ALK-Dependent Control of Hypoxia-Inducible Factors Mediates Tumor Growth and Metastasis

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Abstract

Rearrangements involving the anaplastic lymphoma kinase (ALK) gene are defining events in several tumors, including anaplastic large-cell lymphoma (ALCL) and non–small cell lung carcinoma (NSCLC). In such cancers, the oncogenic activity of ALK stimulates signaling pathways that induce cell transformation and promote tumor growth. In search for common pathways activated by oncogenic ALK across different tumors, we found that hypoxia pathways were significantly enriched in ALK-rearranged ALCL and NSCLC, as compared with other types of T-cell lymphoma or EGFR- and K-RAS-mutated NSCLC, respectively. Consistently, in both ALCL and NSCLC, we found that under hypoxic conditions, ALK directly regulated the abundance of hypoxia-inducible factors (HIF), which are key players of the hypoxia response in normal tissues and cancers. In ALCL, the upregulation of HIF1α and HIF2α in hypoxic conditions required ALK activity and its downstream signaling proteins STAT3 and C/EBPβ. In vivo, ALK regulated VEGFA production and tumor angiogenesis in ALCL and NSCLC, and the treatment with the anti-VEGFA antibody bevacizumab strongly impaired ALCL growth in mouse xenografts. Finally, HIF2α, but not HIF1α, was required for ALCL growth in vivo whereas the growth and metastasis potential of ALK-rearranged NSCLC required both HIF1α and HIF2α. In conclusion, we uncovered an ALK-specific regulation of the hypoxia response across different ALK+ tumor types and propose HIFs as a powerful specific therapeutic target in ALK-rearranged ALCL and NSCLC. Cancer Res; 74(21); 1–13. ©2014 AACR.

Introduction

The anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase involved in chromosomal rearrangements in anaplastic large-cell lymphoma (ALCL), non–small cell lung carcinoma (NSCLC; ref. 1), and other solid cancers (2). Frequently, in ALCL, the Nucleophosmin 1 (NPM1) gene and ALK generate the NPM–ALK fusion, but more than 20 other partners of ALK have been cloned in ALCL (2). In NSCLC, ALK rearrangements occur in 6% to 7% of the cases and mainly involve the echinoderm microtubule–associated protein-like 4 (EML4) gene as a partner (1) but additional ALK rearrangements have also been described previously (3, 4).

The cell-transforming potential of ALK in tumors largely depends on its deregulated tyrosine kinase activity that results from spontaneous dimerization (2). Several downstream pathways are activated by NPM–ALK, with a broad range of signals that lead to increased cell proliferation, survival, motility, and cytoskeletal rearrangements (2, 5). In NSCLC, conversely, downstream pathways are less extensively characterized (6).

Early clinical trials have shown that ALK-rearranged tumors respond quite dramatically to the inhibition of the ALK activity by specific inhibitors such as crizotinib (5). High rate of responses were observed in patients with ALK-rearranged NSCLC (7) and ALCL (8). Unfortunately, the benefit of ALK inhibition is only transient because tumors relapse almost invariably in patients due to the development of resistance to crizotinib (9, 10). Therefore, novel therapeutic targets are needed for potential combination therapies with ALK inhibitors.

Our group and others previously described a link between ALK and angiogenesis in lymphoma (11) and neuroblastoma...
(12). In ALCL, oncogenic ALK was shown to control VEGF expression in vitro in murine cells and human ALCL (11, 13). Furthermore, ALK-dependent regulation of platelet-derived growth factor receptor-β (PDGFRβ) could contribute to tumor angiogenesis in a mouse model of ALK-rearranged lymphoma (14). In neuroblastoma, ALK-regulated activity controlled both tumor growth and tumor vessel formation (12).

Tumor angiogenesis provides oxygen and nutrients to the growing mass (15), and hypoxic conditions are critical to stimulate new vessel formation in tumors (16). The regulators of hypoxic response in the cells are transcription factors called hypoxia-inducible factors (HIF). HIFs are heterodimers composed of an O2-labile α subunit (HIF1α, HIF2α, and the less characterized HIF3α) and a stable β subunit (HIF1β). Whereas HIF1α is ubiquitously expressed, HIF2α and HIF3α have a more restricted pattern of expression, including vascular endothelial cells and type II pneumocytes (17). The role of HIFs in different cancer types is variable and sometimes contradictory, sharing redundant functions but also opposing effects on angiogenesis, metabolism, and other processes that can influence tumor growth (18).

