Human Cancer Biology

Role of ERK-BIM and STAT3-Survivin Signaling Pathways in ALK Inhibitor–Induced Apoptosis in EML4-ALK–Positive Lung Cancer

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Abstract

Purpose: EML4-ALK (echinoderm microtubule-associated protein–like 4 anaplastic lymphoma kinase) was recently identified as a transforming fusion gene in non–small cell lung cancer. The purpose of the present study was to characterize the mechanism of malignant transformation by EML4-ALK. Experimental Design: We established NIH 3T3 cells that stably express variant 1 or 3 of EML4-ALK and examined the signaling molecules that function downstream of EML4-ALK. Results: Forced expression of EML4-ALK induced marked activation of extracellular signal–regulated kinase (ERK) and STAT3, but not that of AKT. Inhibition of ERK or STAT3 signaling resulted in substantial attenuation of the proliferation of cells expressing either variant of EML4-ALK, suggesting that these signaling pathways function downstream of EML4-ALK in lung cancer cells. The specific ALK inhibitor TAE684 induced apoptosis that was accompanied both by upregulation of BIM, a proapoptotic member of the Bcl-2 family, and by downregulation of survivin, a member of the inhibitor of apoptosis protein (IAP) family, in EML4-ALK–expressing NIH 3T3 cells as well as in H3122 human lung cancer cells harboring endogenous EML4-ALK. Depletion of BIM and overexpression of survivin each inhibited TAE684-induced apoptosis, suggesting that both upregulation of BIM and downregulation of survivin contribute to TAE684-induced apoptosis in EML4-ALK–positive lung cancer cells. Furthermore, BIM and survivin expression was found to be independently regulated by ERK and STAT3 signaling pathways, respectively. Conclusions: ALK inhibitor–induced apoptosis is mediated both by BIM upregulation resulting from inhibition of ERK signaling as well as by survivin downregulation resulting from inhibition of STAT3 signaling in EML4-ALK–positive lung cancer cells. Clin Cancer Res; 17(8); 2140–8. ©2011 AACR.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide. Given that the efficacy of conventional chemotherapeutic agents with regard to improving clinical outcome in lung cancer patients is limited, target-based therapies are being pursued as potential treatment alternatives. Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) have been associated with tumor responsiveness to EGFR tyrosine kinase inhibitors (TKI) in a subset of individuals with non–small cell lung cancer (NSCLC; refs. 1–3). Such findings suggest that the use of molecularly targeted therapy in genetically defined subsets of cancer patients may prove to be an effective strategy for the treatment of many cancers including NSCLC. Given that lung cancer is a common type of cancer, the identification of even small subsets of lung cancer patients harboring specific genetic abnormalities will translate into the provision of large cohorts for targeted therapy. A recent study identified a potential driver mutation in NSCLC: fusion of the echinoderm microtubule-associated protein–like 4 gene (EML4) with the anaplastic lymphoma kinase gene (ALK), which results in the production of a fusion protein (EML4-ALK) consisting of the NH2-terminal portion of EML4 and the COOH-terminal region of ALK (4). ALK was originally discovered as the result of characterization of chromosomal translocations that lead to the expression of fusion proteins consisting of the COOH-terminal kinase domain of ALK and the NH2-terminal portion of nucleophosmin (NPM) in patients with anaplastic large cell lymphoma (5, 6). Various break and fusion points within the EML4 locus in NSCLC cells give rise to different isoforms of EML4-ALK, which appear to be present in 5% to 10% of NSCLC cases (4, 7–14). The most common EML4-ALK variants are 1 and 3, which together account for about 60% of EML4-ALK–positive lung cancer cases (14). All EML4-ALK isoforms undergo constitutive oligomerization mediated by the coiled coil domain of the
**Translational Relevance**

