrichment analysis.

Gene set enrichment analysis (GSEA) was performed using GSEA software v2.0.4 (www.broad.mit.edu/gsea/msigdb/msigdb_index.html)[12] using gene expression data publicly available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/gds, GSE 4824)and a hypoxia-related gene set containing 125 probesets from 69 individual genes[1]. From the downloaded CEL files from the 53 NSCLC cell lines already described, including eight with EGFR mutations, raw microarray data were processed using quartile normalization and the RMA algorithm[13].

Reverse phase protein array (RPPA).

RPPA slides were printed from NSCLC cell line lysates, and RPPA studies were performed and analyzed as previously described[14].

Mouse xenograft studies.

Male athymic nude mice (NCI-nu) were obtained from the National Cancer Institute (Frederick Cancer Center, Frederick, MD). Animal studies were conducted under an institutionally approved protocol in compliance with NIH guidelines. Details on xenograft experiments can be found in Supplementary Material and Methods.

Statistical analysis.

Two-tailed paired Student *t*-tests were used to detect differences in data generated from real-time PCR, ELISA assays, HIF protein half-life assays, luciferase assays, and mouse xenograft studies. P-values less than 0.05 were considered significant. Mantel-Cox Log-rank test was used to determine statistical differences and hazard ratios in PFS for PDX studies.

Results

NSCLC cells with *EGFR* activating mutations express higher levels of VEGF than *EGFR* wild-type cells

We evaluated *VEGF* gene expression in a panel of 110 NSCLC cell lines with or without *EGFR* activating mutations. *VEGF* RNA levels were significantly elevated in *EGFR* mutant NSCLC cell lines compared to *EGFR* wild-type cell lines ($p = 0.02$; Fig. 1A). There was no significant difference in the expression of angiogenic factors not regulated by HIF-1α including *FGF2*, *IL6*, *IL8* and *PDGFB* (Fig. 1 B–E) demonstrating that the elevated VEGF expression did not reflect a general upregulation of cytokines and angiogenic factors. Among *EGFR* wild-type cell lines, *VEGF* expression was not not significantly different between cells with or without *EGFR* copy number gains (Supplementary Fig. 1A). Together these data suggest that mutant but not wild-type *EGFR* increase VEGF expression.

EGFR regulates VEGF expression in *EGFR* mutant NSCLC cells

NSCLC cell lines were cultured in serum-free media with or without EGF, the EGFR TKI erlotinib, or EGF plus erlotinib, and VEGF was measured in the conditioned media by ELISA. NSCLC cells harboring *EGFR* activating mutations (HCC827, H3255, and H1975 cells) expressed higher baseline levels of VEGF than A549 cells (EGFR wild-type) (Fig. 1F). Treatment of erlotinib-sensitive HCC827 or H3255 cells with erlotinib for 24 hours significantly reduced VEGF to levels similar to A549 cells. Demonstrating pharmacologic specificity, we found erlotinib did not alter VEGF secretion in erlotinib-resistant H1975 cells which express both L858R and T790M mutations[15] (Fig. 1F). A549 cells, which express wild-type *EGFR*, exhibited the lowest level of VEGF secretion, and VEGF production was not altered by erlotinib. In all NSCLC lines tested, EGF treatment resulted in a slight increase in secreted VEGF, and the effects of EGF were blocked by erlotinib. Treatment of cells with the EGFR TKI osimertinib which has activity against *EGFR* T790M mutations significantly inhibited VEGF secretion by HCC827 and H1975 cells (Fig. 1G). Likewise, poziotinib-sensitive YUL-0019 cells[9], which harbor an *EGFR* exon 20 activating mutation (N771del insFH) were treated with EGF with or without poziotinib. Poziotinib significantly reduced VEGF secretion when used alone or in combination with EGF (Fig. 1H). To confirm that the observed effects of EGFR TKIs on VEGF secretion was not due to changes

in cell viability, we evaluated cell viability after 24 hour treatment with erlotinib, osimertinib or poziotinib. No change in cell viability was observed (Supplementary Fig. 1B–E). Collectively, these data demonstrate that in NSCLC cells with different activating *EGFR* mutations, VEGF secretion is EGFR-dependent.

To further investigate the role of *EGFR* activating mutations on VEGF expression, we analyzed transcriptional activation of the *VEGF* promoter. In HCC827 cells, erlotinib significantly reduced *VEGF* promoter activity (Fig. 1I), while *VEGF* promoter activity was unchanged in hypoxic conditions in HCC827 cells, indicating that *VEGF* promoter activity was predominantly regulated by EGFR signaling rather than hypoxia. In A549 cells, erlotinib had no effect on *VEGF* promoter activity, while hypoxia induced a 2.7-fold increase in *VEGF* promoter activity (Fig. 1I). We next transfected H1299 cells (EGFR wild-type) with wild-type or mutant *EGFR* (E746_A750del; del19) expression constructs, and found expression of mutant, but not wild-type, *EGFR* increased *VEGF* promoter activity (Fig. 1J). Likewise, expression of *EGFR* exon 19 deletion (Ex19del) in H23 cells (*EGFR* wild-type) and expression of *EGFR* Ex19del/T790M or the *EGFR* exon 20 insertion D770insSVD in Ba/F3 cells (EGFR negative) increased VEGF secretion (Supplementary Fig. S1F&G). Together these data indicate that NSCLC cells expressing mutant *EGFR* have diminished hypoxic regulation of VEGF and that mutant EGFR promotes VEGF expression.

Cells with *EGFR* activating mutations have increased HIF-1α expression

Cell lines expressing mutant *EGFR* including L858R and del19 mutations (n = 8) expressed 1.5 fold higher levels of *HIF-1α* mRNA than cell lines expressing wild-type *EGFR* (n = 45) (Fig. 2A). Furthermore, HIF-1α protein levels were higher in cell lines expressing mutant *EGFR* than in cell lines with wild-type *EGFR* (Fig. 2B). *HIF-1α* mRNA levels were not significantly different between cell lines expressing EGFR L858R mutation (n = 2) and cell lines expressing *EGFR* del19 mutation (n = 6).

