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Activating mutations in *ALK* provide a therapeutic target in neuroblastoma

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Abstract

Neuroblastoma, an embryonal tumor of the peripheral sympathetic nervous system, accounts for approximately 15% of all deaths due to childhood cancer1. High-risk neuroblastomas, prevalent in the majority of patients, are rapidly progressive; even with intensive myeloablative chemotherapy,

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relapse is common and almost uniformly fatal2,3. Here we report the detection of previously unknown mutations in the *ALK* gene, which encodes a receptor tyrosine kinase, in 8% of primary neuroblastomas. Five non-synonymous sequence variations were identified in the kinase domain of *ALK*, of which three were somatic and two were germline. The most frequent mutation, F1174L, was also identified in three different neuroblastoma cell lines. *ALK* cDNAs encoding the F1174L and R1275Q variants, but not the wild-type *ALK* cDNA, transformed IL-3-dependent murine hematopoietic Ba/F3 cells to cytokine-independent growth. Ba/F3 cells expressing these mutations were sensitive to a small-molecule inhibitor of ALK, TAE6844. Furthermore, two human neuroblastoma cell lines harboring the F1174L mutation were sensitive to the inhibitor. Cytotoxicity was associated with increased levels of apoptosis as measured by TUNEL-labeling. shRNA-mediated knockdown of ALK expression in neuroblastoma cell lines with the F1174L mutation also resulted in apoptosis and impaired cell proliferation. Thus, activating alleles of the ALK receptor tyrosine kinase are present in primary neuroblastoma tumors and in established neuroblastoma cell lines, and confer sensitivity to ALK inhibition with small molecules, providing a molecular rationale for targeted therapy of this disease.

In a genome-wide analysis of primary neuroblastomas using single-nucleotide polymorphism (SNP) arrays, we noted high-level amplification of the *ALK* (anaplastic lymphoma kinase) gene5. To determine the frequency of this amplification, we analyzed 94 tumors with *MYCN* amplification by fluorescence *in situ* hybridization (FISH), and documented 14 (15%) with concomitant *ALK* amplification (Supplementary Fig.1), which was not detected in 51 tumors without *MYCN* amplification (p=0.0016). None of the tumors had *ALK* rearrangements, such as those that have been found in other tumor types with *ALK* translocations6–9.

We reasoned that in tumors without *ALK* amplification or translocation, acquired somatic mutations or germline sequence variants might contribute to oncogenicity. DNA re-sequencing of the *ALK* open reading frame in primary neuroblastomas identified 5 novel non-synonymous sequence variations in conserved positions in the tyrosine kinase domain in 7 of 93 samples (8%; Table 1; Supplementary Fig.2). None of these variants were previously identified SNPs or known somatic mutations, based on analysis of dbSNP and Sanger databases or by genotyping of 270 samples derived from the International Hap Map Consortium10.

Sequence analysis of matched normal samples from these patients revealed that 2 of the sequence variants were germline and 3 represented somatically acquired mutations (Table 1). The most common mutation, identified in 4.3% (4/93) of the primary tumors, was a recurrent cytosine-to-adenine change in exon 23 that results in a phenylalanine-to-leucine substitution at codon 1174 (F1174L) within the kinase domain. Most of the patients with somatic *ALK* mutations had metastatic disease characterized by *MYCN* amplification, although one patient with the F1174L mutation had localized disease with favorable histology and unamplified *MYCN* (Supplementary Table 1). Four of the 5 *ALK* mutations involve residues that correspond to those affected by known activating mutations in the *EGFR* gene11–14 (Supplementary Figs.2 & 3). The F1174 residue corresponds to V769 in EGFR, which is in a region of frequent mutation in both EGFR11 and ERBB212 genes. The

F1245C mutation corresponds to L833V in EGFR, a gefitinib-resistant mutation in lung cancer (Greulich & Meyerson, unpublished observations). The R1275Q mutation is located adjacent to L858R in EGFR, which is the most common EGFR mutation in lung cancer13,14.