EML4-ALK (echinoderm microtubule-associated protein–like 4 anaplastic lymphoma kinase) was recently identified as a transforming fusion gene in non–small cell lung cancer (NSCLC), and several selective inhibitors of the kinase activity of ALK, such as crizotinib, are currently undergoing clinical trials for the treatment of EML4-ALK–positive NSCLC. Identification of the signaling pathways responsible for malignant transformation by EML4-ALK will likely enhance further development of ALK-targeted therapy for NSCLC patients. We have now shown that both ERK (extracellular signal–regulated kinase) and STAT3 pathways are the principal mediators of EML4-ALK signaling, and we further identified the mediators of apoptosis induced by ALK inhibition. Our preclinical data provide both insight into the pathogenesis of EML4-ALK–positive lung cancer and a potential basis for the development of biomarkers for the efficacy of ALK-targeted therapy in patients with this condition.

EML4 portion, which confers marked transforming activity both in vitro and in vivo (4, 15).

ALK inhibitors have been found to suppress the growth of and to induce apoptosis in EML4-ALK–positive lung cancer cells, suggesting that ALK inhibition is a potential strategy for the treatment of NSCLC patients with this molecular abnormality (9, 16). Indeed, a selective inhibitor of the kinase activity of ALK, crizotinib, is currently undergoing clinical trials and has shown high efficacy in NSCLC patients with EML4-ALK (17). However, the downstream signaling pathways that regulate the proliferation or survival of EML4-ALK–positive lung cancer cells have remained to be well established, and the key mediators of ALK inhibitor–induced apoptosis have not been fully determined. In the present study, we constructed expression vectors for EML4-ALK variants 1 and 3 and then established cells stably expressing these proteins. With the use of these cells, we examined the signaling molecules that function downstream of EML4-ALK. We further investigated the molecular mechanisms underlying ALK inhibitor–induced apoptosis in EML4-ALK–positive lung cancer cells.

**Materials and Methods**

**Cell culture and reagents**

NIH 3T3 cells as well as the human cancer cell lines H2228 and Karpas299 were obtained from American Type Culture Collection. H3122 cells were obtained as previously described (9). NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% FBS and 1% penicillin-streptomycin. H2228, Karpas299, and H3122 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were maintained under a humidified atmosphere of 5% CO₂ at 37°C. U0126 and LY294002 were obtained from Cell Signaling Technology and TAE684 was from ShangHai Biochempartner.

**Cell transfection**

A cDNA for EML4-ALK variant 1 was cloned into pDNA-Dual (Becton Dickinson) as previously described (9). A full-length cDNA fragment encoding EML4-ALK variant 3b was obtained from H2228 cells by reverse transcription and the PCR with the primers EAV-F (5’-AACGCTTCCGAAGATGGACGAGGCCGAGTC-3’) and EAV-R (5’-GCGGCCGCTCAGGGCCCAGGCAGTC-3’). Amplification products were verified by sequencing after their cloning into the pcCR-Blunt II TOPO vector (Invitrogen). The EML4-ALK variant 1 or 3b cDNA was excised from pcCR-Blunt II-TOPO and transferred to either pcDNA3.1-Hygro(+) (Invitrogen) or pMzs (Cell Biolabs). A pBabe-puro vector encoding CA-STAT3 with a COOH-terminal FLAG tag was kindly provided by J. Bromberg (18). A pQCXIH-survivin vector was constructed as previously described (19). All expression vectors were introduced into NIH 3T3 cells as previously described (20, 21).

**Immunoblot analysis**

Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethysulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of cell lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% or 12% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 hour at room temperature before incubation overnight at 4°C with primary antibodies. Rabbit polyclonal antibodies to human phosphorylated ALK (pY1608), to ALK, to phosphorylated extracellular signal–regulated kinase (ERK), to ERK, to phosphorylated STAT3, to STAT3, to phosphatidylinositol 3-kinase (PI3K), to p-AKT, to Bcl-xL, to XIAP, and to BIM, to Mcl-1, to Bcl-xl, to X-linked inhibitor of apoptosis (XIAP), and to STAT3 were from Cell Signaling Technology. Cells were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit IgG (Sigma). Immune complexes were finally detected with chemiluminescence reagents (GE Healthcare).