NSCLC cells with mutant *EGFR* express increased levels of hypoxia-regulated genes

The elevated HIF-1α expression in *EGFR* mutant cells suggested that these cells might exhibit a hypoxic gene signature even in normoxic conditions. We performed GSEA on 53 NSCLC cell lines to determine whether cells with *EGFR* activating mutations were enriched for expression of hypoxia-regulated genes[12]. The probesets of hypoxia-inducible genes revealed a significant enrichment in cell lines expressing EGFR activating mutations (P <0.001, FDR q <0.001; Fig. 2C). We next examined genes specifically upregulated by HIF-1α[16]. *EGFR* mutant cell lines were significantly enriched for expression of HIF-1α-regulated genes (P = 0.037, FDR q < 0.06; Fig. 2D).

HIF-1α is predominantly regulated by EGFR, and not hypoxia, in *EGFR* mutant NSCLC cells

We next investigated whether the regulation of HIF-1α by EGFR impacted the normal hypoxia-induced regulation of HIF-1α. HCC827 cells or A549 cells were treated with erlotinib, EGF, or erlotinib plus EGF under normoxic or hypoxic conditions. HCC827 cells expressed high basal level of HIF-1α, which was only minimally increased by hypoxia (1.5-fold) or EGF (1.7-fold; Fig. 2E). Erlotinib completely diminished HIF-1α expression in HCC827 cells even in the presence of EGF and hypoxia, demonstrating a critical role for

EGFR signaling in the regulation of HIF-1α in EGFR mutant NSCLC cells. By contrast, A549 cells, expressed markedly lower basal levels of HIF-1α compared to HCC827 cells, and HIF-1α expression significantly increased with hypoxia (>5-fold) or EGF stimulation (4-fold; Fig. 2E). When A549 cells were cultured in hypoxia, erlotinib only partially reduced HIF-1α (Fig. 2E). The EGFR TKI osimertinib (200 nM) similarly diminished HIF-1α expression in HCC827 and H1975 cells (Fig. 2F). *EGFR* exon 20 mutant YUL-0019 cells expressed HIF-1α in normoxia, and the addition of poziotinib significantly decreased HIF-1α levels (Fig. 2G), and EGF stimulated an increase in HIF-1α. Hypoxia treatment did not increase HIF-1α levels, but even in hypoxia, poziotinib diminished HIF-1α expression (Fig. 2G). To further confirm these findings, we evaluated protein levels of two hypoxia-regulated genes (hexokinase II and SLC2A1) included in the hypoxia-regulated geneset. Erlotinib decreased EGFR phosphorylation, hexokinase II and SLC2A1 expression in HCC827 cells (Supplementary Fig. S2A). These data indicate that in NSCLC cells harboring *EGFR* activating mutations, HIF-1α expression is predominantly regulated by EGFR signaling and the response to hypoxia is blunted, whereas in NSCLC cells expressing wild-type *EGFR*, HIF-1α expression is predominantly regulated by hypoxia.

Mutant *EGFR* regulates HIF-1α transcription and protein stability in NSCLC cells

To determine whether mutant *EGFR* modulates HIF-1α at the mRNA level, *HIF-1α* transcripts were analyzed by real-time PCR. In HCC827 cells, erlotinib treatment resulted in a 32% ($P = 0.019$) reduction in *HIF-1α* mRNA compared to control-treated cells (Fig. 2H). To determine whether EGFR regulates the protein stability of HIF-1α in NSCLC cells with *EGFR* activating mutations, we treated HCC827 cells with or without erlotinib in the presence of the protein biosynthesis inhibitor, cycloheximide, and evaluated the effect on the half-life of HIF-1α protein. In the absence of erlotinib, HIF-1α protein demonstrated a half-life of greater than 55 minutes (Fig. 2I&J). In the presence of erlotinib, the half-life of HIF-1α was reduced to approximately 20 minutes (Fig. 2I&J). The erlotinib-induced decrease in HIF-1α protein levels was blocked by the proteosome inhibitor MG132 (Supplementary Fig. S2B). Thus, EGFR inhibition increased loss of expression of HIF-1α protein in a proteosome-dependent manner. These data demonstrate that mutant *EGFR* regulates HIF-1α expression both at the mRNA level and through increased protein stability.

Cells expressing *EGFR* activating mutations have elevated expression of CA9 *in vivo*

Carbonic anhydrase 9 (CA9, CA IX) is a HIF-1α–regulated protein used as a marker of hypoxia in clinical and xenograft specimens[17]. We evaluated whether xenografts harboring classical or atypical mutant *EGFR* express higher levels of CA9 protein than xenografts with wild-type *EGFR*. We found that intra-tumoral levels of hypoxia, as quantitated by pimonidazole staining, were similar between xenografts expressing wild-type *EGFR* or mutant *EGFR* (Fig. 3A). Despite the similar levels of hypoxia, both *EGFR* mutant xenografts expressed higher levels of CA9 than *EGFR* wild-type xenografts (20–80% vs. 0–20%, Fig. 3A&B), indicating increased expression of HIF-1α target genes independent of intra-tumoral hypoxia. These data confirmed our *in vitro* findings and further indicate that NSCLC tumors expressing *EGFR* activating mutations upregulate HIF-1α target genes including VEGF and CA9 and that regulation of these genes is uncoupled from the hypoxia, the canonical regulator of this pathway.

