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Identification and characterization of *ALK* kinase splicing isoforms in non-small-cell lung cancer

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Abstract

Purpose: Anaplastic lymphoma kinase (*ALK*) rearrangements are present in an important subset of non-small-cell lung cancer (NSCLC) and predict for response to the tyrosine kinase inhibitor crizotinib. In this study, we evaluated the yet unknown frequency and functional role of *ALK* splicing isoforms in NSCLC.

Experimental Design: We analyzed 270 cases of NSCLC for *ALK* kinase domain splicing aberrations, and in addition generated constructs with full length *EML4-ALK* (E13;A20) and a splicing isoform.

Results: Splicing isoforms of the kinase domain of *ALK* - including complete skipping of exon 23 (*ALK*del23, ALK p.I1171fs*42) and exon 27 (*ALK*del27, ALK p.T1312fs*0) - were identified in 11.1% (30/270 cases) of NSCLC, and these changes co-existed with *ALK* rearrangements, *KRAS* mutations and *EGFR* mutations. *ALK* splicing isoforms were observed with full length *EML4-ALK* in crizotinib-naïve and treated NSCLCs. ALK T1312fs*0 was unable to render cells solely dependent on ALK signaling. Unlike EML4-ALK and EML4-ALK p.L1196M, EML4-ALK T1312fs*0 did not autophosphorylate ALK or other phospho-tyrosine sites. Co-expression

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of equal amounts of EML4-ALK T1312fs*0 and EML4-ALK did not result in resistance to crizotinib, while co-expression of EML4-ALK L1196M with EML4-ALK resulted in resistance to inhibition of ALK by crizotinib.

Conclusions: *ALK* kinase splicing isoforms were present in NSCLC and even if translated seemed to be non-functional variants of ALK.

Keywords

lung cancer; non-small-cell lung cancer; tyrosine kinase; kinase inhibitor; anaplastic lymphoma kinase; ALK; crizotinib; alternative splicing; exon skipping; exon 27; exon 23

INTRODUCTION

Anaplastic lymphoma kinase (*ALK*) rearrangements, either inversions or translocations, are present in approximately 5% of all non-small-cell lung cancers (NSCLCs) at the time of diagnosis [1]. The most frequent inversions event occur within the short arm of chromosome 2 and result in the fusion of the echinoderm microtubule-associated protein-like 4 (*EML4*) with *ALK* [2,3]. The most common *EML4-ALK* fusion variants are E13;A20 and E20;A20 [4].

EML4-ALK variants are transforming tyrosine kinases that activate the phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) downstream pathways [5,6]. Tumors with *EML4-ALK* and other *ALK* rearrangements are oncogene addicted to ALK signaling and can be inhibited by ALK tyrosine kinase inhibitors (TKIs) in preclinical models [5,7,8]. In the clinic, NSCLCs with *ALK* rearrangements are sensitive to the multitargeted ALK TKI crizotinib [9] and this drug received approval by the Food and Drug Administration (FDA) in 2011 to be used in this NSCLC subtype [1]. The reported response rates exceed 60% with a mean progression-free survival of over 7-10 months prior to acquisition of resistance to crizotinib monotherapy [10,11].

ALK kinase domain mutations and non-kinase domain splicing variants, which are present in a subset of neuroblastomas [12], have not been reported in ALK TKI-naïve NSCLCs. Our group reports herein the identification and characterization of splicing isoforms of the kinase domain of *ALK*.

MATERIALS AND METHODS

Patient and tumor characteristics, plus statistical methods

268 Chinese patients from the Queen Mary Hospital (University of Hong Kong) and 2 patients from the Beth Israel Deaconess Medical Center with NSCLC were studied. Approval of this study was obtained from ongoing Institutional Review Board (IRB) approved protocols at these institutions. Clinical, pathologic and molecular characteristics were obtained retrospectively from extraction of medical records [13]. Fisher's exact test was performed to compare categorical variables and Student's t-test for continuous variables.

Sequencing of *ALK*

Total DNA and RNA were extracted using standard techniques. The expression of *ALK* kinase domain was initially screened by reverse transcriptase PCR using forward (5'-GACCATCATGACCGACTACAA-3') and reverse (5'-AGTGGACCATATTCTATCGGC-3') primers flanking *ALK* exons 20 and 29, respectively. Cases showing splicing variations were confirmed by sequencing. Epidermal growth factor receptor (*EGFR*) mutations and *KRAS* mutations were analyzed as previously published [14,15]. *ALK* rearrangements were identified using *ALK* reverse transcriptase PCR primers or *ALK* FISH [9,16].

