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Cancer Biology and Translational Studies

Molecular Cancer Therapeutics

ALK Inhibitor Response in Melanomas Expressing *EML4-ALK* Fusions and Alternate *ALK* Isoforms

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Abstract

Oncogenic *ALK* fusions occur in several types of cancer and can be effectively treated with ALK inhibitors; however, *ALK* fusions and treatment response have not been characterized in malignant melanomas. Recently, a novel isoform of *ALK* (ALK^{ATI}) was reported in 11% of melanomas but the response of melanomas expressing ALK^{ATI} to ALK inhibition has not been well characterized. We analyzed 45 melanoma patient-derived xenograft models for *ALK* mRNA and protein expression. *ALK* expression was identified in 11 of 45 (24.4%) melanomas. Ten melanomas express wild-type (wt) *ALK* and/or ALK^{ATI} and one mucosal melanoma expresses multiple novel *EML4-ALK* fusion variants. Melanoma cells expressing different *ALK* variants were tested for response to ALK inhibitors. Whereas the melanoma expressing *EML4-ALK* were sensitive to ALK inhibitors *in vitro* and *in vivo*, the melanomas expressing wt *ALK* or ALK^{ATI} were not sensitive to ALK inhibitors. In addition, a patient with mucosal melanoma expressing ALK^{ATI} was treated with an ALK/ROS1/TRK inhibitor (entrectinib) on a phase I trial but did not respond. Our results demonstrate *ALK* fusions occur in malignant melanomas and respond to targeted therapy, whereas melanomas expressing ALK^{ATI} do not respond to ALK inhibitors. Targeting *ALK* fusions is an effective therapeutic option for a subset of melanoma patients, but additional clinical studies are needed to determine the efficacy of targeted therapies in melanomas expressing wt *ALK* or ALK^{ATI}. *Mol Cancer Ther; 17(1); 222–31. ©2017 AACR.*

Introduction

Anaplastic lymphoma kinase (*ALK*) can be expressed and activated in cancers as the result of different oncogenic alterations. Chromosomal rearrangements resulting in *ALK* kinase fusions occur in 3%–7% of non–small cell lung cancers (NSCLC) and a subset of other solid tumors including colorectal cancer, breast cancer, and inflammatory myofibroblastic tumors (1–6). In NSCLC, the most common *ALK* fusion includes the 5′ portion of the echinoderm microtubule-associated protein like-4 (*EML4*) gene fused to exons 20–29 (the entire kinase domain) of *ALK* creating a constitutively active fusion protein with potent oncogenic activity (7). Several different *EML4-ALK* variants have been reported, and all identified variants are generally activating and sensitive to ALK inhibition (3, 7–10). Clinically, lung cancer patients with *ALK* fusion–positive tumors generally respond to ALK inhibitors and several inhibitors are FDA approved for the treatment of *ALK*-fusion–positive lung cancers (11–13).

Fusions in *ALK* have been reported in 3% of spitzoid melanomas and up to 15% of all spitzoid neoplasms, but *ALK* fusions were not identified in studies analyzing TCGA data from 374 malignant melanomas (14–16). Also, we did not find any *ALK* rearrangements in our previous analysis of 59 melanomas using FISH (17). One study identified *ALK* genomic rearrangements in acral lentiginous melanomas using FISH and IHC, but the rearrangements were not characterized and ALK inhibitor sensitivity was not tested (18). Therefore, *ALK* fusions and response to targeted therapy remain uncharacterized in malignant melanomas.

Recently, a novel oncogenic alteration in *ALK* was identified in 11% of cutaneous melanomas (19). An alternative transcription initiation site located in intron 19 leads to the expression of a novel *ALK* isoform (ALK^{ATI}), which includes a portion of intron 19 and exons 20–29. In that publication, exogenous overexpression of ALK^{ATI} induced tumorigenesis and sensitized cells to ALK inhibitors, but the inhibitor response was not analyzed in melanoma patient cell lines. A modest clinical response to ALK inhibitor was reported in one melanoma patient with an ALK^{ATI}-positive tumor. However ALK^{ATI} response to inhibitors in melanoma patient samples remains incompletely characterized.