Xenografts expressing mutant EGFR display increased sensitivity to anti-VEGF therapy

We next assessed whether *EGFR* mutant tumors display a greater sensitivity to the VEGF inhibitor, bevacizumab, than *EGFR* wild-type tumors. HCC827 or A549 cells were implanted in nude mice. Bevacizumab treatment began when tumors reached 300 mm^3. Growth of A549 tumors was modestly inhibited by bevacizumab (Fig. 3C). However, bevacizumab completely suppressed the growth of HCC827 tumors (Fig. 3D). At day 14, wild-type *EGFR* tumor growth was reduced by 30%, while *EGFR* mutant tumor growth was reduced by 70% (Fig. 3E). Next, we evaluated the anti-tumor activity of bevacizumab in NSCLC *EGFR* exon 20 mutant PDX models. In PDXs harboring *EGFR* H773insNPH, bevacizumab or poziotinib significantly impaired the growth of EGFR H773insNPH tumors (Log Rank (Mantel-Cox) p = 0.0004 and 0.0008; Fig. 3F–G). The combination of bevacizumab plus poziotinib resulted in near complete tumor regression (Fig. 3F) and significantly prolonged progression free survival (PFS) compared to vehicle or either agent alone (PFS; p = <0.0001; Fig. 3G). Similar results were obtained using an additional *EGFR* exon 20 mutant PDX model. Bevacizumab alone induced a modest but significant improvement in PFS (Log Rank (Mantel-Cox) p = 0.0001) and the combination of bevacizumab plus poziotinib significantly improved the efficacy of poziotinib treatment (p = 0.0107; Fig. 3H). We analyzed RNA from tumors harvested at the end of the experiment (resistance) by qRT-PCTR. In *EGFR* H773insNPH expressing tumors, treatment with bevacizumab caused a modest increase in *CA9* and *VEGF* (Supplementary Fig. 3A&B). Poziotinib, alone or in combination with bevacizumab, caused a significant decrease in *CA9* and *VEGF* RNA levels. In the EGFR S768dupSVD model, the combination of poziotinib and bevacizumab resulted in reduced *CA9* RNA levels (Supplementary Fig. 3C&D). In light of our *in vivo* findings, we assessed whether hypoxia increases tumor cell sensitivity to poziotinib. Poziotinib sensitivity was similar between YUL-0019 cells grown in normoxia or hypoxia (Supplementary Fig. 3E). Collectively, these data suggest that tumor cells expressing mutant *EGFR* are highly sensitive to VEGF inhibition and VEGF blockade may improve the efficacy of EGFR TKIs in this patient population

HIF-1α and VEGF are uncoupled from EGFR in cells with acquired EGFR TKI resistance

While patients with EGFR activating mutations are initially sensitive to EGFR inhibition, resistance inevitably occurs and often times is EGFR-independent[15, 18, 19]. We generated EGFR TKI resistant cells by culturing HCC827 and HCC4006 cells in erlotinib or YUL-0019 cells in poziotinib until erlotinib-resistant (ER) and poziotinib resistant (PR) clones developed. HCC827-ER and HCC4006-ER cells were negative for secondary T790M *EGFR* mutations[20]. EGFR inhibitor treatment reduced EGFR phosphorylation in HCC827, HCC4006, and YUL-0019 parental cells and ER and PR cell lines (Fig. 4A–C). Under normoxic conditions, HCC827-ER HCC4006-ER, and YUL-0019-PR cells expressed high levels of HIF-1α similar to that observed in parental cells (Fig. 4D–E). While EGFR inhibitor treatment significantly reduced HIF-1α levels in HCC827, HCC4006, and YUL-0019 parental cells, EGFR inhibition did not alter HIF-1α levels in HCC827-ER, HCC4006-ER, or YUL-0019-PR cell lines (Fig. 4D–F). In HCC827, HCC4006, and YUL-0019 parental cells EGFR-TKI treatment resulted in a significant reduction in VEGF production (Fig. 4G–I). In contrast, EGFR-TKI treatment did not decreased VEGF levels in any of the ER or PR variants and in fact a trend towards increased VEGF was observed after

erlotinib treatment in the HCC4006-ER lines. In the majority of EGFR TKI cell lines, exposure to hypoxia did not significantly increase HIF-1α protein levels (Supplementary Fig. 4). These data indicate that in NSCLC with acquired EGFR-independent EGFR TKI resistance, HIF-1α expression remains independent of oxygen concentration but de-linked from EGFR signaling.

VEGFR inhibitors do not have direct activity against EGFR TKI resistant cells

We next assessed whether VEGFR inhibitors have direct anti-tumor cell activity on EGFR TKI refractory cell lines. VEGFR2 levels were not altered between parental and EGFR TKI resisitant cells (Supplementary Fig. 5A). We screened parental and resistant variants against bevacizumab and the VEGFR TKIs cederinib, pazopanib, and sorafenib *in vitro* and observed that both parental and resistant cells were resistant to VEGFR2 inhibition (Supplementary Fig. 5B–E).

Discussion

These data offer insight into the mechanism by which *EGFR* activating mutations promote tumor angiogenesis and aggressiveness in NSCLC. Our findings indicate that *EGFR* mutant NSCLC cells display a VEGF-dependent phenotype and that in *EGFR*-mutant tumor cells, EGFR, but not hypoxia, is the predominant regulator of HIF-1α expression, whereas in *EGFR* wild-type NSCLC hypoxia is the primary regulator of HIF-1α. Moreover, in cells with acquired EGFR-independent EGFR TKI resistance, regulation of HIF-1α becomes re-wired and dissociated from EGFR signaling. These findings indicate that patients with *EGFR* mutations may represent a highly VEGF-dependent subgroup that may benefit from combined EGFR and VEGF blockade.