Cell culture

COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS). Ba/F3 cell lines were maintained in RPMI-1640 supplemented with 10% FBS and 5% WEHI-conditioned medium as the source of IL-3.

Generation of constructs/vectors with *EML4-ALK* variants and reagents

The pCDNA3.1 *EML4-ALK* E13;A20 was a kind gift of Dr. Henry Koon (Case Western Reserve University, Cleveland, OH). The pCDNA3.1-Myc-His-Tag (Invitrogen, Carlsbad, CA) vector was used as the template for the introduction of *EML4-ALK* E13;A20 (i.e., *EML4-ALK* variant 1) and mutated constructs for COS-7 experiments. The derived constructs *EML4-ALK ALK* p.L1196M, *EML4-ALK ALK* p.T1312fs*0 and *ALK* p.1312fs*0 were generated by site-directed mutagenesis. Retroviral infection and selection of Ba/F3 cells for IL-3 independence were performed using previously described methods [17]. Crizotinib was purchased from LC Laboratories (Woburn, MA), dissolved in dimethyl sulfoxide (DMSO) and stored at −80°C.

Western blotting, immunoprecipitation and antibodies

Cells were lysed and protein extracts obtained for analysis [17]. For immunoprecipitation, protein G agarose/sepharose beads were used for pre-clearing the cell lysates and capturing the immunocomplex after incubation with the immunoprecipitating antibody (Myc). Phospho-ALK (pTyr1096), ALK, phospho-Tyrosine (pTyr100) and Myc-Tag antibodies were purchased from Cell Signaling Technology (Beverly, MA). β-actin was purchased from Santa Cruz Biotechnology (Dallas, TX). Phospho-ALK was diluted 1:500 and all other primary antibodies were diluted 1:1000, while secondary antibodies were diluted 1:10000.

RESULTS

ALK splicing variants with skipping of exons 23 or 27 in NSCLC

We attempted to identify *ALK* kinase domain splicing abnormalities in NSCLC samples by analyzing cDNA derived from 270 cases of NSCLC (Table 1). Interestingly, the *ALK* kinase domain was solely expressed in 113 of 270 (41.85%) tumors (Figure 1A). *ALK* kinase domain (i.e., exons 20 to 28) splicing variants were noted in 11.1% (30/270) of cases. The major changes observed were either complete skipping of exon 27 of *ALK* (*ALK*del27) in 11

cases or complete skipping of exon 23 (*ALK*del23) of *ALK* in 19 cases (Figures 1B and C, respectively). Both splicing isoforms have a nucleotide sequence that leads to early stop codons (Figure 1D): *ALK*del27 to the protein ALK p.T1312fs*0, and *ALK*del23 to ALK p.I1171fs*42.

The clinical, pathological and molecular characteristics of NSCLCs with or without splicing abnormalities of *ALK* were similar (Table 1). *ALK* splicing variants were observed in cases with or without *ALK* rearrangements, *KRAS* mutations and *EGFR* mutations (Table 1). Out of the 4 cases with *ALK* rearrangements and *ALK* splicing variants, one had *EML4-ALK* with *ALK*del27 and 3 had *EML4-ALK* with *ALK*del23 (Table 1). Only the *EML4-ALK*del27 case received crizotinib. Out of the cases with *EML4-ALK*del23, 2 remain free of disease after curative surgical resection and 1 had recurrence 29 months after surgical resection and died prior to crizotinib therapy.

EML4-ALK positive NSCLC with *ALK*del27

The *EML4-ALK* rearranged NSCLC that harbored *ALK*del27 had crizotinib-resistant malignant lymphatic tissue analyzed in our group's attempt to sequence the *ALK* kinase domain of crizotinib-naïve and crizotinib-resistant NSCLCs. The patient had attained significant symptomatic response and minor tumor regression of his intra-thoracic disease best classified as stable disease for 8 months prior to development of crizotinib-resistant lesions [18]. cDNA derived from crizotinib-resistant *EML4-ALK* positive left axillary node tissue was sequenced to encompass exons 20-28 of *ALK*. We identified an overlapping sequence amidst the wild-type *ALK* kinase domain of this *EML4-ALK* fusion, which corresponded to complete skipping of exon 27 of *ALK* (Figure 2A). cDNA isolated from non-malignant lymphoid tissue from the same patient only contained *ALK* and not *EML4-ALK*; and *ALK* T1312fs*0 was not present (data not shown). Interestingly, the crizotinib-naïve tumor from the patient also disclosed *EML4-ALK* with *ALK* T1312fs*0. Sequence of the exon-intron junction of exons 26-27 and 27-28 using DNA isolated from the crizotinib-resistant sample did not yield abnormal sequences.