The functional consequences of four of the mutations, T1151M, F1174L, A1234T, and R1275Q, were determined by testing their abilities to transform interleukin-3 (IL-3)-dependent murine lymphoid Ba/F3 cells to cytokine-independent growth. Reduction in IL-3 concentration by 100-fold to 0.01 ng/ml resulted in a clear difference in cell proliferation, with the Ba/F3 cells expressing F1174L and R1275Q mutations exhibiting much higher cell numbers relative to those transduced with wild-type ALK or the T1151M mutation (Fig 1a). To generate IL3-independent lines, we reduced the IL-3 concentration by half in successive passages of each transduced Ba/F3 line. After 5 passages, the Ba/F3 cells expressing the F1174L and the R1275Q ALK mutations, as well as *NPM-ALK*, were able to grow in medium completely lacking IL-3, while cells expressing T1151M or wild-type ALK did not survive. Moreover, when expressed in Ba/F3 cells, the F1174L allele, and to a lesser extent, the R1275Q allele, were associated with constitutive phosphorylation of ALK (Fig. 1b). In contrast, neither the T1151M nor A1234T alleles exhibited ALK phosphorylation. Expression of the F1174L ALK protein in IL-3-deprived Ba/F3 cells was also associated with phosphorylation of downstream targets of ALK signaling such as STAT3 and AKT, while R1275Q was associated with phosphorylation of ERK1/2 and AKT (Fig 1b). Together, these studies demonstrate that the ALK mutant proteins F1174L and R1275Q possess gain-of-function kinase activity that can sustain key signaling pathways in the presence of reduced concentrations of IL-3.

The Ba/F3 assay has been validated for a broad spectrum of oncogenic tyrosine kinase alleles including mutant EGFR15 and FLT316, and thus we treated Ba/F3 cells expressing each of the ALK mutations with increasing concentrations of TAE684, a highly potent ALK inhibitor4,17,18. The activating mutation, F1174L was found to be extremely sensitive to TAE684, with an IC_{50} of 8 nM, identical to that of NPM-ALK-expressing Ba/F3 cells. The R1275Q mutation was also sensitive to TAE684, albeit with a much higher IC_{50} of 328 nM. In contrast, Ba/F3 cells expressing FLT3-ITD or wild-type ALK, did not respond to TAE684 (IC_{50} 4.5 μM; Fig 1c).

Analysis of the *ALK* gene in a panel of 30 neuroblastoma cell lines revealed sequence variants in 6, including 3 different cell lines containing the F1174L mutation (Kelly, SH-SY5Y and LAN-1), which was also the most common mutation in the primary tumors (Table 1). An R1275Q mutation, identical to the one found in primary sample 411, was also detected in the SMS-KCNR cell line. We observed dose-dependent growth inhibition of the SH-SY5Y (F1174L) and Kelly (F1174L) neuroblastoma cell lines with increasing concentrations of TAE684, (IC_{50s} of 258 and 416 nM respectively; Fig. 2a). These results are in agreement with data from a recent study showing sensitivity of these cell lines to TAE68419. Of note, the SMS-KCNR cell line expressing the ALK R1275Q mutation was resistant to TAE684 (IC_{50} of 4.9 μM; Fig. 2a), even though Ba/F3 cells expressing this mutation became IL-3 independent and were sensitive to the inhibitor (Fig. 1c). Neuroblastoma cell lines without *ALK* mutations, including IMR-5, were also resistant to

TAE684 (Fig. 2a and Supplementary Fig. 4a). Treatment with TAE684 (200 nM) resulted in increased apoptosis in Kelly (F1174L) and SH-SY5Y (F1174L) cells, but not in the SMS-KCNR (R1275Q) and IMR-5 (WT) cells (Fig. 2b). Cytotoxicity was also associated with G1-phase arrest and substantial reductions in S-phase cell fractions (Supplementary Fig. 4b).

