Priority Report

Cancer Research

Therapeutically Targetable ALK Mutations in Leukemia

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Abstract

Genome sequencing is revealing a vast mutational landscape in leukemia, offering new opportunities for treatment with targeted therapy. Here, we identify two patients with acute myelogenous leukemia and B-cell acute lymphoblastic leukemia whose tumors harbor point mutations in the ALK kinase. The mutations reside in the extracellular domain of ALK and are potently transforming in cytokine-independent cellular assays and primary mouse bone marrow colony formation studies. Strikingly, both mutations conferred sensitivity to ALK kinase inhibitors, including the FDA-approved drug crizotinib. On the basis of our results, we propose that tumors harboring ALK mutations may be therapeutically tractable for personalized treatment of certain aggressive leukemias with ALK inhibitors. *Cancer Res; 75(11); 2146–50. ©2015 AACR.*

Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase in the insulin receptor subfamily with homology to leukocyte tyrosine kinase (LTK), insulin-like growth factor-1 receptor kinase (IGF1R) and insulin receptor kinase (INSR; ref. 1). ALK consists of a large ligand-binding extracellular domain, transmembrane region, and cytoplasmic domain comprised largely of the tyrosine kinase domain. The extracellular domain consists of two MAM (meprin, A5 protein, and receptor protein tyrosine phosphatase mu) domains, a LDLa (low-density lipoprotein), and a glycine-rich region (2). Although its normal physiologic role is not entirely clear, ALK is proposed to play a role in the development of the nervous system based on its high level of expression in embryonic neural tissue (2).

ALK was originally identified as part of a gene fusion in patients with anaplastic large-cell lymphoma (3). This fusion is a result of an in-frame fusion of the cytoplasmic domain of ALK to the N-terminus of nucleolar phosphoprotein (NPM; ref. 3). In anaplastic large-cell lymphoma, STAT3 is a key mediator, which is required for the neoplastic transformation and prevents cell death (4, 5). ALK rearrangements have also been identified in non–small cell lung carcinomas (NSCLC), inflammatory myofibroblastic tumors, and other solid tumors (1). In NSCLC, the most common ALK fusion partner is echinoderm microtubule-associated protein-like 4 (EML4), which was found in 6.7% of cases (6). ALK overexpression has been observed in multiple tumor types (2). In neuroblastoma, activating point mutations are found in the ALK kinase domain (7).

Multiple ALK inhibitors are being developed clinically. Crizotinib (PF-2341066, Xalkori, Pfizer), an ATP-competitive MET, ALK, and ROS1 inhibitor (8), is the most clinically advanced and is now FDA approved for first-line treatment in ALK-positive NSCLC. In a phase III clinical trial, the response rate for patients with ALK-positive NSCLC was 65% (9). ALK is therefore a promising therapeutic target in a variety of tumor types.

We recently sequenced primary samples from leukemia patients and found that, aside from a few relatively frequent mutations, there are large numbers of mutations that occur at low frequency. A similar mutational landscape of cancer is emerging from large datasets produced from other efforts (10). Understanding which of these mutations are oncogenic drivers that can be therapeutically targeted remains a major challenge. We report sequencing from two leukemia patients with somatic mutations in the extracellular domain of ALK that were of unknown significance. Here, we show that these mutations are oncogenic, and cells transformed by these mutant versions of ALK are sensitive to crizotinib and other ALK inhibitors.

Materials and Methods

Sequencing of leukemia patient samples

Primary blood and bone marrow specimens were obtained after written informed consent from patients with hematologic malignancies according to a protocol approved by the Oregon Health and Science University (OHSU) institutional review board. Deep sequencing was performed on 1,862 kinase and

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kinase-associated genes as described previously (11). Patient samples ($n = 185$) were sequenced, including 96 acute myelogenous leukemia (AML), 51 acute lymphoblastic leukemia, and 38 myeloproliferative neoplasms. The ALK A348D mutation was verified by Sanger sequencing using the following M13F- and M13R-tagged primers (ALK-e4-L gtaaaacgacggccagtCCACAGAGCTACTGCTGGTC and ALK-e4-R caggaaacagctatgaccACCAAAAGCCAAATCACCTG) and then sequenced using M13F (gtaaaacgacggccagt) and M13R primers (caggaaacagctatgacc) by Eurofins MWG Operon. The ALK F856S mutation was verified by Sanger sequencing by Genewiz Inc.