In the present study, we show that the hypoxia response is specifically enriched in a large series of human ALK-rearranged lymphoma and NSCLC cases, and we provide evidence that ALK specifically regulates HIF1α and HIF2α expression under hypoxic conditions in both ALCL and NSCLC. In vivo, ALK activity induced VEGF production and tumor angiogenesis and we demonstrated that HIF2α, but not HIF1α, was required for ALCL growth, whereas both HIF1α and HIF2α were essential for NSCLC growth and metastasis. Thus, we have identified an ALK-dependent hypoxic response shared by ALK-rearranged ALCL and NSCLC, and suggest VEGFA and HIFα proteins as powerful therapeutic targets for ALK-rearranged tumors.

Materials and Methods

Gene-expression profiling and GSEA

Gene-expression profiling (GEP) data from T-cell lymphomas were generated by our group (19). Expression data for NSCLC are publicly available (20, 21).

Cell lines and reagents

ALK-rearranged (TS, SU-DHL1, JB6, and Karpas-299) and ALK− (CEM, FePD, JURKAT, and MAC-1) ALCL cell lines were obtained from the DSMZ (German collection of Microorganisms and Cell Cultures) collection and were previously described (22). Methods of characterization from the cell bank include karyotyping and DNA fingerprinting. Cells were passaged for fewer than 6 months after receipt and resuscitation. Cells were internally tested by ALK and p53 mutational status within 6 months after receipt and resuscitation. EML4–ALK-rearranged H228 (variant 2, EML4/ex6–ALK/ex20) and H3122 (variant 1, EML4/ex13–ALK/ex20), B-RAF–mutated H1395 (p.G469A), EGFR-mutated H1975 (p.L858R, T790M) and H325 (p. L858R), and K-RAS–mutated A549 (p.G12S), H441 (p.G12V), and H460 (p.Q61H) lung carcinoma cell lines were obtained from the ATCC and DSMZ collections and were passaged for fewer than 6 months after receipt and resuscitation. These cell lines were further internally tested for the mutational status of their respective characterizing oncogene mutation (EML4-ALK, B-RAF, EGFR, and K-RAS). Cell were cultivated in presence of NVP-TAE684 (synthesized by Axon Medchem), crizotinib (PF-02341066), deferoxamine mesylate (DFX; Sigma), doxycycline hyclate (Sigma), MG-132 (Sigma), and bevacizumab (Genentech/Roche) for the indicated times and concentrations. For experiments in hypoxia, cells were cultured in Heracell 150i CO2 incubator (Thermo Scientific) at 1% O2.

ELISA for hVEGFA was performed on supernatants collected after 8 hours of culture by the Quantikine human VEGF ELISA Kit (R&D Systems) according to the manufacturer’s instructions.

Lentiviral-mediated shRNA targeting

Lentiviral shRNA clones targeting HIF1α and HIF2α were obtained from Sigma. Inducible shRNAs cells were obtained by transduction of pLVtTRKRAB vector followed by pLVTHM vectors containing the cloned shRNA cassettes (23). ALK-, C/EBPβ-, and Stat3-specific shRNAs have been previously described (23, 24). Retrovirus expressing NPM–ALK (25), STAT3C (26) or lentivirus expressing C/EBPβ (23) have been previously described.

Immunoblotting

Total cellular proteins were extracted as previously described (22). Primary antibodies used were: anti-HIF1α (1:800; Cell Signaling Technology), anti-HIF2α (1:800; Novus Biologicals), anti-ALK (1:2,000; Invitrogen), anti–phospho-ALK (1:1,000; Cell Signaling Technology), anti-actin (1:1,000; Sigma), anti–phospho-IBK (1:1,000; Cell Signaling Technology), anti–phospho-EGFR (1:1,000; Cell Signaling Technology), and anti-ERBB2 (1:1,000; Cell Signaling Technology). Secondary antibodies were used: anti-rabbit IgG (1:1,000; Cell Signaling Technology), anti-alk (1:2,000; Invitrogen), and anti-alk (1:2,000; Invitrogen). Immunoblotting for ALK, EGFR, and Stat3 was performed and analyzed as previously described (22). After blocking with 5% BSA, the cell lysates were probed with the primary antibodies, and the signals were detected using an Image Analysis software (Olympus Italia).

Histology, immunohistochemistry, and immunofluorescence

The following primary antibodies were used: CD34 (clone ME14.7; Santa Cruz Biototechnology), α-smooth muscle actin (clone 1A4; DakoCytomation), desmin (clone D3; DakoCyto- mation), VEGFA (clone 26503; R&D Systems), and Ki-67 (clone MIB-1; DakoCytomation). Positive cells were evaluated on randomly selected high power field (HPF; ×100 objective) using an Image Analysis software (Olympus Italia).

Quantitative real-time PCR

Total cDNA was synthesized as previously described (22). Normalization was performed against the housekeeping genes murine actin and mouse samples and human RPLP0 for human samples according to the formula 2−ΔΔCt, where the ΔCt = Ct reference gene−Ct internal control, as indicated by the manufacturer. Primers used in quantitative real-time PCR (qRT-PCR) as indicated in Supplementary Table S1.
Mice strains and in vivo xenograft and metastasis assays

Strains of mice used in this study include K-RasLSLG12V (27), TgEGFRDel19 (28), B-RafLmLV600E (29), and EML4–ALK transgenic mice (C. Voena; submitted for publication).