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Given the success of targeted therapy in NSCLC patients with *ALK* fusion–positive tumors and the availability of clinically effective ALK inhibitors, we sought to identify melanomas with *ALK* alterations and analyze ALK inhibitor responses in melanoma patient tumor samples. We identified a mucosal melanoma expressing multiple novel *EML4-ALK* fusion variants and six melanomas expressing ALK^{ATI}. The *EML4-ALK*–expressing melanoma was sensitive to ALK inhibitors *in vitro* and *in vivo*; however, ALK^{ATI}-expressing melanomas were not sensitive to multiple ALK inhibitors. In addition, a patient with mucosal melanoma expressing ALK^{ATI} did not respond to treatment with an ALK inhibitor. These findings implicate *ALK* fusions as clinically relevant therapeutic targets in melanomas and suggest the need for additional clinical studies to establish the relevancy of ALK inhibitors for the treatment melanomas expressing ALK^{ATI}.

Materials and Methods

Patient samples

Tumor and blood samples were collected from melanoma patients at the University of Colorado Denver Hospital from 2008 to 2016. Samples were obtained with patient consent and approval from the Colorado Institutional Review Board (#05-0309) as part of the International Melanoma Biorepository and Research Laboratory at the University of Colorado Cancer Center. Patient studies were conducted according to the Declaration of Helsinki, the Belmont Report, and the U.S. Common Rule.

Patient-derived xenograft models

Patient derived xenograft (PDX) models were generated from fresh tumor tissue as described previously (20, 21). All animal work and care were performed under the guidelines of the University of Colorado Denver Institutional Animal Care and Use Committee (IACUC).

Cell lines

Melanoma cell lines MB 2141, 3443, 3429 (derived in 2016 by our laboratory) and MB 1692, 1374 (derived in 2015 by our laboratory) were generated from fresh PDX tumor tissues using a human tumor dissociation kit (Miltenyi Biotec). All experiments in this study used MB cell lines within 20 passages from initial derivation. The H3122 cell line (obtained in 2008 from Dr. Paul A. Bunn, University of Colorado, Denver, CO) has been described previously (22). The NIH3T3 cell line was purchased in 2016 from the ATCC and the 293T cell line was purchased in 2016 from Clontech. Cells were cultured in DMEM (NIH3T3 and 293T cells) or RPMI (melanoma and NSCLC cells) with 10% FBS and 1% penicillin–streptomycin, under 5% CO_2. All cell lines were short tandem repeat (STR) profiled and matched >80%, except for NIH3T3 and 293T cell lines which were used within 15 passages of receiving from the company. Cell lines tested negative for mycoplasma (August 2017) using a luminescence-based assay (Lonza).

Inhibitors and treatments

ALK inhibitors (crizotinib, ceritinib, entrectinib, alectinib, and ASP3026) and MEK inhibitor (trametinib) were purchased from Selleck Chemicals. Cells were treated with inhibitor concentrations ranging from 0.3 nmol/L to 10,000 nmol/L for either 72 hours (viability assays) or 2 hours (immunoblots). Controls were treated with DMSO alone.

RNA and cDNA preparation

Total RNA was extracted from frozen tissue or cells using the RNeasy Plus Mini Kit (Qiagen), with on-column DNase digest and tissue homogenization using the TissueLyser II (Qiagen). cDNA was generated using 500-ng input RNA and the Verso cDNA synthesis kit (Thermo Fisher Scientific).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on PDX, cell line, or xenograft cDNAs using PowerUP SYBR Green (Thermo Fisher Scientific) and the StepOne Plus real-time PCR system (Applied Biosystems). Reactions were performed in triplicate and sequences for primers used are listed in Supplementary Table S1.

Subcloning and Sanger sequencing

PCR was performed on PDX cDNA (GoTaq, Promega) using a forward primer in exon 4 of *EML4* and a reverse primer in exon 17 or 20 of *ALK*, with restriction enzyme sites included in each primer (Supplementary Table S1). PCR products were digested and cloned into the pLVX-EF1α-IRES-ZsGreen1 vector. Plasmids were purified from up to 10 colonies using a plasmid mini-prep kit (Zymo), and Sanger sequencing was performed with both forward and reverse primers using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems).

Immunoblots

Cells or tumors were lysed for 10 minutes in ice-cold RIPA buffer with added protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Tumors were homogenized using the TissueLyser II (Qiagen) and all lysates were cleared by centrifugation at 13,000 rpm for 10 minutes. Sixty-microgram protein was separated using SDS-PAGE and transferred to nitrocellulose membranes. Proteins were detected using the following primary antibodies from Cell Signaling Technology: ALK (#3633), phospho-ALK Tyr1604 (#3341), ERK1/2 (#4696), phospho-ERK1/2 Thr202/Tyr204 (#4370), AKT (#2920), phospho-AKT Ser473 (#4060), and β-actin (#3700). Fluorescent rabbit and mouse secondary antibodies (LI-COR Biosciences) were used and blots were imaged using the LI-COR Odyssey. Quantification was performed using ImageJ software (23).