Cloning

A gateway-compatible entry clone containing the ALK cDNA was obtained from Genecopoeia (ALK pDONR, GC-T1863). The ALK A348D and F856S mutations were made by site-directed mutagenesis using the Quikchange II XL Kit (Agilent Technologies, Inc.) and the following primers: ALK_A348D_F cactgcacactggacgtctcggtgcac, ALK_A348D_R gtgcaccgagacgtccagtgtgcagtg, ALK_F856S_F ggccaagacagacacgagccacccagagagactg, ALK_F856S_R cagtctctctgggtggctcgtgtctgtcttggcc.

Mutated cDNAs were then transferred into a Gateway-compatible MSCV-IRES-GFP vector using the LR Clonase enzyme (Life Technologies). Constructs were verified by Sanger sequencing.

Cell culture and virus production

293T17 cells (ATCC) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and amphotericin B (HyClone). To produce murine retrovirus, 293T17 cells were cotransfected with pEcopac helper packaging plasmid and MSCV-IRES-GFP as an empty vector or containing wild-type (WT) or mutant versions of ALK. Viral supernatants were harvested 48 hours after transfection.

Ba/F3 cells were grown in RPMI (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), amphotericin B (HyClone), and 15% WEHI conditioned medium (a source of IL3). Ba/F3-mutant lines were generated by spinoculation with viral supernatants and polybrene at 2500 rpms for 90 minutes. GFP-positive infected cells were then sorted using a FACSAria flow cytometer.

Immunoblot analysis

Immunoblot analysis was performed on the Ba/F3 cell lines as described previously (11). The primary antibodies used were rabbit anti-ALK (Cell Signaling Technology; #3333) and Rabbit anti-GAPDH (Santa Cruz Biotechnology; #25778).

IL3-independent assays

Ba/F3 cells expressing either WT ALK or the ALK mutations were generated by retroviral infection. Ba/F3 lines expressing WT ALK or ALK mutants were washed three times in RPMI medium with 10% FBS (to remove all traces of IL3-containing WEHI conditioned medium). Cells were plated at 5×10^5 cells per mL and counted on a Guava personal flow cytometer every other day and divided as necessary.

Colony assays

Bone marrow was isolated from BALB/c mice, cells were stimulated overnight with IL3, IL6, and stem cell factor, and then spinoculated with ALK-expressing retrovirus, polybrene, and Hepes buffer on 2 successive days. Cells were plated in methylcellulose (Stem Cell Technologies, M3234) and then counted on day 7 after plating. Animal work was conducted in accordance with OHSU Institutional Animal Care and Use Committee protocol number IS00002726.

Table 1. Summary of IC_{50} values for inhibition of Ba/F3 cell growth by ALK inhibitors calculated from data presented in Figs. 2 and 3

	IC_{50} (nmol/L)	
	A348D	**F856S**
Crizotinib	33	6
NVP-TAE684	2.5	0.5
AP26113	3	0.6
LDK378	22	4
GSK1838705	18	2

Results and Discussion

Identification of ALK point mutations in leukemia patient samples

Deep sequencing of 1,862 kinase and kinase-associated genes in 185 leukemia samples revealed oncogenic point mutations in ALK in specimens from two patients diagnosed with hematologic malignancies. The first specimen from a pediatric patient with B-ALL exhibited an ALK A348D mutation that resides within the MAM1 domain of ALK (Fig. 1A). The other specimen was obtained from an adult patient with AML and exhibited an ALK F856S point mutation within the glycine-rich domain (Fig. 1A). The presence of both of these mutations was confirmed by Sanger sequencing, which showed that both mutations were heterozygous (Fig. 1B and C). Interestingly, the B-cell acute lymphoblastic leukemia (B-ALL) sample also harbored the NRAS mutations, G12C and G12S. Oncogenic mutations of known significance were not found in the AML sample, but it should be noted that the capture library is focused on kinase-associated genes, and there may therefore be other mutations present that were not assessed in this platform.

Leukemia-associated ALK point mutations exhibit oncogenic capacity

To determine whether the ALK mutations identified in AML and B-ALL patient specimens carried oncogenic capacity, we employed a cytokine-independent transformation assay. The murine pro–B-cell Ba/F3 cell line normally requires the cytokine IL3 for growth. In the presence of certain transforming oncogenes, Ba/F3 cells can grow indefinitely in the absence of IL3. Both ALK A348D– and

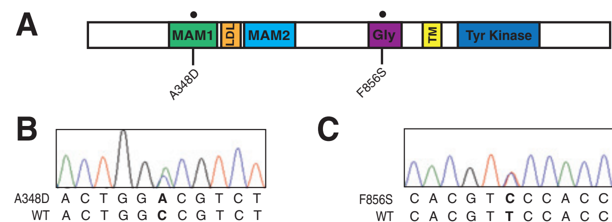

Figure 1.
Identification of ALK mutations in leukemia patient samples. A, schematic of the location of the ALK mutations identified by deep sequencing. The location of the following domains is included: MAM1, LDL-A (LDL), MAM2, glycine-rich (Gly), transmembrane (TM), and tyrosine kinase. B and C, Sanger sequencing confirms that tumor cells from a pediatric B-ALL sample harbored the ALK A348D mutation in the MAM1 domain, and tumor cells from a patient with adult AML exhibited the ALK F856S mutation in the glycine-rich domain.