For in vivo induction of inducible lentiviral constructs, we dissolved doxycycline hydrochloride (MP Biomedicals, 1 g/L) in the drinkable water. For ALK knockdown, samples were collected at 96 hours after induction to achieve the maximum before the induction of apoptosis (24).

Statistical analysis

For GEP, we used false discovery rate (FDR) to calculate the statistical significance of enrichment scores. For the other experiments, statistical significance was calculated with the Student t-test, and only values lower than 0.05 were considered significant.

Results

Hypoxia pathways are significantly enriched in ALK-rearranged ALCL and NSCLC

In the search for pathways significantly enriched in ALK-rearranged ALCL, we performed gene set enrichment analysis (GSEA) of GEP data in a series of 169 T-cell lymphoma cases, which included 54 ALCL samples (30 ALK-rearranged ALCL and 24 ALK− ALCL), 74 peripheral T-cell lymphoma NOS (PTCL-NOS), and 41 angioimmunoblastic T-cell lymphoma. We found that several GSEA hypoxia datasets were significantly enriched in ALCL, as compared with other T-cell lymphoma types (Supplementary Fig. S1A–S1C). Furthermore, when we further separated ALCL into ALK+ and ALK− cases, GSEA indicated that hypoxia pathways enrichment was significantly associated with ALK+ ALCL cases (Fig. 1 and Supplementary Fig. S2), thus suggesting that the deregulated ALK activity in ALCL could be directly related to a hypoxia signature. We next asked whether hypoxia pathways were enriched also in other tumors expressing ALK translocations. We compared the GEP dataset from EML4–ALK-rearranged NSCLC with EGFR-mutated NSCLC, in which EGFR-L858R or EGFR-De19 mutations aberrantly activate the EGFR tyrosine kinase receptor. Hypoxia pathways were significantly enriched in ALK-rearranged NSCLC as compared with EGFR-mutated cases in two independent GEP datasets (Supplementary Fig. S3; refs. 20, 21). Thus, we concluded that ALK rearrangements are associated with an enriched hypoxia signature in both ALCL and NSCLC.

ALK regulates HIFs expression in ALCL via STAT3- and C/EBPβ-dependent transcription

Because hypoxia pathways are largely dependent on the activity of HIFs, we asked whether ALK activity could control HIFs expression in human cell lines derived from patients with ALK-rearranged ALCL. In all NPM–ALK-rearranged ALCL cell lines tested (SU-DHL-1, TS, JB6, and KARPAS-299), levels of HIF1α and HIF2α were low in standard 21% oxygen conditions, but were strongly upregulated in hypoxic condition (1% oxygen; Fig. 2A) or after treatment with the chemical compound that mimics hypoxia DFX (Supplementary Fig. S4). Conversely, in hypoxic condition ALK− ALCL cell lines (FeDP and MAC-1) as well as other T-cell Lymphoma lines (Jurkat and CEM) showed upregulation only of HIF1α and no changes in HIF2α expression (Fig. 2B).

When we treated ALK-rearranged ALCL with the specific ALK inhibitors TAE684 or crizotinib to block the kinase activity of the NPM–ALK fusion, ALK phosphorylation was strongly reduced and HIF1α and HIF2α protein levels were strongly reduced in both normoxic and hypoxic conditions (Fig. 2A and Supplementary Fig. S4). In contrast, HIF1α expression was modestly and inconsistently reduced in ALK− lymphoma cell lines, possibly due to some nonspecific effects of ALK inhibitors (Fig. 2B). To further confirm the role of ALK in HIFs expression, we also performed a genetic knockdown of ALK by shRNA. Again, efficient knockdown of ALK induced a marked reduction of both HIF1α and HIF2α protein levels (Supplementary Fig. S5), thus supporting our conclusions that in ALK-rearranged ALCL the expression of both HIF1α and HIF2α in hypoxic conditions is specifically regulated by the activity of ALK. Furthermore, although HIF1α upregulation in hypoxic conditions was detectable in all lymphoma cell lines, HIF2α upregulation appeared to be a specific feature of ALK+ ALCL. To further support the ALK specificity of HIF2α regulation, we analyzed the expression levels of HIF2α in ALK+ ALCL compared with ALK− ALCL and other T-cell lymphoma. Indeed, HIF2α expression was significantly higher in ALK-rearranged ALCL as compared with the other T-cell lymphoma analyzed (Fig. 2C).