**Cell growth inhibition assay**

Cells were plated in 96-well, flat-bottomed plates and cultured for 24 hours before exposure to various concentrations of drugs for 72 hours. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku) was then
added to each well, and the cells were incubated for 3 hours at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems).

**RNA interference**

Cells were plated at 50% to 60% confluence in 6-well plates or 25-cm² flasks and then incubated for 24 hours before transient transfection for the indicated times with siRNAs mixed with the Lipofectamine reagent (Invitrogen). The siRNAs specific for STAT3 mRNA (STAT3-1, 5'-UCAUUGACCUUGUUGAAA-3'; STAT3-2, 5'-GCAAAAA-GUUUCUACACA-3'), ALK mRNA (ALK-1, 5'-ACACCCAAUUUAUACCAA-3'; ALK-2, 5'-UCACCAAAUUCACCCACCA-3'), ERK mRNA (ERK-1, 5'-CAAGCAAAUUAAUACCAA-3'; ERK-2, 5'-UCAGGCCCCUUGAGCCACCA-3'), or BIM mRNA (BIM-1, 5'-GGAGGGUAUUUUUGAAUAA-3'; BIM-2, 5'-AGGAGGGUAUUUUUGAAUA-3') as well as a nonspecific siRNA (5'-GUUGAGAGAUAUUAGAGUU-3') were obtained from Nippon EGT. The cells were then subjected to immunoblot analysis or the annexin V-binding assay. All data presented were obtained with STAT3-1, ALK-1, ERK-1, or BIM-1 siRNAs, but similar results were obtained with STAT3-2, ALK-2, ERK-2, and BIM-2 siRNAs.

**Annexin V–binding assay**

Binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining kit (Roche). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 x g for 5 minutes. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 minutes at 15°C to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson).

**Statistical analysis**

Quantitative data are presented as means ± SD and were analyzed by Student’s 2-tailed t test. A value of P < 0.05 was considered statistically significant.

**Results**

**Oncogenic EML4-ALK tyrosine kinase activates ERK and STAT3 signaling pathways**

To study the function of oncogenic EML4-ALK, we established nontransformed mouse fibroblast (NIH 3T3) cells that either stably express EML4-ALK variant 1 or 3 (3T3/EAV1 and 3T3/EAV3 cells, respectively) or stably harbor the corresponding empty vector (3T3-Mock cells). Immunoblot analysis revealed that EML4-ALK variant 1 or 3 was detected with antibodies to ALK at positions corresponding to molecular sizes of about 120 and 90 kDa, respectively, in the transfected cells (Fig. 1A). The kinase activity of the EML4-ALK variants was activated as revealed by immunoblot analysis of cell lysates (Fig. 1B). The activation of ERK and STAT3 pathways was also observed in the transfected cells (Fig. 1C). The results of these experiments are shown in Figure 1D. The kinase activity of the EML4-ALK variants was activated as revealed by immunoblot analysis of cell lysates (Fig. 1D). The activation of ERK and STAT3 pathways was also observed in the transfected cells (Fig. 1C). The results of these experiments are shown in Figure 1D.
analysis with antibodies specific for the Tyr\(^{1608}\)-phosphorylated form of ALK. Consistent with previous observations (4, 15), the 3T3/EAV cells exhibited transforming activity both in vitro (Fig. 1B) and in vivo (Fig. 1C). We also found that phosphorylation of both the mitogen-activated protein kinase (MAPK) ERK and STAT3 was markedly increased in the cells expressing either variant of EML4-ALK compared with that in 3T3-Mock cells, whereas the phosphorylation level of the kinase AKT was not affected by expression of EML4-ALK (Fig. 1A). To exclude the possibility that these results were due to nonspecific effects of transfection, we depleted both 3T3/EAV1 and 3T3/EAV3 cells of EML4-ALK by RNA interference (RNAi) with ALK siRNA. The phosphorylation of both ERK and STAT3, but not that of AKT, was markedly suppressed by depletion of EML4-ALK (Fig. 1D). Moreover, similar depletion of endogenous EML4-ALK variant 1 in the lung cancer cell line H3122 resulted in marked inhibition of the phosphorylation of ERK and STAT3 without an effect on that of AKT (Fig. 1D). In contrast, the phosphorylation of ERK, STAT3, and AKT was inhibited by ALK siRNA in the NPM-ALK–positive lymphoma cell line Karpas299 (Fig. 1D), in which activation of the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway has been shown to contribute to malignant transformation (22–25). Together, these data suggested that either variant 1 or 3 of EML4-ALK activates ERK and STAT3 signaling pathways but not the PI3K-AKT signaling pathway.