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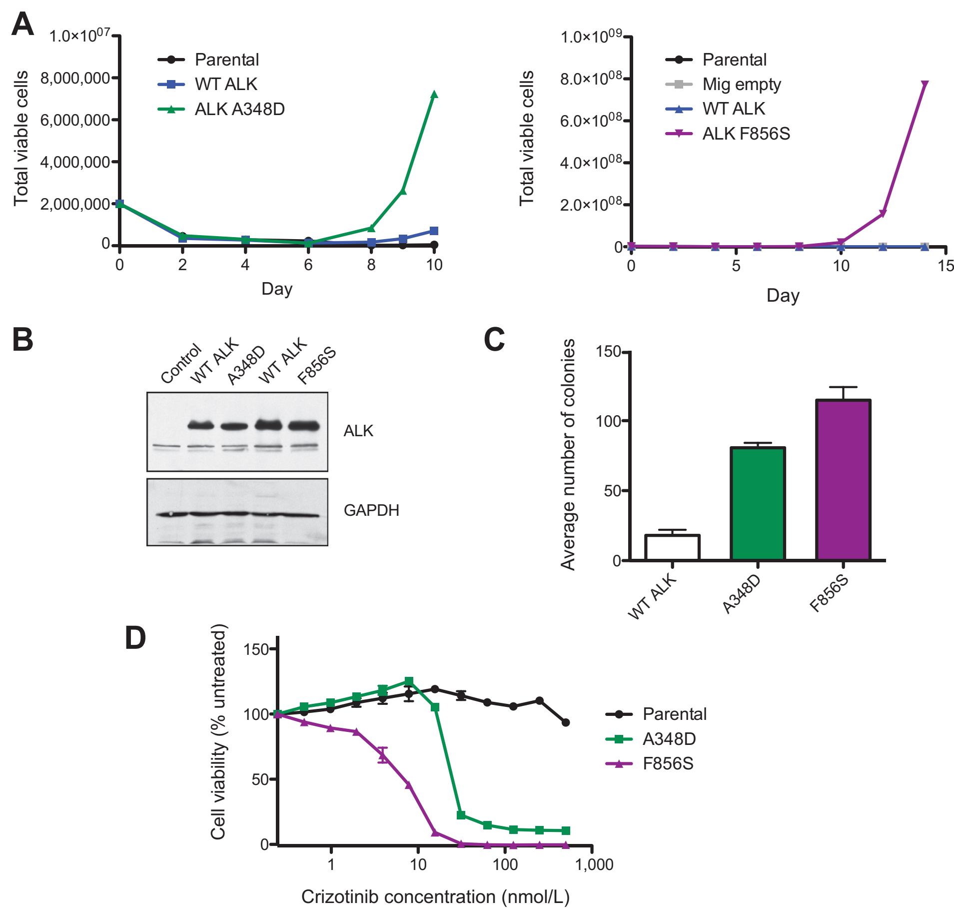

Figure 2.

ALK point mutations found in leukemia samples are oncogenic and sensitive to crizotinib. A, the ALK A348D and F856S mutations transform the murine Ba/F3 pro–B cell line to cytokine-independent growth. Ba/F3 cells expressing WT ALK, ALK A348D, F856S, Ba/F3 cells harboring an empty vector (Mig empty), or parental Ba/F3s were grown in the absence of the cytokine IL3. Total viable cells were plotted over time. B, ALK mutations are expressed at the same level as WT ALK. Immunoblot analysis of Ba/F3 cells expressing ALK mutations along with their respective WT ALK controls from each Ba/F3 IL3 withdrawal experiment. GAPDH served as a loading control. C, ALK point mutations induce colony formation in mouse bone marrow. Mouse bone marrow cells were infected with retrovirus expressing WT ALK, A348D, or F856S and then plated in colony formation medium in the absence of cytokines. The average number of colonies formed from three replicates are shown. Error bars, SEM. D, ALK point mutant–expressing Ba/F3 cells are sensitive to crizotinib. IL3-independent Ba/F3 cells expressing the ALK A348D or F856S mutations were treated with crizotinib in triplicate. Cell viability was determined using a tetrazolamine-based viability assay. Viability is represented as a percentage of the untreated control. The mean of three replicates is shown, along with the standard error.