*EML4-ALK*del27 generates the truncated EML4-ALK T1312fs*0 protein with inability to auto-phosphorylate ALK

Since both *ALK*del27 and *ALK*del23 would potentially lead to truncated proteins lacking the full kinase domain of ALK (Figure 1F), we wanted to understand the biological properties of one of these splicing variants; if translated into protein. We decided to study the skipping of exon 27 of *ALK* (T1312fs*0) in the context of *EML4-ALK*, and to this end generated *EML4-ALK* E13;A20 (*EML4-ALK*) constructs with or without ALK T1312fs*0, and in addition *EML4-ALK* with *ALK* L1196M. The latter is known to represent a crizotinib-resistant gatekeeper mutation [19].

The EML4-ALK and EML4-ALK L1196M constructs expressed proteins of their expected size of close to 117kDa (Figure 2B). However, the EML4-ALK T1312fs*0 protein was identified at a size of ~80kDa (Figure 2B). The latter results indicated that *EML4-ALK*del27 could generate a truncated EML4-ALK protein.

We then decided to study the functional properties of EML4-ALK T1312fs*0. Using an antibody specific for a phosphorylation site within amino acid 1096 (tyrosine 1096) of ALK (which is still present within ALK T1312fs*0), we were only able to detect auto-phosphorylation of ALK in EML4-ALK and EML4-ALK L1196M; while EML4-ALK T1312fs*0 did not have a detectable signal (Figure 2C). We also performed an immunoprecipitation experiment to detect auto-phosphorylation of tyrosine residues within ALK or other proteins bound to EML4-ALK using a phospho-tyrosine antibody. EML4-ALK T1312fs*0 was unable to generate a measurable signal (Figure 2C). These results confirmed that EML4-ALK T1312fs*0 was unable to phosphorylate tyrosine sites and was a non-functional kinase when expressed alone. We attempted to transform a cell line system with ALK T1312fs*0 but it was ineffective in making Ba/F3 cells interleukin-3 (IL-3) independent (data not shown). Since ALK p.I1171fs*42 lacks all the amino acid sites of ALK T1312fs*0 in addition to other areas of the ALK kinase, we can foretell it will similarly be unable to transform cell line systems. EML4-ALK and EML4-ALK L1196M can make Ba/F3 cells IL-3 independent [20,21].

EML4-ALK T1312fs*0 and response to the ALK TKI crizotinib

Although we demonstrated that EML4-ALK T1312fs*0 was potentially non-functional as a “driver” oncogene, we also tested the possibility that this truncated protein could affect the exquisite sensitivity of EML4-ALK towards the ALK TKI crizotinib. When EML4-ALK was transfected alone, we were able to observe that the inhibition of phosphorylation of ALK by crizotinib occurred at doses of 0.1μM (Figure 4C). When we co-transfected equal amounts of EML4-ALK and EML4-ALK T1312fs*0 we observed that the inhibition of phosphorylation of ALK within EML4-ALK was unaffected and crizotinib continued to inhibit ALK at doses of 0.1μM (Figure 4C). When EML4-ALK and EML4-ALK L1196M were co-transfected in equal amounts, then a shift in the pattern of inhibition of phosphorylation of ALK was noted (Figure 5C); confirming that EML4-ALK L1196M is a crizotinib-resistant mutation. Taken together these results led us to speculate that EML4-T1312fs*0 was not involved in the pattern of either sensitivity or resistance of EML4-ALK to crizotinib.