FISH

Dual-colored FISH assays were performed on formalin-fixed paraffin-embedded (FFPE) slides from PDX tissue using commercially available *ALK* break-apart probes and reagents (Abbott Molecular) as described previously (5). A total of 50 cells were analyzed and signals physically separated by $\geq$1 signal diameter were scored as split. The specimen was considered positive for *ALK* rearrangement if >15% of the cells showed split signals.

Targeted RNA sequencing

Library preparation and targeted sequencing were performed on PDX RNA by the University of Colorado Denver Genomics and Microarray Core. Libraries were generated using the Ovation cDNA module and Ovation Fusion Panel Target Enrichment System (NuGEN) with 500 ng RNA input as described previously (24). Paired-end sequencing (2 × 150 bp) was performed on a

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HiSeq 4000 instrument (Illumina) and approximately 10 million total reads were collected. Fusions were detected using the BaseSpace data analysis hosting platform (Illumina) and the NuGEN Ovation Fusion Target App (NuGEN).

Whole-exome sequencing

Whole-exome sequencing was performed on tumor and normal DNA from patient tissue and blood samples as previously described (17). Briefly, genomic DNA was isolated using the DNeasy Blood and Tissue kit or the QiaAmp DNA FFPE kit (Qiagen). Exome cDNA libraries were constructed using the Agilent SureSelect XT Target Enrichment System for Illumina Paired End Multiplexed Sequencing Library (cat# G9641B) and libraries were sequenced on the Illumina HiSeq 2000 with 125 bp pair-end reads. Data were analyzed using the IMPACT pipeline (25).

Viability assays

Cells were plated in triplicate in 96 well plates (1,000–2,500 cells per well) 24 hours prior to treatment. Cells were treated with inhibitors for 72 hours and viability was measured with an ATP-content based assay by luminescence using CellTiter Glo (Promega) and Synergy 2 plate reader (BioTek). Triplicate wells were analyzed for each condition and normalized to DMSO-treated controls.

Lentiviral constructs and expression

cDNA inserts for *ALK* fusions, wt *ALK*, and ALK^{ATI} were synthesized (GenScript) and cloned into the pLVX-EF1α-IRES-ZsGreen1 lentivirus expression vector (Clontech). Lentivirus was produced by transfecting 293T cells using the Lenti-X single shot system (Clontech) and virus was collected and filtered 48 hours post-transfection. NIH3T3 cells were infected for 24 hours using virus and 8 μg/mL polybrene, and ZsGreen1–positive cells were sorted 2 weeks after infection using a MoFfo XDP 100 instrument (Beckman Coulter).

siRNA knockdown

Cells were transfected in suspension using 350 nmol/L On-Target Plus siRNAs (Dharmacon, Thermo Fisher) and DMRIE-C transfection reagent (Invitrogen) in OptiMEM reduced serum media (Invitrogen). After 6 hours, media were replaced with normal growth media. siRNAs included a nontargeting control (catalog no. D-001810-01-20) and two siRNAs targeting the kinase domain of ALK (catalog no. J-003103-10; catalog no. J-003103-11).

Mouse xenografts

Eight-week-old female nude mice were injected with 5 million cells subcutaneously (100 μL) in the hind flanks in 1:1 media plus Matrigel solution (Corning). After tumors reached approximately 150 mm^3, mice were randomized into three groups (7 mice and at least 11 tumors per group) and treated with vehicle (0.5% Tween 80 + 0.5% hydroxypropyl methylcellulose), crizotinib (LC Laboratories, 100 mg/kg once daily, 100 μL, orally), or ceritinib (LC Laboratories, 50 mg/kg once daily, 100 μL, orally) for 25 days. Tumor volume [equation or volume = (length × $width^2$) × 0.52] was evaluated twice per week with digital calipers using the Study Director software package (Studylog Systems). Body weights were measured twice weekly. An additional two MB 2141 xenograft mice were generated and once tumors reached 500 mm^3 they were treated with crizotinib or ceritinib for 7 days for further genetic and pharmacodynamic analyses.