HIFα proteins are regulated in normoxic and hypoxic cells through various mechanisms that include proteasome-dependent degradation and transcription (16, 18, 30). In well-oxygenated conditions, hydroxylation of HIFα subunits induces their proteosomal degradation by an E3 ubiquitin ligase, the von Hippel–Lindau protein (pVHL) complex.

In contrast, in hypoxic conditions, HIFα proteins are stabilized by a decreased proteasome-dependent degradation. Therefore, we asked whether the low levels of HIFα proteins during ALK inhibition could be explained by an increased proteasome-dependent degradation. To test this hypothesis, we treated ALCL with the proteasome inhibitor MG132. The inhibition of the proteasome-dependent degradation by MG132 clearly stabilized the known degradation target phospho-IκBα but it was unable to restore high levels of HIFα proteins in ALCL treated with the ALK inhibitor, thus ruling out proteasome-dependent degradation as a major mechanism of ALK-dependent HIFα regulation (Supplementary Fig. S6).

Next, we asked whether ALK regulated HIFα proteins by a transcription-dependent mechanism. By qRT-PCR, ALCL cells treated with an ALK inhibitor showed a significant reduction of both HIF1α and HIF2α mRNA levels (Fig. 2D), thus indicating that ALK controls HIFα expression at a transcriptional level. Because we and others previously showed that both STAT3 and C/EBPβ are key transcription factors in the oncogenic ALK signaling (2, 23, 31–33), and STAT3 has been shown to regulate HIF1α transcription (13), we tested whether they could be involved in ALK-dependent HIFα protein regulation. In ALCL cells, shRNA-mediated
knockdown of either STAT3 or C/EBPβ resulted in a marked reduction of both HIF1α and HIF2α protein levels (Supplementary Fig. S7). Consistently, ectopic overexpression of C/EBPβ and/or a constitutively active form of STAT3 (STAT3C; ref. 34) partially rescued HIF2α upregulation under hypoxic conditions even in the presence of the ALK inhibitor (Supplementary Fig. S8). In contrast, in ALK− cell lines, basal levels of STAT3 phosphorylation and C/EBPβ were low but increased after ectopic expression of NPM–ALK. Still, HIF2α expression remained undetectable even under hypoxic conditions, possibly suggesting a block of HIF2α expression in ALK− lymphoma cells mediated by other mechanisms such as epigenetic silencing (Supplementary Fig. S9). Therefore, we concluded that in ALK-rearranged ALCL cells both STAT3 and C/EBPβ are key transcription factors in the ALK-mediated regulation of HIFα proteins.
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**C**

- **ALK (P = 0.0001)**
- **TNFRSF8/CD30 (P = 0.39)**
- **HIF1α (P = 0.055)**
- **HIF2α (P = 0.005)**

**D**

- **TS**
- **SU-DHL1**

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**HIFs in ALK-Rearranged Lymphoma and Lung Carcinoma**

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HIF2α, but not HIF1α, is essential for tumor growth in ALCL

As we showed that HIF1α and HIF2α are regulated by the ALK activity in ALK-rearranged ALCL, we next seek to establish their biologic roles in ALCL growth. In a previous in vitro study in ALK-rearranged ALCL cell lines, HIF1α depletion has been shown to increase cell proliferation but to decrease VEGF synthesis (13), thus suggesting potentially contrasting effects for ALCL growth in vivo. Indeed, both HIF1α and HIF2α have been shown to possess contradicting roles in vitro and in vivo, mostly due to the different types of tumor analyzed (18, 30). Therefore, we decided to test directly in vivo the role of HIF1α and HIF2α in ALK-rearranged ALCL tumor growth by xenograft assays. We generated inducible lentiviral vectors to specifically knockdown HIF1α or HIF2α proteins upon treatment with doxycycline (Supplementary Fig. S10). When HIF1α was knocked down by treating mice with doxycycline, we did not observe any impairment of tumor growth in vivo as compared with control untreated mice (Fig. 3A and C), consistent with previous in vitro results (13). In contrast, HIF2α knockdown strongly impaired the growth of ALCL tumors in vivo (Fig. 3B and D and Supplementary Fig. S11). Remarkably, the knockdown of HIF1α or HIF2α did not reduce tumor growth in ALK− lymphomas (Supplementary Fig. S12). Finally, when we induced the HIF2α knockdown in established tumors, they arrested their growth and partially regressed (Fig. 3B and D, arrows). In sum, our data show that ALK specifically

Figure 3. HIF2α is essential for the growth of ALK-rearranged ALCL in vivo. SU-DHL-1 (A and B) and TS (C and D) ALCL cell lines were transduced with a doxycycline-inducible shRNA against HIF1α (A–C) or HIF2α (B–D) and injected s.c. in the flank of NOD/SCID mice (n = 6 for each group). For treated mice (+ Doxy), treatment with doxycycline started the same day of injection. Tumor diameter was measured over time. To study the role of HIF2α in already established tumors, we administered doxycycline to the untreated control group when the tumor size reached 8 to 10 mm in diameter, as indicated by an arrow; error bars, the SEM; *, P < 0.05; **, P < 0.0001.