Uncoupling of HIF expression and hypoxia in cells expressing mutant *EGFR* demonstrates an important mechanism by which cells can upregulate HIF and its target genes in the absence of hypoxia. This aberrant upregulation can trigger an overall hypoxic gene signature within the tumor microenvironment creating pseudohypoxia, which has been proposed as a mechanism of EGFR inhibitor resistance[21]. Hypoxia has been shown to lead to more aggressive tumors through a number of biological mechanisms and is a principal regulator of tumor angiogenesis. Hypoxia has also been shown to contribute to therapeutic resistance[22–24] and enhanced invasion and metastases[24, 25]. We have previously demonstrated a role for HIF-1α in the upregulation of c-Met[4], which when activated leads to increased invasiveness in *EGFR* mutant NSCLC[26, 27]. Therefore, the presence of *EGFR* activating mutations would give cells a “head start” in hypoxia-induced tumor aggressiveness and therapeutic resistance.

While ligand-induced activation of EGFR has been shown to modulate HIF-1α levels[1], our finding that *EGFR* activating mutations regulate HIF-1α in a manner that is dominant over hypoxia-controlled HIF expression has important biological implications. In tumors driven primarily by the EGFR pathway, targeting HIF or key HIF-regulated genes (i.e. VEGF) may further inhibit tumor growth and invasiveness beyond the effect of EGFR inhibition alone, and delay drug tolerance. Moreover, VEGFR and EGFR activate common downstream signaling pathways including the PI3K/Akt and MAPK pathways in endothelial and tumor

cells expressing VEGF receptors (VEGFR). Therefore, VEGF overexpression may further drive signal bypass-mediated resistance to EGFR TKIs, which is supported by preclinical findings that VEGF expression is further elevated *in vivo* following EGFR TKI resistance[28] and that blockade of both VEGFR2 and EGFR has greater anti-tumor activity than the respective monotherapies[29]. While the data presented here using preclinical models indicates that EGFR activating mutations promote increased levels of tumor-derived VEGF, the study is limited by the fact that it does not compare tumor VEGF levels in EGFR wild-type and mutant NSCLC clinical specimens. The comparison of VEGF levels is challenging as publicly available datasets are not controlled for tumor size which would impact tumor hypoxia and VEGF levels.

To date, several clinical studies have provided evidence supporting the dual targeting of EGFR and VEGFR pathways. In an exploratory analysis from the double-blinded, placebo-controlled Phase 3 BeTa trial, it was observed that that patients with EGFR mutant, but not EGFR wild-type, tumors demonstrated a trend towards benefit from the addition of erlotinib to bevacizumab (overall survival hazard ratio 0.44 for EGFR mutant vs 1.11 for EGFR wild-type)[30]. Further clinical evidence is provided in clinical study J025567 where the addition of bevacizumab with erlotinib significantly improved progression free survival (PFS) in NSCLC patients harboring *EGFR* mutations[31]. A subgroup analysis included in the phase 3 maintenance study, ATLAS, revealed that patients with *EGFR* mutations had an improved PFS and overall survival (OS) when treated with bevacizumab plus erlotinib[32]. Moreover, in the RELAY study (NCT02411448) the VEGFR2 antagonist, ramucirumab, plus erlotinib yielded a significantly improved PFS compared to erlotinib treatment alone in metastatic EGFR mutant NSCLC[33]. Our preclinical data indicates that *EGFR* mutant tumors have a VEGF-dependent phenotype and that VEGF is regulated by EGFR in *EGFR*-mutant NSCLC, providing a mechanism for the clinical observations that *EGFR* mutant patients benefit from VEGF blockade.

While studies of combination EGFR and VEGF inhibition have yielded encouraging results in patients with classical mutations, no clinical studies of combination EGFR and VEGF inhibition have been completed with patients harboring exon 20 mutations due to a lack of an effective EGFR TKI for this subpopulation. Our data indicate that not only would patients with classical *EGFR* mutations receive clinical benefit from the addition of VEGF inhibition, but that also patients with EGFR exon 20 insertion may benefit with the addition of up-front VEGF inhibition in combination with an EGFR TKI.

In models of acquired EGFR TKI resistance, we observed that the hypoxia-independent expression of HIF-1α was re-wired and no longer EGFR-dependent. The finding that all resistant cells tested retained elevated expression of HIF-1α and VEGF supports the notion that the HIF-1α/VEGF axis is an important pathway in *EGFR* mutant NSCLC and further suggests that targeting VEGF may be clinically useful in delaying the emergence of EGFR TKI resistance. It is worth noting, however, that the specific role that VEGF plays in these tumors is not definitively addressed in this study. We do not see evidence that the survival or growth of *EGFR* mutant tumor cells are directly impacted by VEGF pathway inhibition in a cell autonmomous manner (Supplementary Fig. 5B–E), and we previously observed that tumor expression of VEGFR-2 (KDR) was not a predictor of benefit from vandetinib[34],

providing evidence that the primary effect of VEGF is unlikely to be directly on the tumor cells. We and others have reported that bevacizumab[28, 35] and other VEGFR TKIs[36] do cause a marked reduction in tumor microvessel density and alterations in tumor vessel tortuosity in human EGFR mutant xenografts *in vivo*, suggesting that the effects are likely due, at least in part, to effects on tumor vasculature. These preclinical models conducted in immunodeficient models, and it is possible that VEGF may exert additional immunomodulatory effects not captured in these preclinical models.