Clinical trial

STARTRK-1 (NCT02097810) is a phase I open-label, multicenter study of entrectinib (RXDX-101) in patients with locally advanced or metastatic cancer that have a confirmed alteration in *ALK*, *ROS1*, *NTRK1*, *NTRK2*, *NTRK3*. This study is approved by institutional review boards at all institutions enrolling patients, and all patients provided written informed consent. The study is sponsored by Ignyta, Inc. and is conducted in accordance with the Declaration of Helsinki and Good Clinical practices. Entrectinib is an oral ATP-competitive inhibitor of the tyrosine kinases tropomyosin receptor kinase ALK, ROS1, and TRK A/B/C. Expression of the *ALK* kinase domain was confirmed in the melanoma patient FFPE tumor specimen using an anchored multiplex PCR (AMP) method (ArcherDx) as described previously (26). The patient in this study was enrolled in the phase I portion of the clinical trial and received 600 mg oral daily doses of entrectinib, CAS registry number 1108743-60-7, in 200-mg tablets provided by the study sponsor. Efficacy was evaluated using RECIST 1.1 criteria.

Results

Melanomas express multiple different *ALK* variants

To identify melanoma patient tumors expressing *ALK* that could be tested for ALK inhibitor sensitivity, we used qRT-PCR to analyze a set of 45 melanoma PDX tumors including melanomas from distinct subtypes (mucosal, acral, nodular, superficial spreading, and unknown primary). Analysis with primers designed in early exons (E) or exons located within the kinase domain (K1, K2) of *ALK* identified 12 melanomas which express full-length *ALK* or the *ALK* kinase domain only (Fig. 1A; Supplementary Fig. S1). Four out of 12 tumors had similar expression of early and kinase domain exons (MB 2040, 1245, 1374, and 1588) and immunoblotting confirmed expression of wt ALK protein in these tumors (Fig. 1B). Eight of 12 tumors (MB 3443, 2141, 2724, 2204, 3429, 2661, 2223, and 1692) showed imbalanced expression of *ALK* early and kinase domain exons, with predominantly kinase domain exons expressed. Immunoblotting showed potential ALK^{ATI} protein expression in all 8 samples (Fig. 1B). Expression of ALK^{ATI} in the 8 tumors was confirmed using qRT-PCR with primers for *ALK* intron 19, which are specific to the ALK^{ATI} transcript (Fig. 1C). ALK^{ATI} expression by both intron 19 qRT-PCR and immunoblotting was low and nearly undetectable in MB 2204 therefore we did not include this tumor in further analysis (Fig. 1B and C).

Interestingly, three tumors (MB 2661, 2223, and 2141) coexpress ALK^{ATI} and another form of ALK. MB 2661 and 2223 coexpress wt ALK (although wt ALK expression is low in MB 2661). MB 2141 coexpresses an ALK protein of approximately 100 kDa, indicative of a potential ALK fusion protein (Fig. 1B). We confirmed the presence of an *ALK* rearrangement in MB 2141 (mucosal melanoma) by break-apart FISH analysis (Fig. 1D). To characterize the *ALK* rearrangement, we performed fusion-specific targeted RNA sequencing on MB 2141 PDX RNA and identified multiple, novel *EML4-ALK* fusion variants (Supplementary Fig. S2A). PCR analysis indicated three different fusion variants were present (Supplementary Fig. S2B), and these PCR products were subcloned and sequenced to identify the exon breakpoints in