DISCUSSION

We identified *ALK* splicing variants, involving the complete skipping of exons 23 and 27 of *ALK*, in samples with NSCLC. To the best of our knowledge, these results represent the first attempt to understand the biological and clinical significance of *ALK* kinase domain splicing variants in NSCLC. In our cohort of 270 patients, more than 10% of NSCLCs harbored either *ALK*del23 (~7% of cases) or *ALK*del27 (~4% of cases). However, it is noteworthy that most tumors (58.15% [157/270]) did not even demonstrate expression of the *ALK* kinase domain. This observation is supported by the known low to absent expression of *ALK* in most lung tumors that do not harbor *ALK* rearrangements [11,22,23]. More interestingly, *ALK* splicing variants were noted to co-exist not only with *ALK* rearrangements in some samples but also with other oncogenic driver mutations (in specific *EGFR* and *KRAS* mutations). The latter may be indicative that *ALK* kinase domain splicing variants are “passenger” events in NSCLCs.

The other known tumor type with oncogenic *ALK* abnormalities that has been shown to display *ALK* splicing variants is neuroblastoma. In this tumor type, characterized by mutations within the tyrosine kinase of *ALK* in a subset of samples, truncated ALK proteins with complete skipping of exon 2-3 (*ALK*del2-3) and exons 4-11 (*ALK*del4-11) of *ALK* have been described and characterized [24,25]. Different than the *EML4-ALK*del27-derived protein EML4-ALK T1312fs*0 described herein by us, these truncated extracellular domain proteins led to increased constitutive kinase activity of ALK and transformation potential (i.e., *ALK*del2-3 and *ALK*del4-11 were putative driver events). It is possible to speculate that the opposite nature of these proteins is based on the fact that *ALK*del2-3 and *ALK*del4-11 retain the sequence of the active kinase domain of *ALK*, whereas *ALK*del27 leads to a stop codon that occurs within the tyrosine kinase domain. The lack of a functional complete tyrosine kinase domain may explain the non-functional nature of the EML4-ALK T1312fs*0 truncated protein. Therefore, *ALK*del27 seems to be a silent variant that indeed is a passenger alternative splicing form of *ALK*. ALKdel23 (ALK I1171fs*42), due to its even more significant lack of residues within the ALK kinase domain, is also expected to be a truncated and non-functional variant if translated.

Interestingly, the currently approved and/or used methods of detection of *ALK* rearrangements in clinical practice are unable to obtain sequence reads of the introns and exons spanning the kinase domain of *ALK* [1,9,16]. Even robust targeted next-generation sequencing techniques [26] using tumor-derived genomic DNA as a template would not be able to identify a splicing mutation or exon skipping variant within *ALK*. These limitations highlight the possible lack of reporting of *ALK* splicing variants in NSCLC.

The identification of mechanisms of resistance to crizotinib and other ALK TKIs in *ALK* rearranged NSCLC is of utmost importance, and to date only *ALK* kinase mutations and/or bypass tracks due to activation of other oncogenes (such as EGFR) have been described [11,17,27-29]. *ALK* kinase mutations – which are present in only around one third of crizotinib-resistant samples – have been characterized *in vitro* as crizotinib-resistant mutations using EML4-ALK driven models [29]. We were able to confirm that EML4-ALK with the gatekeeper *ALK*-L1196M indeed is resistant to crizotinib. Our preclinical characterization of *EML4-ALK*del27 and the resulting truncated EML4-ALK T1312fs*0 mutated protein add to these prior results by annotating a non-functional and non-resistant inducing variant of EML4-ALK. The translation of these results into the clinic would inform future investigators not to alter their ALK TKI treatment strategies in the presence of *ALK*del27. The patient in which we identified *EML4-ALK*del27 had this variant present together with wild-type *EML4-ALK* in his crizotinib-naïve and resistant samples. His marked response to crizotinib [18] does not seem to have been affected by *EML4-ALK*del27.

In summary, our group report that splicing isoforms of the kinase domain of *ALK* (either *ALK*del23 or *ALK*del27) were identified in 11.1% of NSCLCs, and these *ALK* kinase splicing variants were non-functional genomic events in NSCLC.