Figure 2. Effects of inhibition of ERK, PI3K, or STAT3 signaling on the growth of cells expressing EML4-ALK. A, the indicated cell lines were incubated in complete medium for the indicated times, after which cell viability was assessed as described in the "Materials and Methods" section. Data are expressed relative to the absorbance value for 3T3-Mock cells at time 0. B and C, cells were incubated in complete medium with or without 10 μmol/L U0126 (B) or 10 μmol/L LY294002 (C) for 72 or 24 hours, after which cell viability was assessed (left) or cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins (right), respectively. D, cells were transfected with nonspecific or STAT3 siRNAs for 72 or 48 hours, after which cell viability was assessed (left) or cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins (right), respectively. The abundance of β-actin was examined as a loading control. All quantitative data are means ± SD from 3 independent experiments. *, P < 0.05 versus the corresponding value for 3T3-Mock cells or for the indicated comparisons. NS, not significant.
EML4-ALK promotes cell proliferation through ERK and STAT3 signaling pathways

We next examined the effect of EML4-ALK on cell proliferation. Both 3T3/EAV1 and 3T3/EAV3 cells proliferated significantly faster than did 3T3-Mock cells (Fig. 2A). To determine the role of intracellular signaling pathways in this action of EML4-ALK, we first examined the effects of chemical inhibitors. We found that U0126, an inhibitor of the ERK kinase MEK, had little effect on the growth of 3T3-Mock cells but that it significantly inhibited the proliferation of both 3T3/EAV1 and 3T3/EAV3 cells at a concentration (10 μmol/L) that resulted in marked inhibition of ERK phosphorylation (Fig. 2B). These data thus suggested that the MEK-ERK signaling pathway contributes to the regulation of cell proliferation by EML4-ALK. We also found that the specific PI3K inhibitor LY294002 had no significant effect on the growth of 3T3-Mock cells or on that of 3T3/EAV1 and 3T3/EAV3 cells at a concentration (10 μmol/L) at which the phosphorylation of AKT was largely abolished (Fig. 2C). To examine the effect of STAT3 inhibition on cell proliferation in cells expressing EML4-ALK, we transfected the cells with an siRNA specific for STAT3 mRNA. Transfection of 3T3-Mock, 3T3/EAV1, or 3T3/EAV3 cells with STAT3 siRNA resulted in marked depletion of STAT3 (Fig. 2D). Whereas such depletion of STAT3 did not affect the proliferation of 3T3-Mock cells, it significantly inhibited that of 3T3/EAV1 and 3T3/EAV3 cells (Fig. 2D).
second siRNA targeted to a different region of STAT3 mRNA yielded similar results (data not shown). These observations thus suggested that EML4-ALK promotes cell proliferation through both MEK-ERK and STAT3 signaling pathways but not through the PI3K-AKT signaling pathway.