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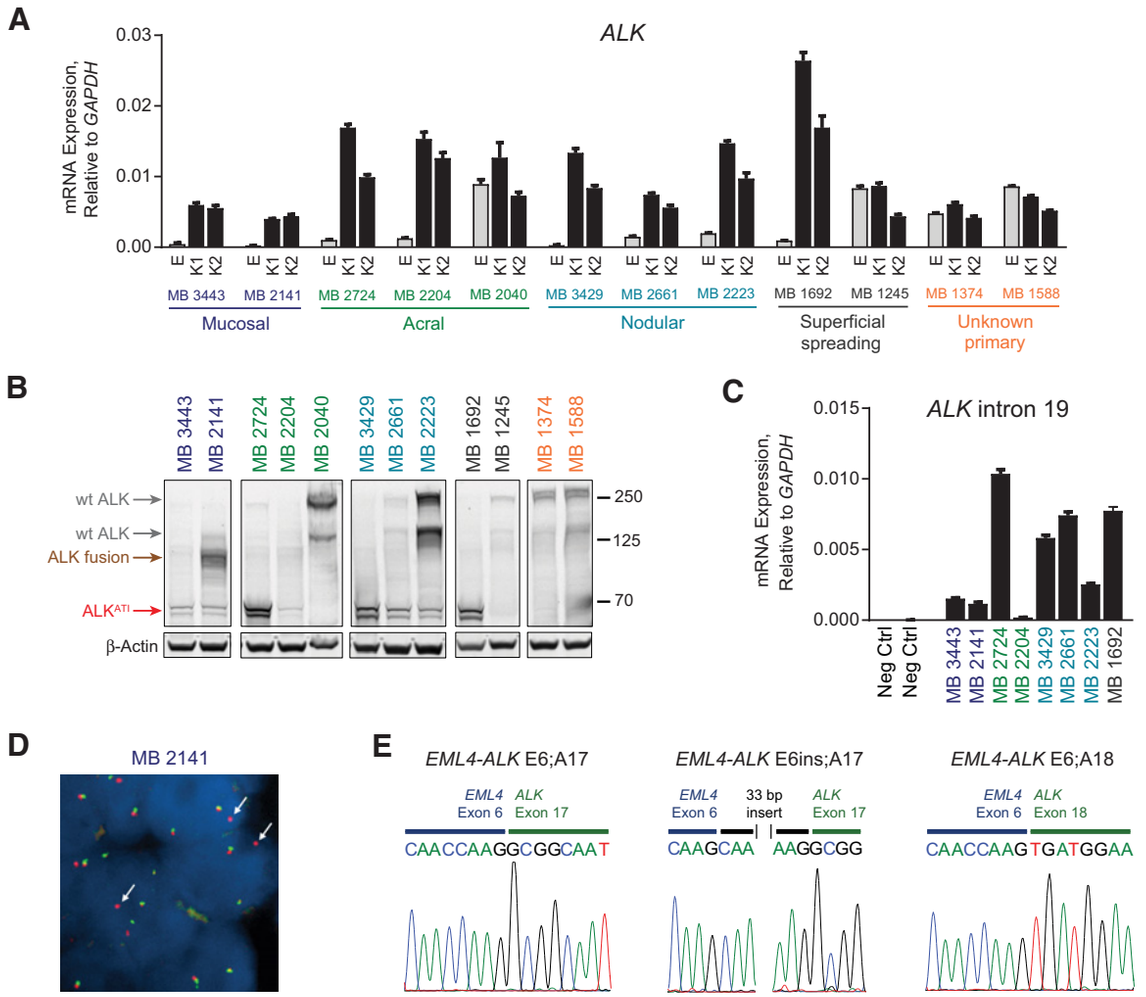

Figure 1.

Identification of melanoma tumors expressing wt ALK, ALK^{ATI}, and an EML4-ALK fusion. **A,** *ALK* mRNA expression was quantified in melanoma PDX tumors by qRT-PCR analysis with primers located in early exons (E) or exons within the kinase domain (K1, K2) of *ALK*. Expression was normalized to *GAPDH* and error bars represent SEM of triplicate reactions. The 12 *ALK* expression–positive tumors out of 45 total PDX tumors analyzed are shown. **B,** Immunoblot analysis of *ALK*-positive PDX tumor lysates. **C,** Expression of *ALK* intron 19 was quantified by qRT-PCR. Expression was normalized to *GAPDH* and error bars represent SEM of triplicate reactions. **D,** FISH analysis of the MB 2141 PDX tumor using *ALK* specific 5′ (green) and 3′ (red) break-apart probes. **E,** Sanger sequencing analysis of *EML4-ALK* fusion variant transcripts. PCR products were generated from MB 2141 PDX cDNA using an *EML4*-exon 4 forward primer and *ALK* exon 17 or 20 reverse primers. PCR products were subcloned and analyzed by Sanger sequencing.

each fusion variant (Fig. 1E; Supplementary Fig. S2C). The three variants include *EML4* exon 6 fused to *ALK* exon 17 (E6;A17), *EML4* exon 6 fused to *ALK* exon 17 with a 33-bp insert from *EML4* intron 6 (E6ins;A17), and *EML4* exon 6 fused to *ALK* exon 18 (E6;A18; Fig. 1E). This is the first report of an *ALK* fusion in a malignant melanoma and, to our knowledge, the first report of *EML4-ALK* fusion variants including exon 17 of *ALK*.

Altogether, we determined 11 of 45 (24.4%) melanomas express *ALK*. Four of 45 (8.9%) express wt *ALK*, 7 of 45 express *ALK*^{ATI} (15.6%), and 1 of 45 (2.2%) expresses an *EML4-ALK* fusion. Analysis of whole-exome sequencing data for these 11 melanoma patient tumors shows the *EML4-ALK* fusion occurs in a driver mutation negative tumor (lacking mutations in *BRAF, NRAS, KIT, GNAQ, GNA11*, and *NF1*), whereas the wt *ALK*- and *ALK*^{ATI}-expressing tumors all contain a mutation or previously identified fusion in a common melanoma driver gene (Table 1; ref. 17). This raised the question of the possible role for different *ALK* molecular variants as drivers in melanoma and potential therapeutic response.