After treatment with TAE684 (100nM), the sensitive cell lines SH-SY5Y (F1174L) and Kelly (F1174L) demonstrated reduced phosphorylation of ALK, and of ERK1/2 and AKT and to a lesser extent, STAT3 (Supplementary Fig 4c). By contrast, there was no apparent effect on phospho-AKT and STAT3 in the resistant cell line IMR-5 (WT), although there was a slight reduction in phospho-ERK1/2. Moreover, knockdown of ALK in the Kelly and SH-SY5Y cell lines (F1174L), but not the inhibitor-resistant SMS-KCNR line (R1275Q), was associated with a reduction in cell proliferation and increased apoptosis (Fig. 2c, d). In neuroblastoma, ALK is detected as both a 220 kDa protein, reflecting the glycosylated protein encoded by the transduced ALK cDNA and a second protein of ~140 kDa (inset, Fig. 2c), which has been documented by multiple investigators and most likely represents an as-yet-uncharacterized splice variant-encoded isoform of ALK20–22. The resistance of SMS-KCNR cell line is apparently not due to any difference between the R1275Q and F1174L mutations in ALK activation *per se*, because both of these mutations transform Ba/F3 cells to IL-3 independence and the transformed cells respond to the inhibitor (Fig 1c). Rather, we suspect that other molecular aberrations, such as coactivation of other receptor tyrosine kinases23,24, could have been acquired during culture that render this cell line independent of activated ALK for growth and viability.

We observed that ALK was expressed at substantially lower levels in the TAE684-sensitive neuroblastoma cell lines [Kelly (F1174L), and SH-SY5Y (F1174L)] than in the remaining cell lines harboring either wild-type *ALK* or the R1275Q mutation (Fig. 3a). However, TAE684 inhibition of ALK kinase activity resulted in an increase in the ALK protein level in the sensitive Kelly (F1174L) cell line, but not in IMR-5 (WT) (Fig. 3b). Blockade of protein degradation by the proteasome inhibitor MG-132 resulted in increased ALK levels in Kelly (F1174L) cells, but not in IMR-5 cells (Fig 3c), consistent with a higher turnover rate in cells with the constitutively activated mutant ALK protein.

The studies reported here demonstrate previously unrecognized activating mutations affecting critical residues within the ALK kinase domain and indicate that ALK has potential as a novel therapeutic target in neuroblastoma. Our results with the ALK small-molecule inhibitor, TAE684, demonstrate that most of the neuroblastoma cell lines harboring activating ALK mutations are dependent on the altered ALK protein for survival. One exception is the activating R1275Q allele, which when expressed by itself in Ba/F3 cells is sensitive to treatment with TAE684, but not in the SMS-KCNR neuroblastoma background. This observation, together with the lack of transforming activity of the T1151M and the A1234T alleles, supports the emerging concept that mutations affecting critical domains of cancer genes must be studied both genetically and functionally to validate their potential as therapeutic targets16. Thus, it will be important to test the ability of each mutant ALK protein identified in patient tumor samples to confer IL-3 resistance in Ba/F3 cells and mediate sensitivity to ALK inhibitory drugs as they move into therapeutic trials. One sensitive cell line, Kelly (F1174L), harbored both an activating *ALK* mutation and *MYCN*

amplification, suggesting that a subset of very high risk *MYCN*-amplified neuroblastomas may respond to treatment with an ALK inhibitor. Although established neuroblastoma cell lines with high levels of wild-type ALK expression did not respond to ALK inhibitors in our study, it will be important to evaluate whether the larger group of neuroblastoma patients expressing wild-type ALK proteins have responses when ALK inhibitors are tested *in vivo* in the natural tumor microenvironment, which includes exposure to the effects of ALK ligands25,26.

METHODS SUMMARY

DNA sequencing

Primers were designed to cover the 29 exons of *ALK* and characterized using 3 Coriell DNAs. Passing primers and samples were PCR amplified and sequenced bidirectionally on an ABI 3730xl automated sequencer (Applied Biosystems). Automated analysis and coverage statistics were generated with SNP Compare [(an in-house package using PolyPhred27 and PolyDHAN (D. Richter et al., manuscript in preparation)]. Bidirectional sequence traces were analyzed with Mutation Surveyor (SoftGenetics, version 3.10) and manual review. Genotyping was performed by primer extension mass spectrometry.

Cell culture

Neuroblastoma cell lines were cultured in RPMI-1640 containing L-glutamine and 10% fetal bovine serum (FBS; Sigma-Aldrich). Ba/F3 cells were maintained in RPMI-1640 supplemented with 10% FBS and 0.5 ng/ml murine IL-3 (Millipore).