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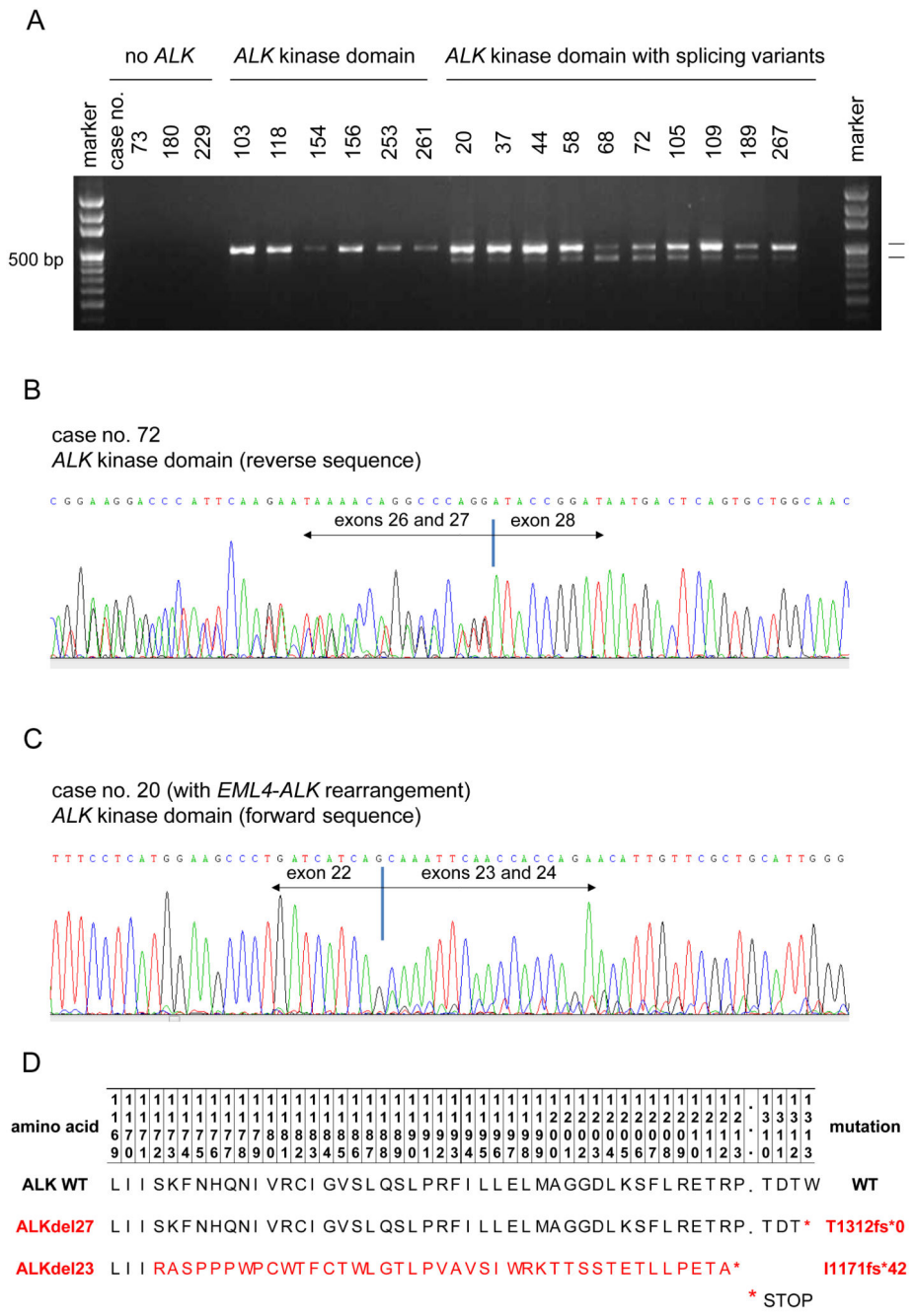

FIGURE 1.

Screening for *ALK* kinase domain splicing variants in non-small-cell lung cancer (NSCLC). **A.** Reverse transcriptase PCR using primers that flank exons 20 to 29 of *ALK* (encompass the kinase domain). Cases with or without *ALK* kinase domain expression, and those expressing *ALK* slicing variants;

B. Sequence chromatogram of a tumor specimen with complete skipping of exon 27 of *ALK* (*ALK*del27). Representative sequences from cDNA isolated from case no. 72 highlighting the ALK exon 26-exon 27 and ALK exon 26-exon 28 co-existing sequences;

C. Sequence chromatogram of a tumor specimen with complete skipping of exon 23 of *ALK* (*ALK*del23). Representative sequences from cDNA isolated from case no. 20 highlighting the ALK exon 22-exon 23 and ALK exon 22-exon 24 co-existing sequences. Reference *ALK* gene sequence (NM_004304.3; homo sapiens *ALK* mRNA);
D. Proposed amino acid sequence of wild type (WT) ALK, ALKdel27 and ALKdel23. The latter truncated proteins generate early stop codons. *denotes a STOP codon sequence.