Melanomas expressing *EML4-ALK* fusions, but not wt *ALK* or *ALK*^{ATI}, respond to ALK inhibitors *in vitro*

We established cell lines from melanoma PDX tumors expressing wt *ALK* (MB 1374), *EML4-ALK* fusion and *ALK*^{ATI} (MB 2141), or *ALK*^{ATI} only (MB 3443, 3429, 1692). Interestingly, only MB 1692 retained expression of ALK^{ATI} when cultured *in vitro*, whereas ALK^{ATI} was nearly undetectable in MB 2141, 3443, and 3429 (Fig. 2A). Analysis of phospho-ALK showed strong bands corresponding with total ALK expression for MB 2141 and H3122, an NSCLC cell line, which expresses an *EML4-ALK* (E13;A20) fusion

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Table 1. Driver gene mutations in *ALK* expressing melanomas

Sample	ALK status	Subtype	*BRAF*	*NRAS*	*KIT*	*GNAQ*	*GNA11*	*NF1*
MB 2141	EML4-ALK + ALK^{ATI}	Mucosal	—	—	—	—	—	—
MB 3443	ALK^{ATI}	Mucosal	—	Q61H	—	—	—	—
MB 2724	ALK^{ATI}	Acral	P402H[a]	—	—	—	—	—
MB 3429	ALK^{ATI}	Nodular	G596C	—	—	—	R183C	—
MB 1692	ALK^{ATI}	SS	AGK-BRAF	—	—	—	—	—
MB 2661	ALK^{ATI}+ wt ALK	Nodular	G466E	—	—	—	—	—
MB 2223	ALK^{ATI}+ wt ALK	Nodular	—	—	—	—	—	Q1255X[a]
MB 2040	wt ALK	Acral	V600E	—	—	—	—	—
MB 1245	wt ALK	SS	V600E	—	—	—	—	—
MB 1374	wt ALK	Unk Pri	ARMC10-BRAF	—	—	—	—	—
MB 1588	wt ALK	Unk Pri	—	—	—	—	—	E844X[a]

NOTE: "—" indicates no mutation detected.

Abbreviations: SS, superficial spreading; Unk Pri, unknown primary.

[a]Specific mutation has not been functionally characterized.

(Fig. 2A; ref. 27). No corresponding phospho-ALK bands were observed for MB 1692 (ALK^{ATI}) or MB 1374 (wt ALK) suggesting ALK may not be activated in these tumor cells (bands appearing in MB 1692 and 1374 in the phospho-ALK blot are non-specific; Supplementary Fig. S3). In addition, we expressed different *EML4-ALK* fusion variants, ALK^{ATI}, and wt *ALK* in NIH3T3 cells (Supplementary Fig. S4A). Similar to the patient-derived cell lines, we observed strong phospho-ALK expression in *EML4-ALK* fusion cells, and only weak expression of phospho-ALK in ALK^{ATI} and wt *ALK* cells (Supplementary Fig. S4A and S4B).

To determine the response of different *ALK* molecular variants to ALK inhibitors, we treated cell lines with crizotinib and ceritinib, both of which are FDA-approved drugs for ALK^+ NSCLC. Cell lines expressing *EML4-ALK* fusions, but not wt *ALK* or ALK^{ATI}, responded to both ALK inhibitors and showed decreased viability in a dose-dependent manner (Fig. 2B). We treated cell lines with three additional ALK inhibitors (entrectinib, ASP3046, and alectinib) to confirm the lack of response was not specific to crizotinib and ceritinib (Supplementary Fig. S5). Only MB 2141 and H3122 showed submicromolar IC_{50} values, and the responses in MB 1692 and 1374 were similar to MB 3429 control cells which do not express ALK (Fig. 2B; Supplementary Fig. S5). Immunoblotting of downstream signaling pathways showed decreased activity of ALK and downstream targets AKT and ERK1/2 in MB 2141 after crizotinib treatment, but no changes in signaling activity were observed after treatment of MB 1692 which expresses ALK^{ATI} (Fig. 2C). We also knocked down ALK^{ATI} expression in MB 1692 using siRNA and did not observe an effect on cell viability (Supplementary Fig. S6). Altogether, our data demonstrate cells expressing *EML4-ALK* fusions, but not ALK^{ATI} or wt *ALK*, respond to ALK inhibitor treatment *in vitro*.