Effects of ALK inhibition on cell growth and intracellular signaling in EML4-ALK–positive lung cancer cells

To investigate the effects of inhibition of the kinase activity of ALK on cell growth and intracellular signaling in cells expressing EML4-ALK, we used TAE684, a selective and highly potent ALK inhibitor (26). The human lung cancer cell line H3122 expresses endogenous EML4-ALK variant 1 and its growth was found to be highly sensitive to TAE684 (Fig. 3A). Treatment with TAE684 also induced a large increase in the number of apoptotic H3122 cells, as revealed with an annexin V–binding assay (Fig. 3B). Consistent with these results, both 3T3/EAV1 and 3T3/EAV3 cells exhibited a sensitivity to TAE684 that was about 100 times as great as that of 3T3-Mock cells (Fig. 3A), and the level of apoptosis induced by this drug was markedly greater in both 3T3/EAV1 and 3T3/EAV3 cells than in 3T3-Mock cells (Fig. 3B). Immunoblot analysis revealed that TAE684 inhibited the phosphorylation of EML4-ALK in 3T3/EAV1, 3T3/EAV3, and H3122 cells at a concentration (30 nmol/L) at which it substantially inhibited the growth of these cells (Fig. 3C). We further found that TAE684 inhibited the activation of ERK and STAT3, without affecting that of AKT, in all 3 of these cell lines (Fig. 3C). These data thus suggested that the ALK inhibitor induced growth suppression and apoptosis in EML4-ALK–positive lung cancer cells, and that these effects were accompanied by inhibition of ERK and STAT3 signaling pathways but not by that of the PI3K-AKT signaling pathway.

Effects of ALK inhibition on the expression of apoptosis-related proteins in EML4-ALK–positive lung cancer cells

Given that TAE684 induced apoptosis in cells expressing EML4-ALK, we examined the effects of this drug on the expression of apoptosis-related proteins in such cells. TAE684 induced cleavage of PARP, a characteristic of apoptosis, in H3122 cells as well as in 3T3/EAV1 and 3T3/EAV3 cells (Fig. 4A). TAE684 also increased the abundance of BIM, a proapoptotic member of the Bcl-2 family of proteins, in cells expressing EML4-ALK, whereas the amounts of the Bcl-2 family members Mcl-1 and Bcl-xL remained unaffected (Fig. 4A). In contrast, TAE684 induced downregulation of the expression of survivin, a member of the IAP family, in cells expressing EML4-ALK, whereas the expression of XIAP, another IAP family member, remained unaffected (Fig. 4A). To investigate the possible roles of the ERK and STAT3 signaling pathways in the induction of BIM and downregulation of survivin by TAE684, we examined the effects of EML4-ALK, ERK, or STAT3 depletion by RNAi in 3T3/EAV1, 3T3/EAV3, and H3122 cells. Similar to the effects of TAE684 (Fig. 3C), depletion of EML4-ALK with an ALK siRNA resulted in inhibition of both ERK and STAT3 phosphorylation in all 3 cell lines (Fig. 4B). The amount of BIM was increased as a result of EML4-ALK or ERK depletion but was not affected by STAT3 depletion (Fig. 4B). In contrast, the expression of survivin was inhibited by depletion of EML4-AKT or STAT3 but not by that of ERK (Fig. 4B). Similar results were obtained with a second set of ALK, ERK, and STAT3 siRNAs targeted to different regions of the corresponding mRNAs (data not shown). These data thus suggested that ALK inhibition results in upregulation of BIM expression through inhibition of the ERK signaling pathway as well as in downregulation of survivin expression through inhibition of the STAT3 signaling pathway.
Role of ERK-BIM and STAT3-survivin signaling pathways in TAE684-induced apoptosis in cells expressing EML4-ALK