Figure 2. Oncogenic ALK controls HIF1α and HIF2α expression by transcription-dependent mechanisms. A, NPM–ALK-rearranged ALCLs were incubated for 8 hours in normoxic (N, 21% O2) or hypoxic (H, 1% O2) conditions with or without the ALK inhibitors TAE684 (300 nmol/L) and crizotinib (300 nmol/L), as indicated. Western Blot analyses were performed with the indicated antibodies. HIF1α and HIF2α levels in cells in hypoxic conditions and treated with ALK inhibitors were quantified by densitometry, normalized to actin, and represented as fold changes compared with the corresponding expression levels in cells without ALK inhibitors. B, ALK+ lymphoma cell lines were treated and analyzed as in A. C, ALK+ ALCL cases show higher expression levels of HIF2α as compared with ALK− ALCL. Box-plot showing the expression of ALK, TNFRSF8/CD30, HIF1α, and HIF2α, in 30 ALK+ and 24 ALK− ALCL, calculated on the basis of GEP data. The paired t test P value is indicated for each gene transcript. D, qRT-PCR for HIF1α and HIF2α mRNA was performed in ALK-rearranged ALCL cell lines treated with TAE684 (300 nmol/L) for 8 hours; error bars, the SD of the mean; *, P < 0.05; **, P < 0.005; ***, P < 0.0001.
regulated HIF2α levels in lymphoma cells and that HIF2α is essential for ALCL growth and maintenance in vivo.

**ALK-mediated VEGFA production is a therapeutic target in ALCL**

The induction of hypoxia pathways mediated by ALK could contribute to the regulation of angiogenesis and metabolism of ALCL cells. Indeed, previous reports have shown that patients with ALK-rearranged ALCL have higher VEGFA levels than ALK− ALCL (11), that ALK regulates VEGFA secretion in fibroblasts *in vitro* (11), and that HIF1α contributes at least partially to VEGFA secretion (13). However, it is not known whether ALK-dependent VEGFA secretion has a role on tumor angiogenesis *in vivo* and whether the therapeutic blockade of VEGFA could be an effective therapeutic approach in ALCL. To address these points, we first confirmed *in vitro* that VEGFA mRNA and protein levels are regulated by ALK also in ALCL (Supplementary Fig. S13). Next, we showed that expression of VEGFA was downregulated in ALCL when ALK was knocked down by specific shRNA *in vivo* (Fig. 4A–C). Because VEGFA regulates tumor vessel formation (35), we expected that a reduced production of VEGFA would decrease the formation and the number of tumor vessels. Indeed, ALCL tumors in which ALK was knocked down, and therefore VEGFA was reduced, showed a significant decrease in tumor vessels, as measured by CD34, SMA, and Desmin stainings (Fig. 4D).

**Figure 4.** VEGFA production is regulated by ALK and is a target for therapy in ALCL. A, TS and SU-DHL-1 ALCL cells were transduced with lentivirus expressing doxycycline-inducible shRNA against ALK (shALK) or a mutated control sequence (shCtrl), as we previously published (24). B and C, tumor xenografts were obtained by s.c. injection in NOD/SCID mice of 1 × 10^7 ALCL cells. Immunohistochemistry for VEGFA was performed in TS or SU-DHL-1 tumor xenografts 96 hours after *in vivo* treatment of mice with doxycycline. C, histograms, counts of VEGFA-positive cells/HFP in at least three independent areas for each tumor. Data were collected from at least four tumors in each group; error bars, SD;*, P < 0.001. D, in the same tumor xenografts as in C from SU-DHL-1 cells immunofluorescence stainings for CD34, SMA, and Desmin were performed to measure tumor vessel formation; error bars, SD;*, P < 0.001. E, a total of 1 × 10^7 TS and SU-DHL-1 ALCL cells were injected s.c. in NOD/SCID mice. Treatment with bevacizumab started at day 3 and continued twice a week for 3 weeks. Data are from at least six tumors for each group; ***, P < 0.005.
To exploit ALK-induced VEGFA production as a target for therapeutic strategy, we treated mice that received ALCL xenografts with the VEGFA blocking antibody bevacizumab that is currently used in several clinical trials for different cancer types (36). Remarkably, ALCL tumors in mice treated with bevacizumab grew significantly slower than in control mice, thus indicating an important role for the ALK-dependent VEGFA production in ALCL growth (Fig. 4E). The growth of ALK/C0 lymphoma xenografts was also impaired by bevacizumab (Supplementary Fig. S14), thus indicating that in ALK/C0 lymphoma VEGFA secretion could be regulated by different mechanisms but still be important for tumor growth. We thus concluded that VEGFA production induced by oncogenic ALK regulates tumor vessels formation in ALCL and can be a potential target for ALCL therapy.