Collectively, our results indicate that in *EGFR* mutant NSCLC, *EGFR* is the predominant regulator of VEGF and HIF-1α independent of hypoxia and EGF ligand binding, likely contributing to the enhanced sensitivity to VEGF inhibitors observed clinically, while hypoxia is the primary driver of HIF-1 α in the *EGFR* wild-type NSCLC models studied. Mutant *EGFR*–mediated HIF-1α upregulation is initially completely attenuated by inhibition of EGFR, raising the possibility that the high efficacy of EGFR TKIs in *EGFR* mutant NSCLC may be due not only to the blockade of EGFR-driven survival pathways, but also to their effects on abolishing HIF-1α and HIF-1α-mediated VEGF expression. These data suggest that *EGFR* mutant NSCLC patients may receive greater benefit for VEGF blockade than patients with wild-type *EGFR* and supports the clinical testing and use of VEGF inhibitors in combination with EGFR TKIs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interests/Disclosures:

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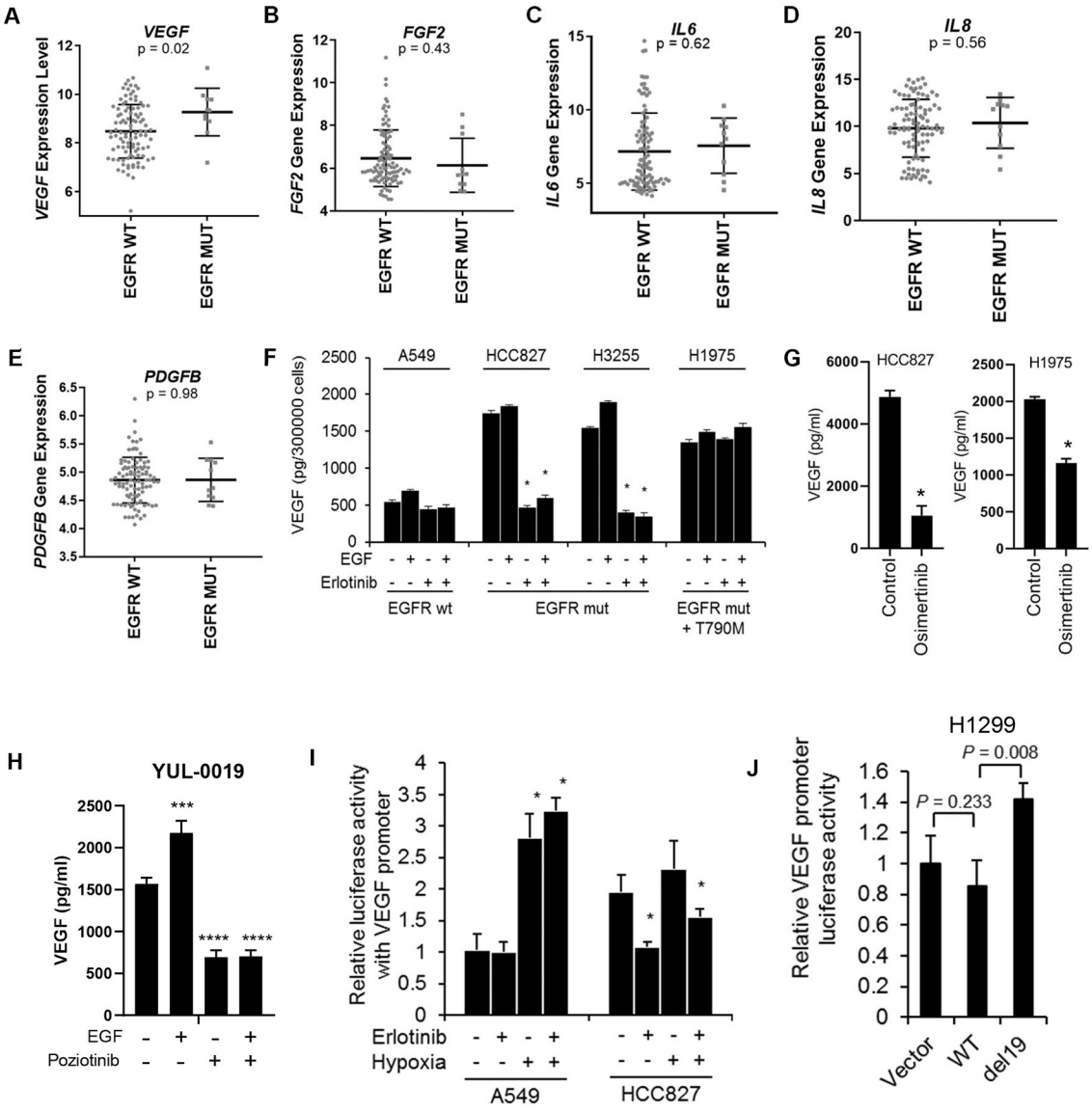

Fig 1. In NSCLC cells with *EGFR* activating mutations, VEGF is elevated and regulated by EGFR.

(**A-E**) Gene expression of *VEGF* (**A**), *FGF2* (**B**), *IL6* (**C**), *IL8* (**D**), and *PDGFB* (**E**) in a panel of 110 NSCLC cell lines with or without EGFR activating mutations. (**F**) VEGF secretion as determined by ELISA in *EGFR* mutant (mut) or wild-type (wt) NSCLC cells treated with EGF or erlotinib. Bars are mean ± SEM; *p<0.05. (**G**) VEGF production following osimertinib treatment as determined by ELISA. *p<0.01. (**H**) VEGF secretion as determined by ELISA in YUL-0019 EGFR exon 20 mutant NSCLC cells treated with EGF or poziotinib. ***p = 0.0002; ****p <0.0001. (**I**) *VEGF* promoter activity was inhibited by erlotinib in cells with mutant *EGFR* (HCC827) but not in cells with wild-type *EGFR* (A549) in normoxic conditions. Cells transfected with a *VEGF*-luciferase construct were treated with or without erlotinib in normoxic or hypoxic conditions (1% O_2). *p <0.05. (**J**) Expression of mutant *EGFR* (del19) increased *VEGF* promoter activity in H1299 cells. WT: wild-type *EGFR*; del19: del19 mutant *EGFR*. Data in panels A–J are shown as mean ± SD.