Melanoma cells respond to targeted MEK inhibition against other oncogenic drivers

As ALK^{ATI} is expressed in melanomas which contain other potential oncogenic drivers of the MAPK pathway, we tested whether these cell lines were sensitive to MAPK inhibition. MB 1692 (*AGK-BRAF* fusion), MB 3443 (*NRAS* Q61H), and MB 3429 (*BRAF* G596C, *GNA11* R183C) were treated with trametinib (MEK inhibitor) or vemurafenib (mutant-specific BRAF inhibitor). All three cell lines responded to trametinib and, as expected, did not respond to vemurafenib (Fig. 3A and B). This suggests that other alterations, not ALK^{ATI}, are likely to be the oncogenic driver in these melanomas. As MB 1692 cells express both ALK^{ATI} an *AGK-BRAF* fusion protein, we speculated that expression of the *BRAF* fusion may prevent response to ALK inhibitors by alternative activation of the MAPK pathway. However, cotreatment with ceritinib in the presence of trametinib did not show any additive or synergistic effect suggesting the lack of ALK inhibitor response is not due to alternate oncogenic signaling pathway activation (Fig. 3C and D).

Melanoma xenografts expressing *EML4-ALK* fusions, but not ALK^{ATI}, respond to targeted treatment *in vivo*

We next tested the response of *EML4-ALK* or ALK^{ATI} expressing melanomas to ALK inhibitors *in vivo*. Xenograft tumors were generated from MB 2141 and MB 3429 cell lines and treated with crizotinib or ceritinib (Fig. 4A). Although MB 2141 and MB 3429 PDX tumor cells lost expression of ALK^{ATI} when cells were cultured *in vitro*, ALK^{ATI} was reexpressed in the xenograft tumors (Fig. 4B). Expression of ALK^{ATI} in the MB 3429 xenografts was similar to ALK^{ATI} original MB 3429 PDX model (Supplementary Fig. S7). In addition, the expression specific *EML4-ALK* isoforms was maintained in both MB 2141 cells and xenograft tumors compared with the original PDX model (Supplementary Fig. S7). MB 2141 xenografts expressing the *EML4-ALK* fusions (and ALK^{ATI}) demonstrated a strong response to both inhibitors, whereas MB 3429 xenografts expressing only ALK^{ATI} did not respond (Fig. 4A). Analysis of downstream signaling targets shows decreased activity of AKT and ERK1/2 in MB 2141 tumors treated with crizotinib or ceritinib, whereas treated MB 3429 tumors show no change in activity of these pathways. These data confirm our *in vitro* observation and show the lack of targeted inhibitor response to ALK^{ATI}-expressing melanomas occurs both *in vitro* and *in vivo*.

A patient with mucosal melanoma expressing ALK^{ATI} does not respond to targeted therapy with entrectinib

The melanoma PDX tumor MB 3443, which was identified as an ALK^{ATI}-expressing melanoma in our screen, was developed from a metastatic tumor of a 62-year-old female diagnosed with mucosal melanoma. The patient presented with an 8-mm vulvar melanoma on the left labia majora and underwent a partial vulvectomy and bilateral inguinal lymph node dissection. Mutational analysis revealed the tumor was wild-type for *BRAF* and *KIT*, but harbored an *NRAS* Q61H mutation. There was residual invasive melanoma to a depth of 2 mm and melanoma *in situ*, but the surgical margins were widely clear and the patient opted for clinical observation instead of adjuvant therapy. In July 2015, the patient noticed enlargement of inguinal lymph nodes and was found to have bilateral metastatic disease to lungs. She received 3 cycles of combination ipilimumab (anti CTLA4, 3 mg/kg) and