DNA constructs and retrovirus production

ALK mutations were engineered using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The mutant cDNAs were subcloned into the pMSCV-Neo-luc retroviral vector. Cotransfection of 293T cells and infection of Ba/F3 cells with retroviral supernatants were performed as described previously16. Transduced Ba/F3 cells were selected with G418 for 7 days and subjected to Ficoll separation to isolate surviving cells.

Cytokine independence assays

Ba/F3 cells transduced with each of the pMSCV-Neo-luc constructs were seeded at 1×10^5/ml and treated with 1, 0.1, 0.01 ng/ml IL-3 for 72 hours. The number of viable cells was determined by trypan blue exclusion using a Vi-CELL Series Cell Viability Analyzer (Beckman Coulter).

Drug sensitivity assay

Cell viability was tested 72 hours following addition of the compound by CellTiter-Glo Luminescent Cell Viability Assay (Promega). IC_{50s} were calculated by nonlinear regression (variable slope) using Graphpad Prism 5 software.

Immunoblotting

Immunoblotting was performed as described previously16. Antibodies are listed in Full Methods.

Proteasome inhibitor treatment

1×10^6 cells were treated with the proteasome inhibitor MG-132 (Sigma-Aldrich) for 1 hour, washed in PBS and immunoblotting performed as described previously16.

Full Methods

are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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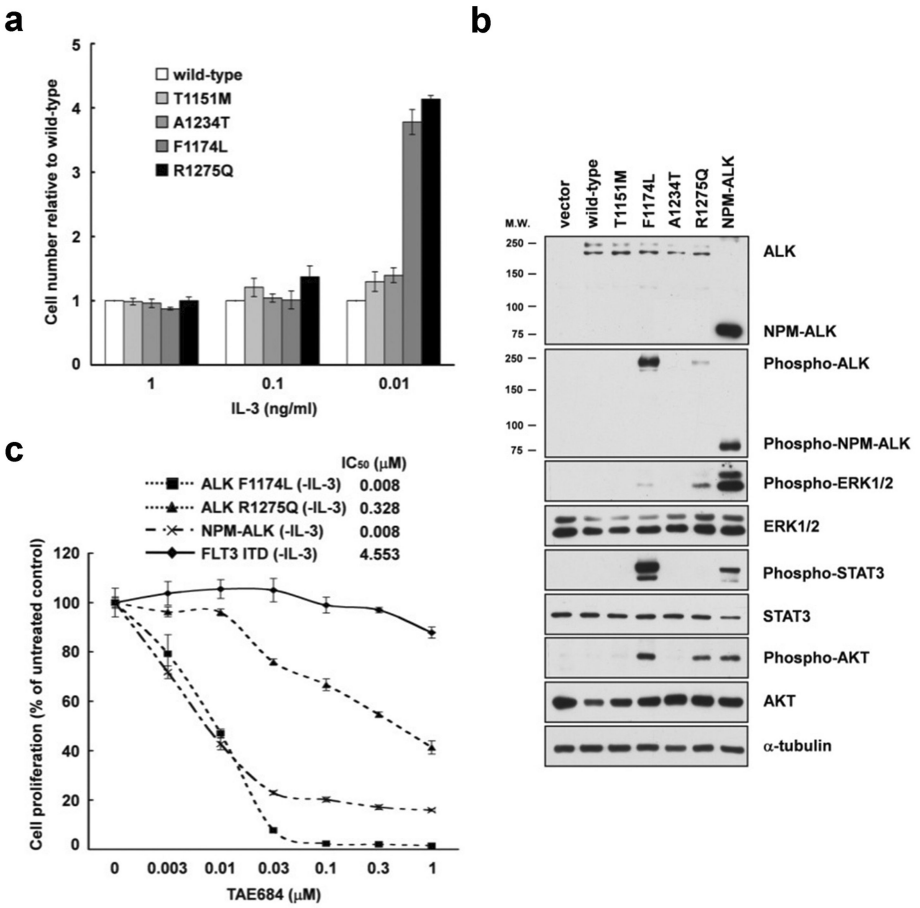