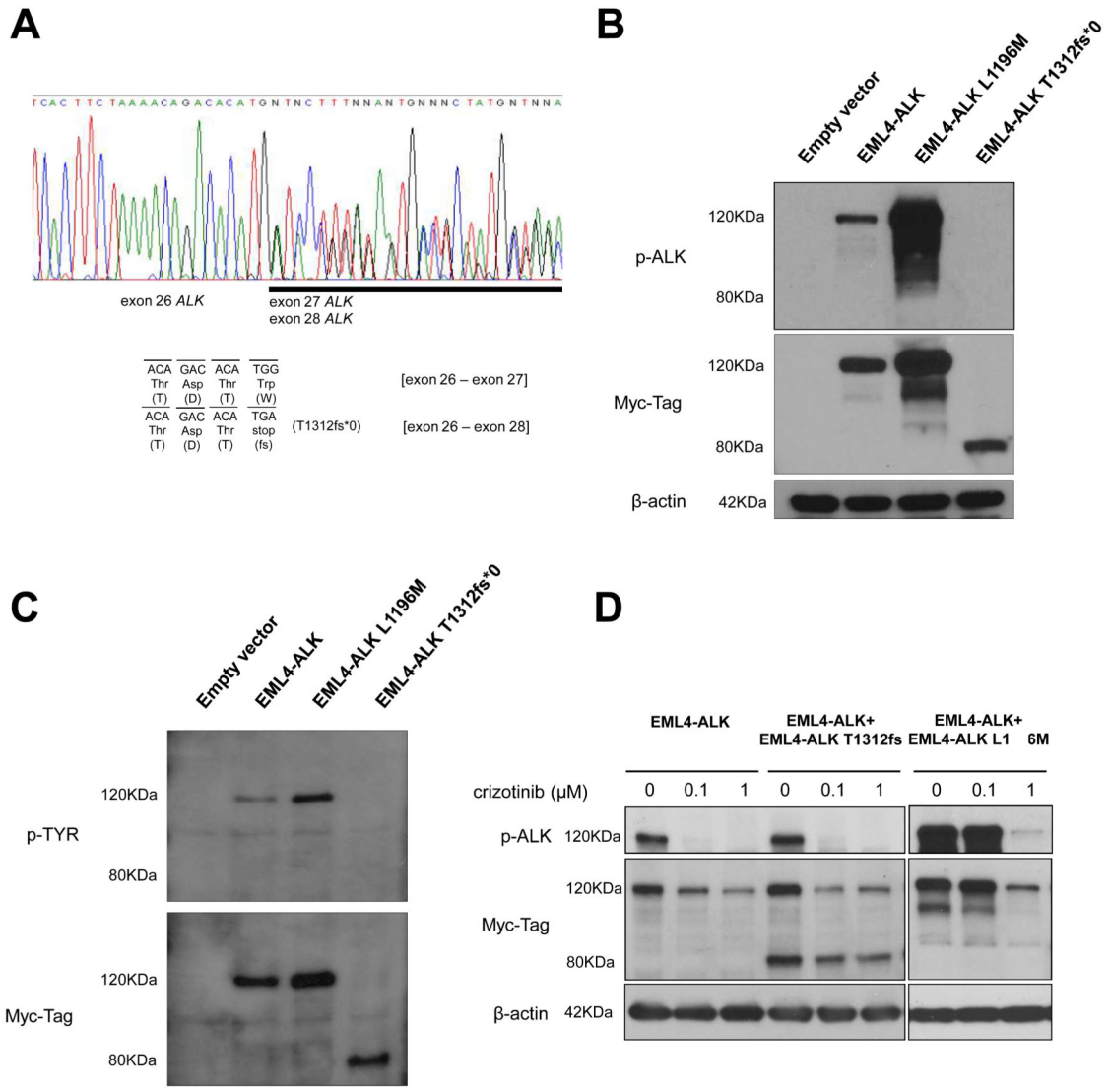

FIGURE 2.

Evaluation of EML4-ALK T1312fs*0 in preclinical models.