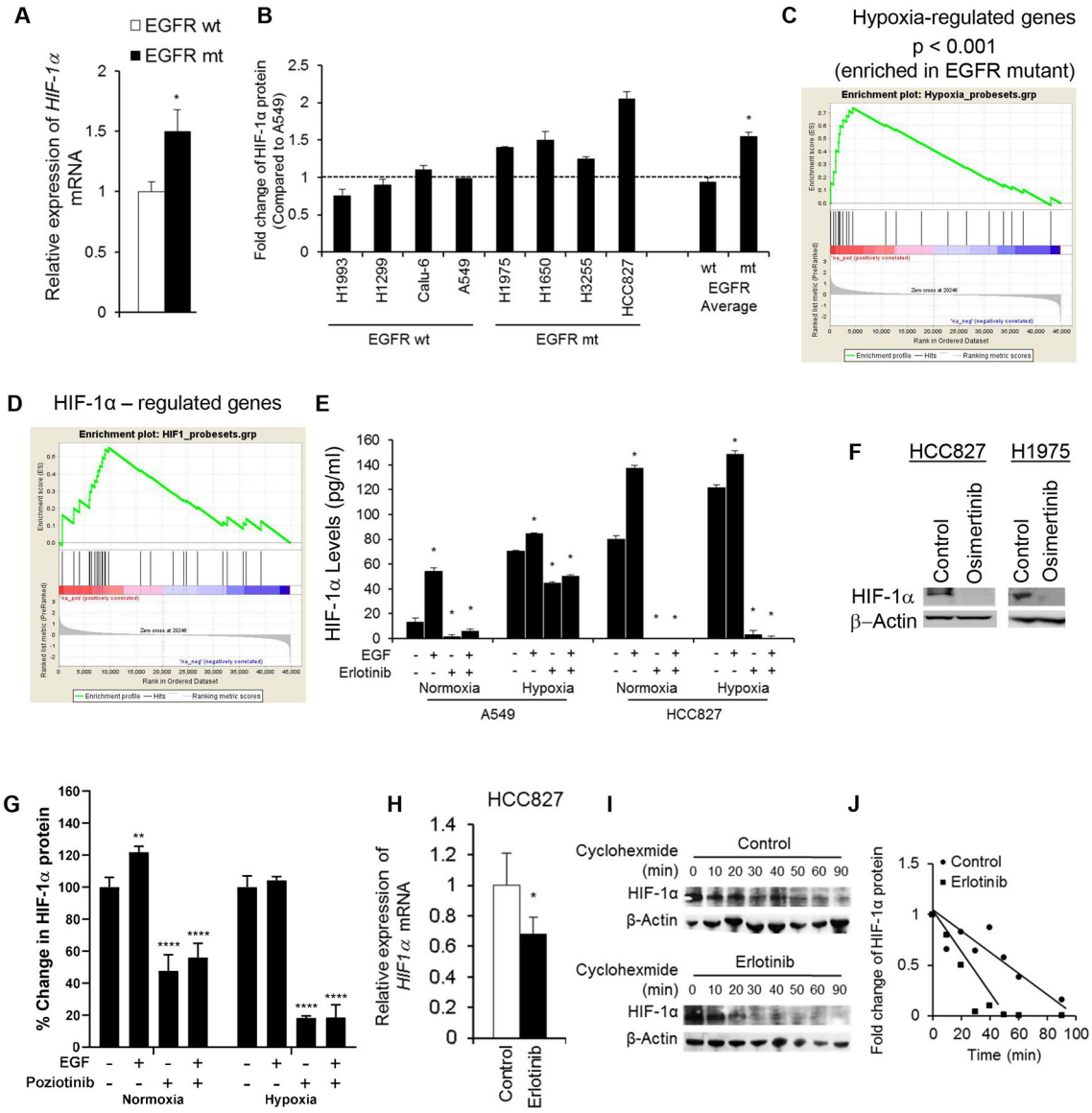

Fig 2. HIF-1α expression is elevated in *EGFR* mutant NSCLC cells and is dependent on EGFR.

(**A**) Fifty-three *EGFR* wild-type (wt) or mutant (mt) NSCLC cell lines were analyzed by Affymetrix genechip analysis. Mean expression levels of *HIF-1α* mRNA are shown. * p <0.05. (**B**) HIF-1α expression as determined by ELISA in whole cell lysates from NSCLC cell lines grown under normoxic conditions. *p < 0.05. (**C & D**) GSEA analysis revealed that NSCLC cells with *EGFR* activating mutations displayed enriched expression of hypoxia- responsive genes and HIF-1α-regulated genes. (**E**) HIF-1α protein expression as measured by ELISA in A549 and HCC827 cells treated with erlotinib (1 μmol/L) and/or EGF (60 ng/mL) for 24 hours in normoxic or hypoxic conditions. *p <0.05 vs untreated control. (**F**) HIF-1α expression as determined by Western blotting after treatment with 200 nM osimertinib. (**G**) HIF-1α protein expression as measured by ELISA in YUL-0019 cells treated with EGF (60 ng/ml) or poziotinib (0.1 μmol/L). **p <0.0098, ***p <0.0001 vs untreated control. (**H**) *HIF-1α* mRNA expression was measured by real-time PCR in

HCC827 cells treated with or without 1 μmol/L erlotinib in normoxic conditions. * $p < 0.05$. (**I**) HIF-1α protein expression was analyzed by Western blotting in HCC827 cells treated with cycloheximide. One representative blot of three is shown. (**J**) Densitometry of HIF-1α expression in (**I**) was plotted to determine its half-life. Data in panels B, E, G, H are shown as mean ± SD.