Oncogenic ALK regulates HIF1\(\alpha\) and HIF2\(\alpha\) and VEGFA expression in NSCLC

We showed that hypoxia pathways are enriched also in other ALK-rearranged tumor such as EML4–ALK-rearranged NSCLC (Supplementary Fig. S3). Therefore, we asked whether oncogenic ALK regulated HIF\(\alpha\) proteins and VEGF expression also in NSCLC. Treatment with DFX to mimic hypoxic conditions induced increased levels of both HIF1\(\alpha\) or HIF2\(\alpha\) in several NSCLC cell lines, including EML4–ALK-rearranged H2228 and H3122 (Fig. 5A), K-Ras–mutated A549, H460, and H441 (Fig. 5B), and EGFR-mutated H1975 and H3255 (Fig. 5C), whereas the B-RAF–mutated cell line H1395 showed only HIF1\(\alpha\) upregulation (Fig. 5D). However, treatment of NSCLC cell lines with the ALK inhibitors TAE684 or crizotinib-impaired HIF1\(\alpha\) and HIF2\(\alpha\) protein upregulation only in

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\caption{ALK kinase activity regulates HIF1\(\alpha\) and HIF2\(\alpha\) in NSCLC cells. EML4–ALK-rearranged H2228 and H3122 (A), K-RAS–mutated A549, H460, and H441 (B), EGFR-mutated H1975 and H3255 (C), B-RAF–mutated H1395 (D) NSCLC cell lines were incubated for 16 hours with DFX (100 mmol/L) to mimic hypoxic conditions and treated with TAE684 (300 nmol/L) or crizotinib (300 nmol/L) as indicated. Western blot analyses were performed with the indicated antibodies. HIF1\(\alpha\) and HIF2\(\alpha\) levels were normalized to actin and represented as fold changes compared with the corresponding expression levels in cells without ALK inhibitors. E and F, HIF1\(\alpha\) and HIF2\(\alpha\) mRNAs are higher in tumors from EML4–ALK transgenic mice than from other oncogenic mutations as demonstrated by qRT–PCR analysis for HIF1\(\alpha\) (E) and HIF2\(\alpha\) (F) mRNA levels performed on cDNA from five independent tumors dissected for each GEM; \(\ast\), \(P < 0.05\); \(\ast\ast\), \(P < 0.005\).}
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ALK-rearranged H2228 and H3122 (Fig. 5A). Consistently, ALK knockdown by specific shRNA reduced HIF1α or HIF2α upregulation under DFX treatment (Supplementary Fig. S15).

Given that in ALK-rearranged NSCLC, the EGFR family members are active and share common signaling pathways with ALK that can compensate for an inhibited ALK signaling (6, 37), we next investigated whether inhibition of EGFR signaling would affect HIF1α or HIF2α expression. In contrast with ALK inhibitors, HIF1α and HIF2α protein levels were minimally affected by treatment with gefitinib, an EGFR-specific inhibitor (Supplementary Fig. S16A), or with lapatinib, a broader EGFR and ERBB2 inhibitor (Supplementary Fig. S16B). Overall, these data indicate a specific role for ALK in the regulation of HIF1α and HIF2α in ALK-rearranged NSCLC.

To further prove the specific role of oncogenic ALK in HIFα regulation, we studied HIFα expression in genetically engineered mice (GEM) harboring the most common mutations found in NSCLC. We compared HIF1α and HIF2α mRNA levels in tumors isolated from GEM carrying the EML4–ALK translocation or the K-RasV12G, EGFRL858R, or B-RafV600E mutations (Supplementary Fig. S17A). We observed high HIF1α expression in K-Ras–mutated tumors that confirmed previous observations that oncogenic K-Ras increases HIF1α expression likely by reactive oxygen species generation (Fig. 5E; refs. 30, 38). Strikingly, HIF1α transcripts in EML4–ALK tumors were almost as high as K-Ras–mutated tumors, and significantly higher than HIF1α transcripts in normal lung. EGFRL858R- or B-RafV600E–mutated tumors (Fig. 5E). When we analyzed HIF2α mRNA levels, as expected, we found high expression in normal lung given that HIF2α is highly expressed in vessels and alveolar type II pneumocytes (17). Remarkably, EML4–ALK mice showed significantly higher HIF2α transcripts than all the other lung cancer genotypes (Fig. 5F).

Consistent with these data on HIFα regulation mediated by ALK in NSCLC, in GEM models VEGFA levels were significantly higher in EML4–ALK than in K-RasV12G, EGFRL858R, or B-RafV600E–mutated tumors (Supplementary Fig. S17B) and the inhibition of ALK decreased VEGFA expression in human NSCLC (Supplementary Fig. S17C), thus indicating a direct control of VEGFA by oncogenic ALK also in NSCLC, as we showed in ALCL.