To investigate further whether induction of BIM is related to TAE684-induced apoptosis, we transfected 3T3/EAV3 or H3122 cells with an siRNA specific for BIM mRNA. Such transfection largely blocked BIM induction by TAE684 (Fig. 5A). Staining with annexin V revealed that RNAi-mediated attenuation of BIM induction resulted in significant inhibition of TAE684-induced apoptosis in both cell lines (Fig. 5A), implicating upregulation of BIM expression in the induction of apoptosis by TAE684 in EML4-ALK–positive cells. We obtained similar results with a second siRNA targeted to a different sequence within BIM mRNA (data not shown). Given that TAE684 inhibited STAT3-survivin signaling in cells expressing EML4-ALK, we next investigated the contribution of such signaling to TAE684-induced apoptosis by transfecting 3T3/EAV3 or H3122 cells with an expression vector encoding a FLAG epitope–tagged constitutively active (CA) form of human STAT3. Expression of CA-STAT3 increased the abundance of survivin (Fig. 5B), consistent with the notion that survivin expression is upregulated by activation of STAT3 signaling. Furthermore, expression of CA-STAT3 inhibited the downregulation of survivin induced by TAE684, without affecting BIM induction (Fig. 5B), and it significantly inhibited TAE684-induced apoptosis (Fig. 5B). These data suggested that inhibition of the STAT3 signaling pathway contributes to TAE684-induced apoptosis in EML4-ALK–positive cells. To confirm that TAE684–induced apoptosis mediated by STAT3 inhibition was attributable to downregulation of survivin expression, we transfected 3T3/EAV3 or H3122 cells with an expression vector for human survivin. Survivin overexpression resulted in substantial inhibition of the TAE684-induced downregulation of survivin in both 3T3/EAV3 and H3122 cells (Fig. 5C), and this effect was associated with significant inhibition of apoptosis.

Figure 5. Effects of BIM depletion as well as forced expression of CA-STAT3 and survivin on apoptosis induced by TAE684 in 3T3/EAV3 or H3122 cells. A, cells were transfected with nonspecific (control) or BIM siRNAs for 24 hours and then incubated in complete medium with 30 nmol/L TAE684 or DMSO vehicle for 48 hours, after which cells either were lysed and subjected to immunoblot analysis with antibodies to the indicated proteins or were evaluated for apoptosis by staining with annexin V and PI followed by flow cytometry. B, cells were transfected with an expression vector for FLAG-tagged CA-STAT3 or with the corresponding empty vector for 24 hours and were then incubated with or without 30 nmol/L TAE684 for 48 hours and analyzed as in A. C, cells were transfected with an expression vector for survivin or with the corresponding empty vector for 24 hours and were then incubated with or without 30 nmol/L TAE684 for 48 hours and analyzed as in A. All quantitative data are means ± SD from at least 3 independent experiments. *, P < 0.05 for the indicated comparisons.
of TAE684-induced apoptosis (Fig. 5C). These results thus suggested that inhibition of STAT3-survivin signaling by TAE684 contributes substantially to the induction of apoptosis by this drug. Collectively, our results thus suggested that inhibition of both the ERK-BIM and STAT3-survivin signaling pathways contributes to the induction of apoptosis associated with ALK inhibition in EML4-ALK–positive lung cancer cells.

Discussion

EML4-ALK was only recently identified as a transforming fusion gene in NSCLC (4). Although EML4-ALK was shown to possess marked oncogenic activity both in vitro and in vivo (4, 15), the signaling pathways underlying malignant transformation by the fusion protein have remained unclear. We have now shown that phosphorylation of both ERK and STAT3 was similarly and markedly increased in NIH 3T3 cells by forced expression of either variant 1 or variant 3 of EML4-ALK, whereas phosphorylation of AKT remained unaffected. Similar effects were observed in different clones of these cells stably transfected with a vector for either variant of EML4-ALK (data not shown). We further showed that the growth of both 3T3/EAV1 and 3T3/EAV3 cells was significantly attenuated by inhibition of ERK or STAT3 signaling but not by that of PI3K signaling. NPM-ALK has also been shown to activate ERK and STAT3 signaling pathways (6, 27–33), both of which are thought to be essential downstream mediators of the oncogenic action of NPM-ALK. In the present study, we found that ALK siRNA markedly abrogated the phosphorylation of AKT in the NPM-ALK–positive lymphoma cell line Kaspa299, consistent with previous results implicating activation of PI3K-AKT signaling in malignant transformation by NPM-ALK (22–25). In contrast, we found that ALK siRNA did not suppress AKT phosphorylation in the EML4-ALK–positive lung cancer cell line H3122. Together, our results thus suggest that both ERK and STAT3 signaling pathways, rather than the PI3K signaling pathway, are the principal downstream pathways activated by EML4-ALK in lung cancer cells. Oncogenic ALK fusion proteins therefore may activate downstream pathways in a manner dependent on the fusion partner (Supplementary Fig. S1).