Figure 1. *ALK* mutant alleles F1174L and R1275Q are activating in Ba/F3 cells and are sensitive to pharmacologic inhibition

a, Growth of Ba/F3 cells expressing wild-type or mutant ALK in 10-and 100-fold-reduced concentrations of IL-3. The values are means ± standard deviations (SD) of triplicate experiments. **b,** Western blot analysis of ALK proteins and their downstream effectors in wild-type or mutated ALK-expressing Ba/F3 cells depleted of IL-3 for 6 hours. The mobilities of molecular weight (M.W.) standards are shown on the left. **c,** Growth of mutated ALK-expressing Ba/F3 cells exposed to TAE684 for 72 hours. The values are means ± SD of triplicate experiments.

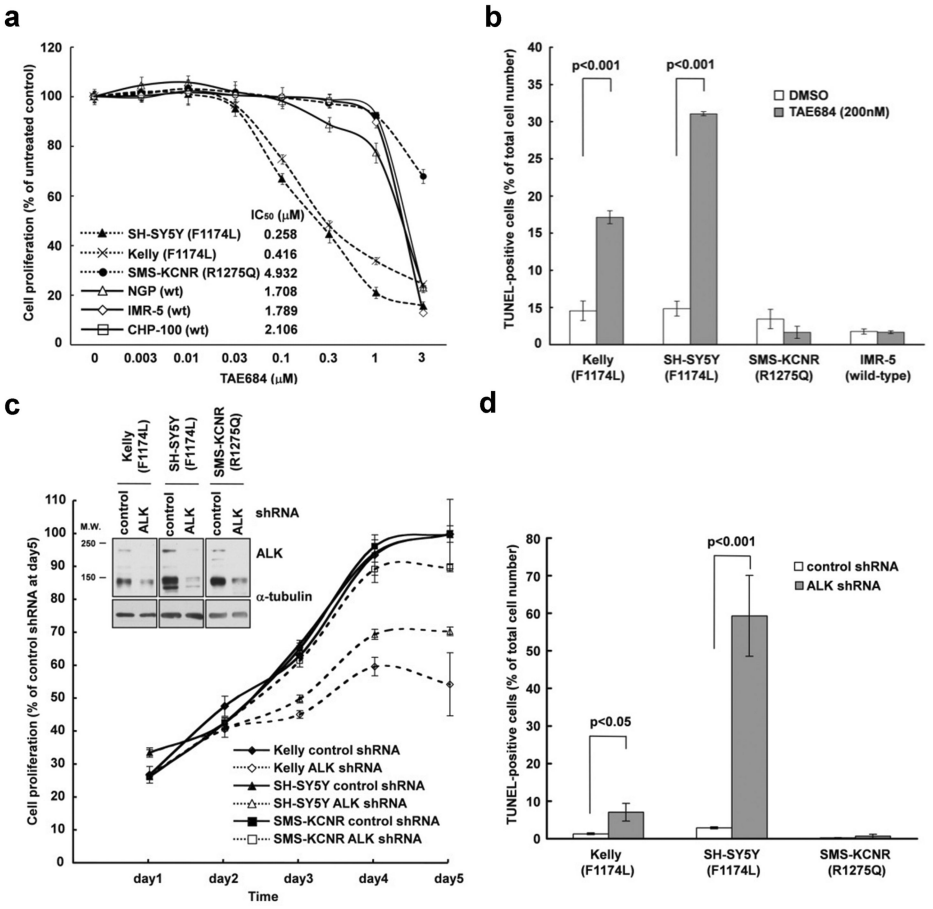

Figure 2. Neuroblastoma cell lines harboring the F1174L, but not the R1275Q ALK mutation, are dependent on the altered protein for growth and survival

a, Growth rates of neuroblastoma cell lines with and without ALK mutations after a 3-day exposure to varying concentrations of TAE684. The values are means ± SD of triplicate experiments. **b,** Induction of apoptosis in the TAE684-sensitive and resistant cell lines as determined by TUNEL assay. **c,** Growth of ALK-mutant neuroblastoma cell lines Kelly and SH-SY5Y (F1174L) and SMS-KCNR (R1275Q) in which ALK expression was downregulated using shRNA. The inset panel shows western blot analysis of ALK expression in the control and shRNA transduced lines. The mobilities of molecular weight (M.W.) standards are shown on the left. **d**, Induction of apoptosis by ALK shRNA knockdown as determine by TUNEL assay.