**HIF1α and HIF2α regulate tumor growth, vessel formation, and metastasis in ALK-rearranged NSCLC**

We next wished to examine the functions of HIFα proteins in ALK-rearranged NSCLC growth and metastasis formation. Knockdown of both HIF1α and HIF2α did not have significant effect on cell growth in vitro (data not shown) but significantly impaired the growth of ALK-rearranged NSCLC xenografts in both H2228 and H3122 EML4–ALK NSCLC cell lines (Fig. 6A, C, E and G). Reduced growth was associated with reduced proliferation, as determined

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**Figure 6.** HIF1α and HIF2α are essential for the growth of ALK-rearranged NSCLC tumors. H2228 and H3122 EML4–ALK NSCLC cell lines were transduced with lentiviral vectors expressing doxycycline-inducible shRNA against HIF1α (A and E) and HIF2α (C and G) and were injected s.c. (1 × 10⁶ cells) in NOD/SCID mice. Treatment with doxycycline started the same day of injection. Tumor growth was measured over time; error bars, SEM from six independent tumors for each group. B, D, F, and H, histograms, the percentages of Ki-67–positive cells in tumors isolated from the corresponding groups of mice. Mean and SDs are calculated on the basis of six independent areas from at least six independent mice for the group; *, P < 0.05; **, P < 0.005; ***, P < 0.001.
Importantly, in ALCL oncogenic ALK not only specifi- 
cally controls hypoxia pathways and tumor vessels 
formation in ALK-rearranged ALCL and NSCLC. Together with 
the evidences on the role of ALK in neuroblastoma tumor 
angiogenesis (12), collectively these data support the concept 
that controlling the hypoxia response could be one major 
function of oncogenic ALK in various tumor types. Both HIF1α 
and HIF2α were potently regulated by ALK in hypoxic condi-
tions in ALK– ALCL. Of note, we propose that HIF2α regulation 
is a specific feature of ALK-rearranged ALCL, as ALK– 
ALK and cell lines showed significantly lower levels of HIF2α and 
a lack of HIF2α regulation under hypoxic conditions. Remark-
ably, we showed that both STAT3 and C/EBPβ transcription 
fac tors are required to sustain hypoxia-induced HIF2α upregu-
lation in ALK-rearranged 

Because HIF1α and HIF2α have unique and sometimes 
opposing functions in tumor biology (18), their precise role 
as oncogenes or tumor suppressor cannot be predicted and 
needs validation in each tumor in vivo. For example, in a 
mouse model of K-Ras–mutated NSCLC, HIF2α deletion 
increased tumor progression, thus acting as a tumor sup-
pressor rather than an oncogene (39). In contrast, HIF2α 
supports tumor growth in renal cell carcinoma (42). We 
showed that HIF1α was dispensable for ALCL growth, thus 
confirming previous results in vitro (13), whereas HIF2α was 
required not only for ALCL growth but also for its main-
tenance. Because it has been shown that ALK regulates 
lymphoma proliferation by sustaining c-myc levels in ALCL 
(43) and that HIF2α regulates c-myc expression (44), one 
intriguing possibility is that ALK could promote lymphoma 
proliferation by an HIF2α-mediated c-myc regulation. 
Importantly, in ALCL oncogenic ALK not only specifically 
regulated HIF2α but lymphoma cells were addicted to its 
functions, suggesting that HIF2α targeting could work as an 
additional therapeutic option for ALCL.

With striking similarities to ALK-rearranged ALCL, 
EML4–ALK rearranged NSCLC showed enriched hypoxia 
pathways when compared with NSCLC carrying other com-
mon driver mutations, such as EGFR and K-RAS mutation. 
Regulation of HIF1α and HIF2α in ALK-rearranged NSCLC 
was less potent but consistent with that observed in ALK-
rearranged ALCL. One possible explanation for such differ-
ce could be that rearranged ALK is expressed at higher 
levels in ALCL than in NSCLC (2, 45). As a result, oncogenic 
ALK activity is more dominant in ALCL than in NSCLC 
in terms of signaling pathway activation. In contrast with 
ALCL, in which oncogenic ALK activity dominates the 
signaling, the ALK blockade results in a milder inhibition 
of the same downstream pathways and in milder biologic 
effects in NSCLC (46). Indeed, STAT3 phosphorylation is 
only minimally impaired by ALK inhibitor treatment (10), 
whereas C/EBPβ is not expressed in ALK-rearranged NSCLC 
(data not shown), indicating that other pathways are likely 
involved in HIFs regulation in NSCLC. In this context, it is 
known that several additional tyrosine kinases can contrib-
ute to the biology of ALK-rearranged NSCLC (9). We exclu-
ded a role for EGFR family receptors by gefitinib or lapatinib 
treatment as well as for c-MET because the selective ALK 
inhibitor TAE684 or the specific ALK shRNA knockdown had 
similar effects to the dual ALK–MET inhibitor crizotinib but 
it is possible that other tyrosine kinases, such as PDGFR or 
c-KIT, or other signaling pathways active in NSCLC would 
partially compensate for ALK inhibition. This “by pass” 
compensation of the ALK activity is also a recurrent mecha-
anism that explains resistance to ALK inhibitors in NSCLC 
cells (9). Of note, knockdown of either HIF1α or HIF2α 
inhibited not only tumor growth but also metastasis forma-
tion in EML4–ALK-rearranged NSCLC, therefore indicating 
that, in contrast with ALCL, in ALK-rearranged NSCLC both 
HIFα proteins act as oncogenes and could serve as potential 
targets for therapy.