Preclinical studies have shown that treatment of NSCLC cell lines expressing EML4-ALK with ALK inhibitors suppresses cell proliferation and induces apoptosis (9, 34), although the underlying mechanisms of these effects were not well characterized. We have now shown that TAE684, a specific inhibitor of the kinase activity of ALK, significantly inhibited the phosphorylation of ERK and STAT3, but not that of AKT, in EML4-ALK–positive lung cancer cells, supporting the notion that ERK and STAT3 signaling pathways function downstream of EML4-ALK. BIM is a key proapoptotic member of the Bcl-2 family of proteins and initiates apoptosis signaling by binding to and antagonizing the function of prosurvival members of the Bcl-2 family (35). We found that TAE684 induced upregulation of BIM in EML4-ALK–positive lung cancer cells. With the use of RNAi-mediated depletion of ERK, we also found that BIM expression is regulated by the ERK signaling pathway. We further showed that knockdown of BIM by RNAi resulted in significant inhibition of TAE684-induced apoptosis in EML4-ALK–positive cells, suggesting that BIM induction mediated by inhibition of the ERK pathway plays a pivotal role in ALK inhibitor–induced apoptosis in EML4-ALK–positive lung cancer cells. These findings are consistent with the previous observation that inhibition of the ERK pathway contributes to EGFR-TKI–induced BIM upregulation, which is essential for the induction of apoptosis by these agents, in EGFR mutation–positive NSCLC cells (36–38).

Survivin is a member of the IAP family and protects against apoptosis by either directly or indirectly inhibiting the activation of effector caspases (39). We have now shown that TAE684 inhibited the expression of survivin in EML4-ALK–positive lung cancer cells. Furthermore, depletion of STAT3 resulted in downregulation of survivin expression, whereas expression of a constitutively active form of STAT3 resulted in upregulation of survivin expression. These data indicate that expression of survivin is regulated primarily through the STAT3 signaling pathway, consistent with the results of a previous study (40). We further found that expression of CA-STAT3 blocked the TAE684-induced downregulation of survivin, indicating that ALK inhibition results in survivin downregulation through inhibition of the STAT3 signaling pathway. Forced expression of either CA-STAT3 or survivin attenuated TAE684-induced apoptosis in 3T3/EAV3 or H3122 cells, suggesting that inhibition of STAT3-survivin signaling contributes to ALK inhibitor–induced apoptosis in EML4-ALK–positive lung cancer cells. Our present data thus suggest that ALK inhibitor–induced apoptosis is mediated both by upregulation of BIM through inhibition of the ERK pathway and by downregulation of survivin through inhibition of the STAT3 pathway in EML4-ALK–positive lung cancer cells.

In conclusion, our results have identified both ERK and STAT3 signaling pathways as key mediators of the transforming activity of EML4-ALK in lung cancer cells positive for this fusion protein. We further demonstrated that inhibition of both ERK-BIM and STAT3-survivin signaling pathways is responsible for ALK inhibitor–induced apoptosis in these cells. Our results thus provide a basis for the further development of ALK-targeted therapy in EML4-ALK–positive lung cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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