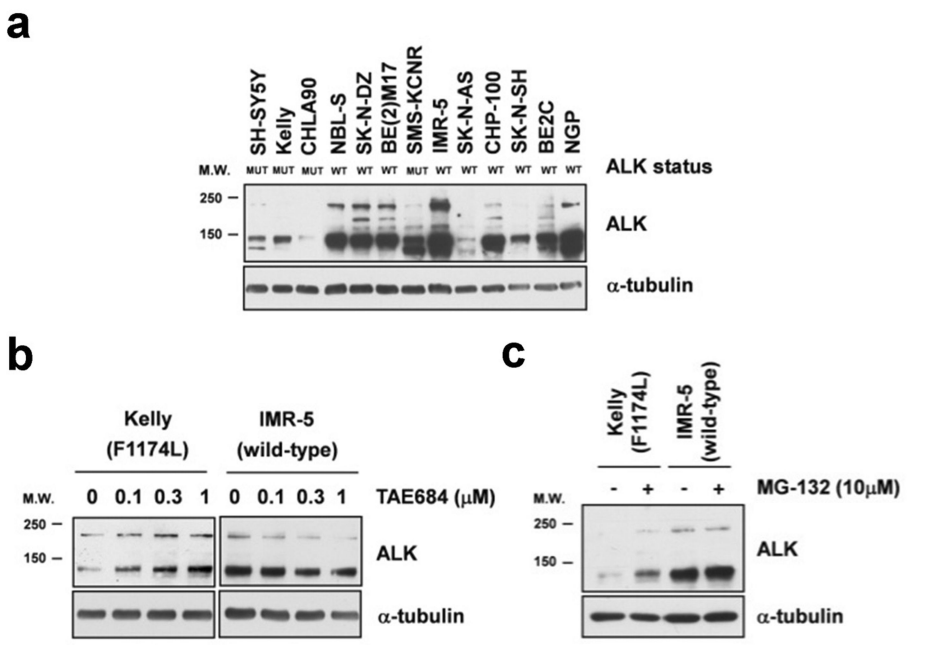

Figure 3. The constitutively activated F1174L ALK protein has a higher turnover rate than wild-type ALK in neuroblastoma cell lines

a, Analysis of ALK expression in ALK wild-type and mutated neuroblastoma cell lines. **b,** Western blot depicting ALK expression in the mutated cell line Kelly (F1174L), and in the ALK wild-type line IMR-5 following exposure to increasing doses of TAE684. **c,** Western blot of ALK expression in the ALK mutated cell line Kelly (F1174L), and in IMR-5, harboring wild-type ALK, following treatment with the proteasome inhibitor MG-132.

Table 1

Nonsynonymous Sequence Variants of *ALK* in 93 patients and 30 cell lines.

Patient sample	Exon	DNA	Protein	Domain	Germline/ Somatic
443	22	C3452T	T1151M	kinase	germline
472	23	C3522CA	F1174L	kinase	somatic
1034	23	C3522CA	F1174L	kinase	somatic
1110	23	C3522CA	F1174L	kinase	somatic
50	23	C3522CA	F1174L	kinase	somatic
50	24	G3700GA	A1234T	kinase	somatic
157	24	T3734TG	F1245C	kinase	somatic
411	25	G3824GA	R1275Q	kinase	germline
Cell line	**Exon**	**DNA**	**Protein**	**Domain**	
LAN 6	20	G3271A	D1091N	juxtamembrane	
Kelly	23	C3522CA	F1174L	kinase	
SH-SY5Y	23	C3522CA	F1174L	kinase	
LAN 1	23	C3522CA	F1174L	kinase	
CHLA 90	24	T3733G	F1245V	kinase	
SMS-KCN R	25	G3824GA	R1275Q	kinase	

(Sequence numbering follows the Ensemble Transcript/Peptide ID: ENST00000389048).