Oncogenic ALK activity is also required for VEGFA produc-
tion by ALCL and NSCLC cells. ALK knockdown significa-
tly reduced VEGFA production and vessel formation in tumors in vivo. EML4–ALK lung tumors in GEM had signifi-
cantly higher levels of VEGFA as compared with EGFR–, K-Ras–, 
and B-Raf–mutated tumors, thus reinforcing the specificity of the ALK-
hypoxia-angiogenesis axis. In ALCL, the mechanisms of ALK-
mediated VEGF regulation could be quite complex because 
ALK controls at least four factors that regulate VEGF expres-
sion, such as HIF1α, HIF2α, STAT3, and C/EBPβ as well as 
miRNA-16 (11). Single knockdown of each of these four factors 
did not significantly change VEGF expression (data not 
shown). To this end, more complex combinatorial knockdown 
experiments are needed to precisely unveil the mechanisms of 
ALK-mediated VEGF regulation.

Blockade of VEGFA by the VEGFA-specific antibody bev-
acizumab in ALCL reduced vessels formation and tumor 
growth, thus indicating a potential efficacy of antiangiogenic 
drugs in ALK-rearranged lymphoma. The use of bevacizumab 
in lymphoma patients is still under debate due to the limited 
efficacy and associated toxicities (47) and should be restricted 
to lymphoma types with proven efficacy (48). In this context,
Figure 7. Knockdown of HIF1α or HIF2α impairs VEGFA production and vessels formation as well as metastatic diffusion in EML4–ALK-rearranged NSCLCs. A, H2228 cells expressing a doxycycline-inducible shRNA for HIF1α or HIF2α were injected (10 × 10⁶) s.c. in NOD/SCID mice. Tumors were collected after 31 days (HIF1α) or 35 days (HIF2α) of treatment with doxycycline. Immunohistochemistry for VEGFA (A) and immunofluorescence for CD34 and Desmin (B) were performed and percentages of positive cells were calculated. Histograms, the mean and SDs of at least five tumors for each group; *, P < 0.05; **, P < 0.001. C–F, H3122 NSCLC was transduced with doxycycline-inducible lentiviral shRNA against HIF1α (C and D) or HIF2α (E and F) and 1 × 10⁶ cells were injected i.v. into NSG mice. Mice were sacrificed after 45 days (for HIF1α) or 35 days (for HIF2α) from i.v. injections and histologic sections of the lungs (C–E) and liver (D–F) were obtained (objective magnification, ×20). Histograms, the mean numbers of tumor foci counted in the entire lungs and livers of mice. Data are from six independent mice each group; *, P < 0.05; **, P < 0.005.
our data indicate that patients with ALK-rearranged ALC may indeed benefit with treatment with bevacizumab. In solid cancers, bevacizumab has been approved as treatment for several late-stage advanced metastatic cancers (35). However, the rate of response to bevacizumab in NSCLC is still unpredictable for unclear reasons. It has been suggested that a stratification based on the molecular genetics of NSCLC could improve the efficacy of bevacizumab, possibly by restricting its use in NSCLC with selected genetic lesions (36, 49). On the basis of the results of the present study, ALK-rearranged NSCLC could represent a subset of tumors that could mostly benefit from such antiangiogenic therapy.

In summary, in this work, we present evidences for an ALK-dependent hypoxia-angiogenesis regulation in both ALC and NSCLC. ALK regulates HIF1α and HIF2α, which in turn become essential for tumor growth. Thus, treatments aimed at blocking the ALK-driven VEGFA production in ALC and NSCLC, or directly at targeting HIF2α in ALC and both HIF1α and HIF2α in NSCLC, have potentials to become additional therapeutic strategies in ALK-rearranged tumors.

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

References

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HIFs in ALK-Rearranged Lymphoma and Lung Carcinoma


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