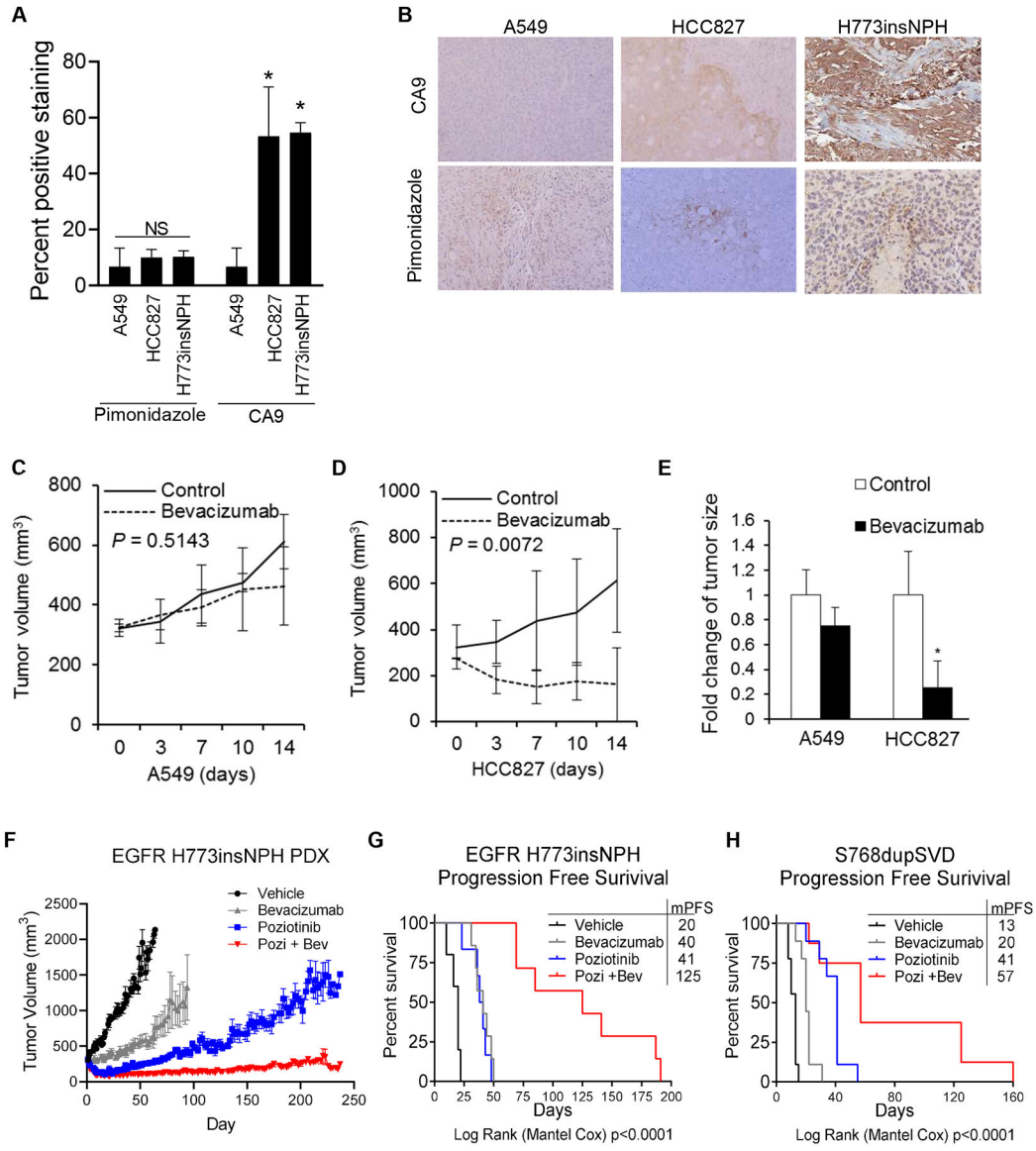

Fig. 3. *EGFR* mutant xenografts have elevated expression of HIF-1α target genes and are sensitive to VEGF inhibition.

(**A**) HCC827 xenografts and H773insNPH EGFR exon 20 PDX tumors display elevated CA9 expression compared to A549 xenografts, although pimonidazole staining was similar. *p <0.05. (**B**) Representative CA9 immunohistochemical staining at 100× magnification. (**C&D**) Tumor growth curves for A549 (**C**) or HCC827 (**D**) tumor-bearing animals treated with bevacizumab (10 mg/kg) or vehicle for 14 days. (E) Fold-changes in size of A549 and HCC827-derived xenografts, compared to controls, at the end of bevacizumab treatment. *p <0.05. (**F**) Tumor growth curve of NSCLC PDX harboring EGFR H773insNPH mutation treated with vehicle (black), bevacizumab (5mg/kg twice per week, grey), poziotinib (2 mg/kg five times per week, blue) or combination of bevacizumab and poziotinib (red) for 225 days. Tumor size was measured three to five times per week. (**G-H**) Kaplan-Meier curves of PFS for PDX models expressing (**G**) EGFR H773insNPH or (**H**) EGFR S768dupSVD treated with vehicle, bevacizumab (5 mg/kg twice per week), poziotinib (2

mg/kg five times per week) or combination of bevacizumab and poziotinib. Progression was defined as time until tumor doubling from best response. Log-rank (Mantel Cox) was used to determine statistical differences between groups. In panels A, C, D, E, F data are shown as mean ± SEM.