A. Sequence chromatogram of an EML4-ALK positive tumor specimen. Representative sequences from cDNA isolated from the aforementioned lymph node. Nucleotide areas corresponding to anaplastic lymphoma kinase (*ALK*) exon 26, 27 and 28 are highlighted. Reference *ALK* gene sequence (NM_004304.3; homo sapiens *ALK* mRNA); the expected nucleotide and amino acid sequences for the wild-type ALK exon 26-exon 27 junction, and for the splicing variant with ALK exon 26-exon 28 junction are shown. The latter generates the early stop codon sequence p. T1312fs*0 (n.TGG->TGA).

B. Western blot analysis of protein extracts from COS-7 cells transfected with EML4-ALK constructs and empty vector. EML-ALK E13;A20 constructs with a 3' Myc-His-Tag were cloned into pcDNA3.1. COS-7 cells were transfected with 1 μg of DNA and protein extracts were collected 24 hours later. The radiograph shows the expression of phosphorylated ALK (p-ALK Tyr1096) and EML4-ALK detected by using a Myc-Tag antibody. EML4-ALK

T1312fs*0 results in an 80 KDa protein, different from the expected size - 117 kDa - of EML4-ALK WT and EML4-ALK containing the L1196M crizotinib-resistant mutation.

C. Western blot analysis of protein extracts from COS-7 cells transfected with EML4-ALK-Myc-His-Tag constructs or empty vector and immunoprecipitated with Myc-Tag antibody. The upper figure shows the expression of phospho-tyrosine (p-TYR) and the lower figure shows the expression of EML4-ALK detected by using a Myc-Tag antibody. EML4-ALK T1312fs*0 results in an 80 KDa protein that is not phosphorylated.

D. Western blot analysis of protein extracts from COS-7 cells co-transfected with different DNA amounts of EML4-ALK constructs and empty vector and treated with crizotinib or vehicle (DMSO). The left panel with E13;A20 EML4-ALK (0.4 μg) + empty vector (0.6 μg); the middle with EML4-ALK (0.4 μg) + empty vector (0.2 μg) + EML4-ALK T1312fs*0 (0.4 μg) in which EML4-ALK T1312fs*0 does not result in resistance to crizotinib as verified by ALK phosphorylation inhibition using Tyr1096 phospho-ALK antibody; the right with EML4-ALK (0.4 μg) + empty vector (0.2 μg) + EML4-ALK L1196M (0.4 μg) in which the co-transfection of EML4-ALK L1196M is shown in the right panel and discloses resistance to inhibition of phospho-ALK by crizotinib. Blots were cropped to highlight the referenced molecular weight.

TABLE 1

Baseline patient, tumor and molecular characteristics of non-small-cell lung cancers with or without anaplastic lymphoma kinase (*ALK*) kinase domain splicing variants

	Total (n=270)		
	***ALK* kinase domain splicing variant expression (n=30)**	**Lack of *ALK* kinase domain splicing variant expression (n=240)**	***p*-value**
Age median(range)	61 (29-81)	64 (33-79)	0.110
Women n (%)	13 (43.3%)	123 (51.2%)	0.444
Race n (%) Asian White	29 (96.7%) 1 (3.3%)	239 (99.6%) 1 (0.4%)	0.210
Smoking status n (%) Current smoker Former smoker Never smoker	12 (40.0%) 4 (13.3%) 14 (46.7%)	77 (32.1%) 33 (13.8%) 130 (54.1%)	0.660
Stage n (%) I II III IV	16 (53.3%) 2 (6.7%) 9 (30.0%) 3 (10.0%)	138 (57.5%) 45 (18.8%) 52 (21.7%) 5 (2.1%)	0.033
Histology n (%) Adenocarcinoma Squamous cell carcinoma Other (NOS)	24 (80.0%) 4 (13.3%) 2 (6.7%)	189 (78.8%) 30 (12.5%) 21 (8.7%)	0.925
EGFR mutation n (%) Positive/Mutated Negative/Wild-type	9 (30%) 21 (70%)	116 (48.3%) 124 (51.7%)	0.079
KRAS mutation n (%) Positive/Mutated Negative/Wild-type	3 (10%) 27 (90%)	19 (7.9%) 221 (92.1%)	0.721
ALK rearrangement n (%) Positive Negative	4 (13.3%) 26 (86.6%)	15 (6.2%) 225 (93.8%)	0.244
ALK splicing variant n (%) exon 23 skipping exon 27 skipping	19 (63.3%) 11 (36.7%)	0 (0%) 0 (0%)	NA

n, number; EGFR, epidermal growth factor receptor; KRAS, v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog; NOS, not otherwise specified