ALK F856S–mutant-expressing Ba/F3 cell lines proliferated in the absence of IL3, whereas overexpression of WT ALK did not confer IL3-independent growth (Fig. 2A). The mutant forms of ALK were expressed at similar levels to WT ALK (Fig. 2B). To confirm that these mutations carried transformative capacity by an independent assay, we infected murine bone marrow with ALK mutants and tested their ability to form colonies in methylcellulose. The ALK A348D and F856S mutations were able to induce mouse bone marrow colony formation in the absence of exogenous cytokines, providing additional evidence that these mutations have oncogenic capacity (Fig. 2C).

We next wanted to determine if ALK was expressed in these samples. Although material was not available for expression analysis from the AML sample, we were able to analyze ALK expression in the B-ALL sample with the ALK A348D mutation by Affymetrix exon arrays. We utilized a set of 25 samples, which had expression measurements using the Affymetrix Exon array. To determine expression, we used a metric known as the probeset detected above background measure (PSDABG), which tests whether a given probeset displays significantly higher intensity than background regions with similar GC content (12, 13). We computed the PSDABG measure for the 40 probesets belonging to the high-confidence "core" annotation group defined by Affymetrix. We found that 25 of 40 (62.5%) of the probesets exhibited a PSDABG value of <0.05, with 2 of them surviving a Bonferroni correction (Supplementary Fig. S1). As each probeset was designed to target an exon, this suggests that the majority of the exons of ALK are expressed above background for this sample.

ALK inhibitors are being used therapeutically for patients with ALK rearrangements in lung cancer (9, 14). We tested the most clinically advanced ALK inhibitor crizotinib against the ALK-mutant Ba/F3 cell lines. The growth of Ba/F3 cells expressing both mutants was highly sensitive to crizotinib, with IC_{50} of 33 nmol/L for ALK A348D cells and 6 nmol/L for the ALK F856S cells (Fig. 2D; Table 1).

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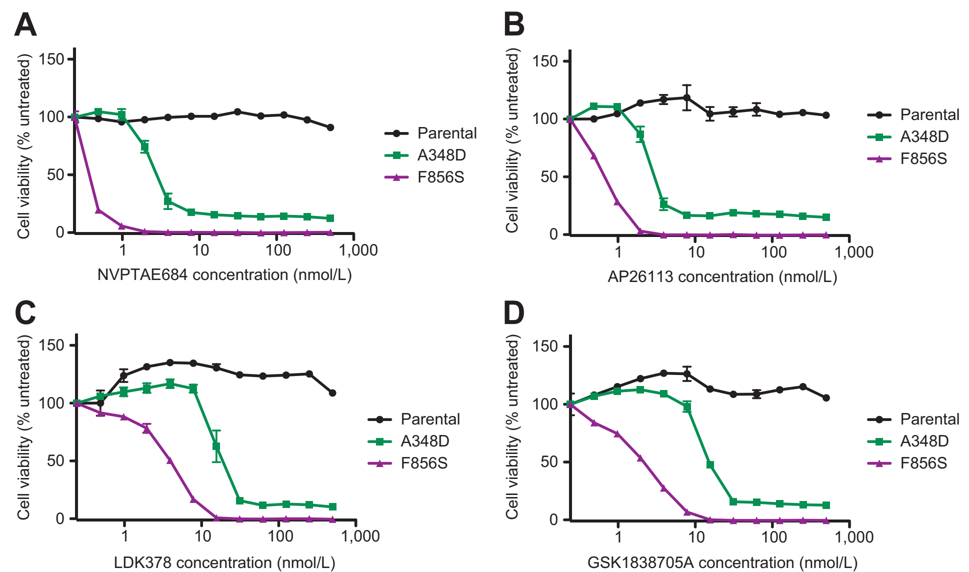

Figure 3.
The ALK A348D and F856S mutations confer sensitivity to ALK inhibitors. IL3-independent Ba/F3 cells expressing the ALK A348D or F856S mutations were treated with NVPTAE684 (A), AP2631 (B), LDK378 (C), or GSK1838705A (D). Parental BaF3 cells in IL3-containing medium were used as a control. All drugs were used at the following concentrations: 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.98, 0.49, and 0 nmol/L. Cell viability was determined using a tetrazolamine-based viability assay. Viability is represented as a percentage of the untreated control. The mean of three replicates is plotted, along with the standard error.