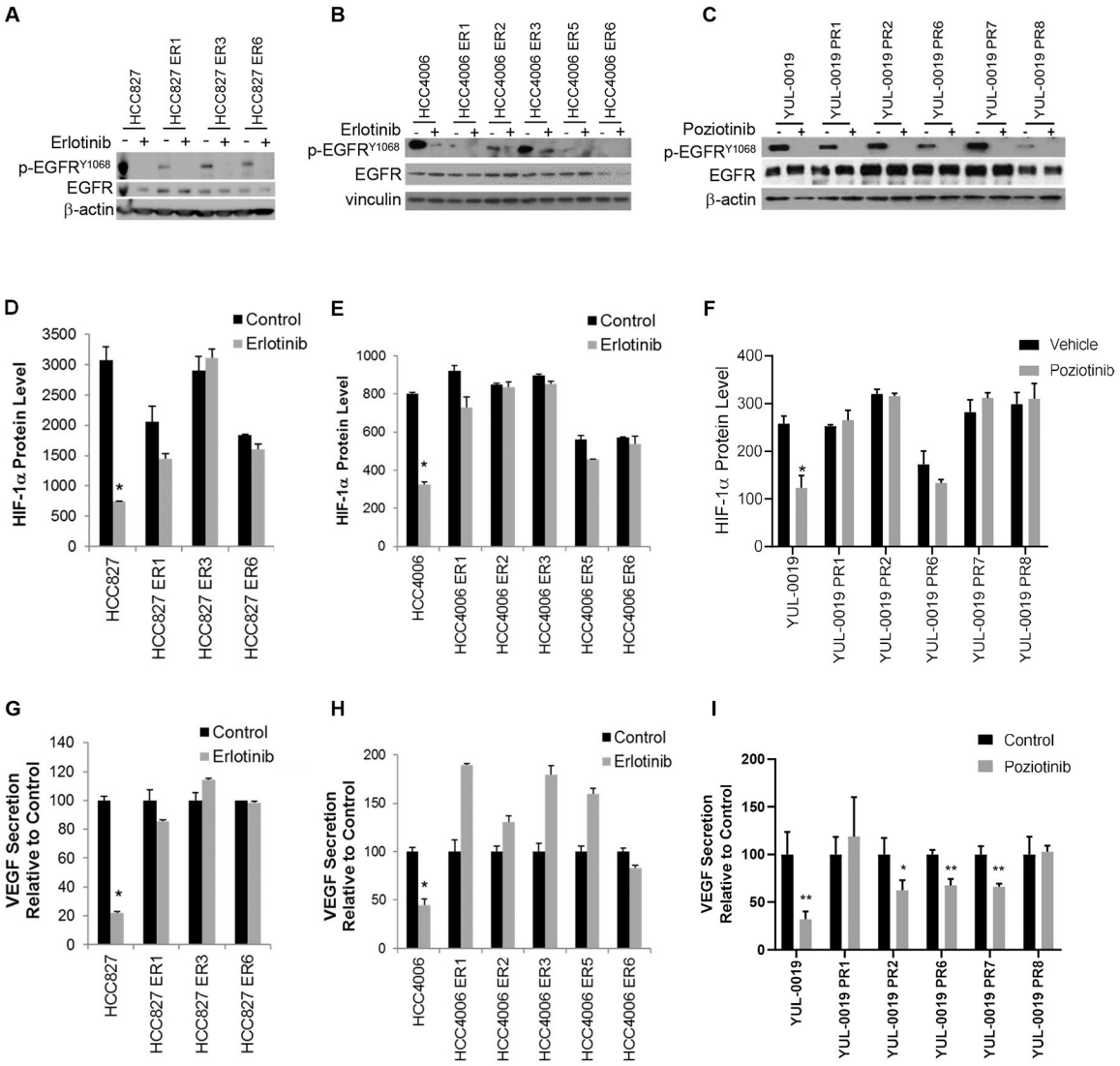

Fig 4. HIF-1α expression is uncoupled from EGFR in NSCLC cells with acquired resistance to EGFR TKIs.

(**A&B**) Erlotinib (1 μM) decreased protein levels of pEGFR in HCC827 and HCC827 ER cells and HCC4006 and HCC4006 ER cells as determined by Western blotting. (**C**) Poziotinib (1 μM) decreased protein levels of pEGFR in YUL-0019 and YUL-0019 PR cells as determined by Western blotting. (**D&E**) Effect of erlotinib (1μM) on HIF-1α protein levels in HCC827 and HCC4006 parental and ER cells as determined by ELISA. *$p < 0.05$ versus control. (**F**) Effect of poziotinib (1μM) on HIF-1α protein levels as determined by ELISA. *$p = 0.002$ versus control. (**G&H**), Effect of erlotinib (1 μM) on VEGF secretion by HCC827 and HCC4006 ER cells as determined by ELISA. *$p < 0.05$ versus control. (**I**) Effect of poziotinib (1 μM) on VEGF secretion by YUL-0019 and YUL-0019 PR cells as determined by ELISA. *$p = 0.03$, **$p < 0.01$ versus control. Data in panels D-I are shown as mean ± SD.

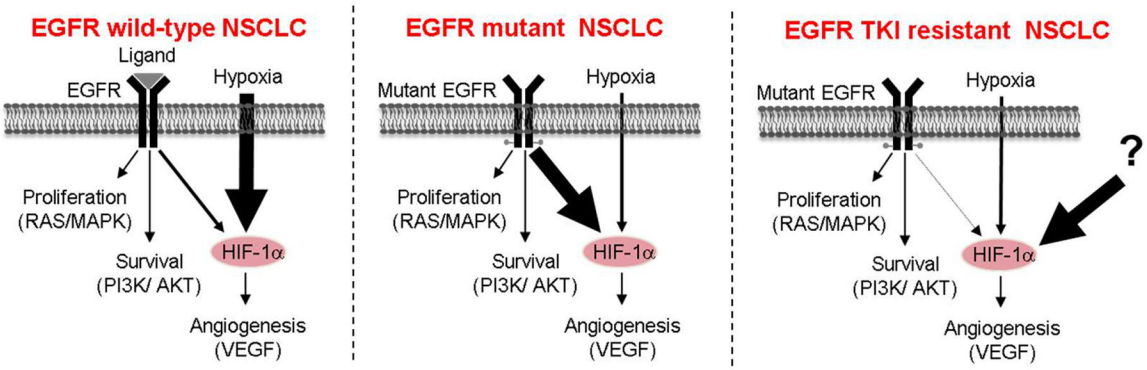

Fig. 5.

In NSCLC cells with EGFR activating mutations, mutant EGFR is the predominant regulator of HIF-1α, while hypoxia is the major regulator of HIF-1α in cells expressing wild-type EGFR. NCLC cells with acquired resistance to EGFR TKIs become rewired and HIF-1α expression is no longer coupled to EGFR signaling.