ALK point mutations are sensitive to multiple ALK inhibitors

To further test the sensitivity of the ALK A348D and F856S mutations to ALK inhibitors, we used four additional ALK inhibitors. The NVP–TAE684 compound was developed as a potent and specific inhibitor of NPM–ALK fusion that blocks the proliferation of lung cancer cell lines harboring ALK gene fusions (15). NVP–TAE684 inhibited the cell survival and proliferation of Ba/F3 ALK A348D and ALK F856S cells with IC_{50} of 2.5 nmol/L and approximately 0.5 nmol/L, respectively. The second-generation ALK inhibitor AP26113 was developed to overcome crizotinib resistance (14) mediated by the emergence of ALK L1196M mutation (16, 17) and is in phase I/II development. Treatment of ALK mutant–transformed Ba/F3 cells with AP26113 potently inhibited cell growth with an IC_{50} of 3 nmol/L for the A348D mutant and 0.6 nmol/L for the F856S mutant.

LDK378 is highly selective for ALK with 30- to 40-fold sensitivity for ALK over IGF1R and INSR, and shows an IC_{50} for growth of NPM–ALK-transformed Ba/F3 cells at 26 nmol/L as well as an IC_{50} of 0.2 nmol/L in cell-free kinase assays (18). This compound inhibited ALK A348D cell growth with an IC_{50} of 22 nmol/L and F856S cell growth with an IC_{50} of 4 nmol/L. Finally, the GSK1838705A compound is potent against IGF1R, INSR, and ALK (with an IC_{50} of 0.5 nmol/L; ref. 19). It was also highly potent in inhibiting growth of ALK-transformed cell lines with IC_{50} of 18 nmol/L and 2 nmol/L for the A348D and F856S cells, respectively. In aggregate, these data show that the ALK point mutations found in leukemia patient samples are highly sensitive to a variety of inhibitors directed against ALK (Fig. 3; Table 1).

The identification of oncogenic ALK point mutations in acute leukemia (AML and B-ALL) is unexpected, given that ALK fusions are exceedingly rare in leukemia. RANB2–ALK fusions have been detected in an adult patient with acute myelomonocytic leukemia (20), and three cases of childhood myeloid leukemia (21). Here, we demonstrate that these ALK point mutations are potently oncogenic. It is interesting that both mutations reside in the extracellular domain of ALK. In neuroblastoma, ALK point mutations are found primarily in the kinase domain (2), while they can occur in both the kinase domain and the extracellular domain in patients with lung cancer, although due to the lack of expression of ALK in the lung, the significance of these mutations to cancer progression is not yet clear (22). These extracellular domain mutations have been identified in both the MAM domains and the glycine rich domain, similar to the mutations described here (22). The extracellular mutations identified in lung cancer were able to increase tumor burden in xenograft models (22). These extracellular domain mutations had variable activation of downstream STAT3, AKT, and ERK signaling pathways (22). Although the function of the MAM and glycine-rich domains is still under investigation, it is clear that these regions are important for ALK's role in the proper development of the Drosophila gut (23). Interestingly, the neuroblastoma cell line NB1 had an activating deletion in the first MAM domain, leading to increased STAT3 signaling, possibly due to receptor mislocalization (24). Future structural studies of the extracellular regions of ALK would provide a framework for analysis of the structural impact of individual point mutations. Although oncogenic, the ALK mutations likely require other cooperating mutation in the progression to leukemia, as evidenced by the presence of known oncogenic NRAS mutations in the B-ALL sample.

The ALK A348D and F856S extracellular mutations were highly sensitive to the approved ALK inhibitor crizotinib and a variety of other ALK inhibitors in clinical development. As we move toward individualized cancer therapy based on mutational profiles of patient tumors, ALK point mutations, although present in a small percentage of samples, represent an exciting therapeutic target. Characterization of additional ALK mutations and demonstration of clinical efficacy will be required to fully implement ALK-directed therapy for leukemia patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.E. Maxson, S.K. McWeeney, J.W. Tyner
Development of methodology: J.E. Maxson, M.A. Davare, S.B. Luty, S.K. McWeeney, J.W. Tyner
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Maxson, S.B. Luty, B.H. Chang, B.J. Druker, J.W. Tyner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.E. Maxson, S.B. Luty, C.A. Eide, D. Bottomly, B. Wilmot, S.K. McWeeney, J.W. Tyner

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