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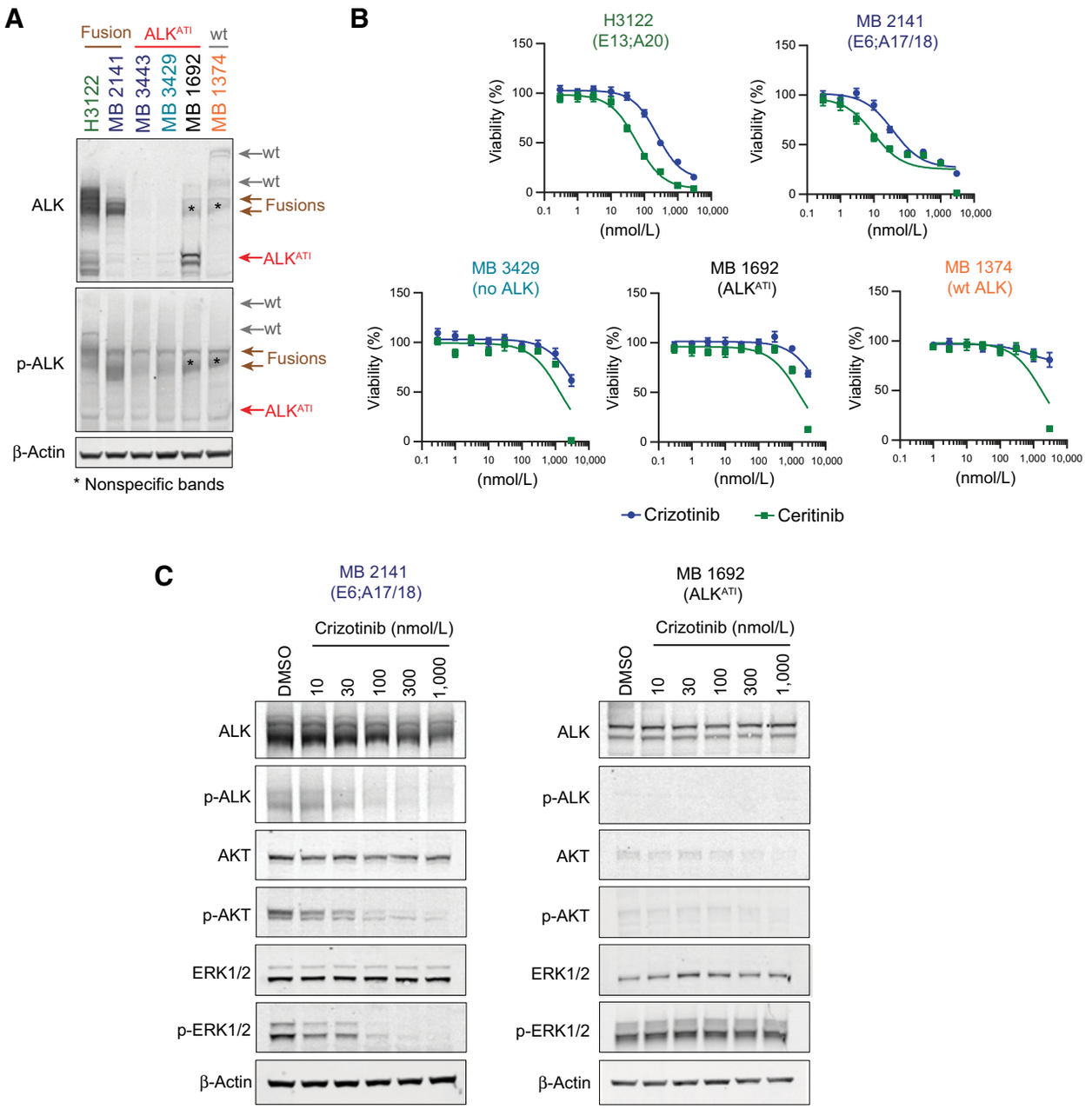

Figure 2.

ALK inhibitors reduce viability in cells expressing EML4-ALK fusions, but not in cells expressing ALK^{ATI} or wt ALK. **A,** Immunoblot analysis of lysates from H3122 NSCLC cells and melanoma cells. **B,** Cells were treated with increasing concentrations of ALK inhibitors crizotinib or ceritinib. Viability was analyzed after 72 hours and normalized to DMSO-treated controls. **C,** Cells were treated with increasing concentrations of crizotinib for 2 hours and analyzed by immunoblotting.

nivolumab (anti PD-1, 1 mg/kg) and demonstrated a mixed response, but treatment was discontinued after the patient developed grade 3 colitis. The patient was enrolled in subsequent clinical trials with mixed responses, including a clinical trial with a pan-RAF inhibitor and a trial with combination pembrolizumab (anti PD-1) with a CpG-C class oligonucleotide inhibitor. However, her disease eventually progressed to multiple metastatic locations.

After identifying *ALK^{ATI}* transcript expression in the corresponding MB 3443 PDX tumor, expression of *ALK* kinase domain was confirmed in a formalin-fixed paraffin embedded (FFPE) patient tumor specimen using an RNA-based, targeted next-generation sequencing assay (Fig. 5A). The patient was enrolled in a phase I clinical trial with entrectinib, an orally available ALK, ROS1, and TRKA/B/C inhibitor which has previously demonstrated activity in patients with *ALK* fusions (28). Unfortunately, the

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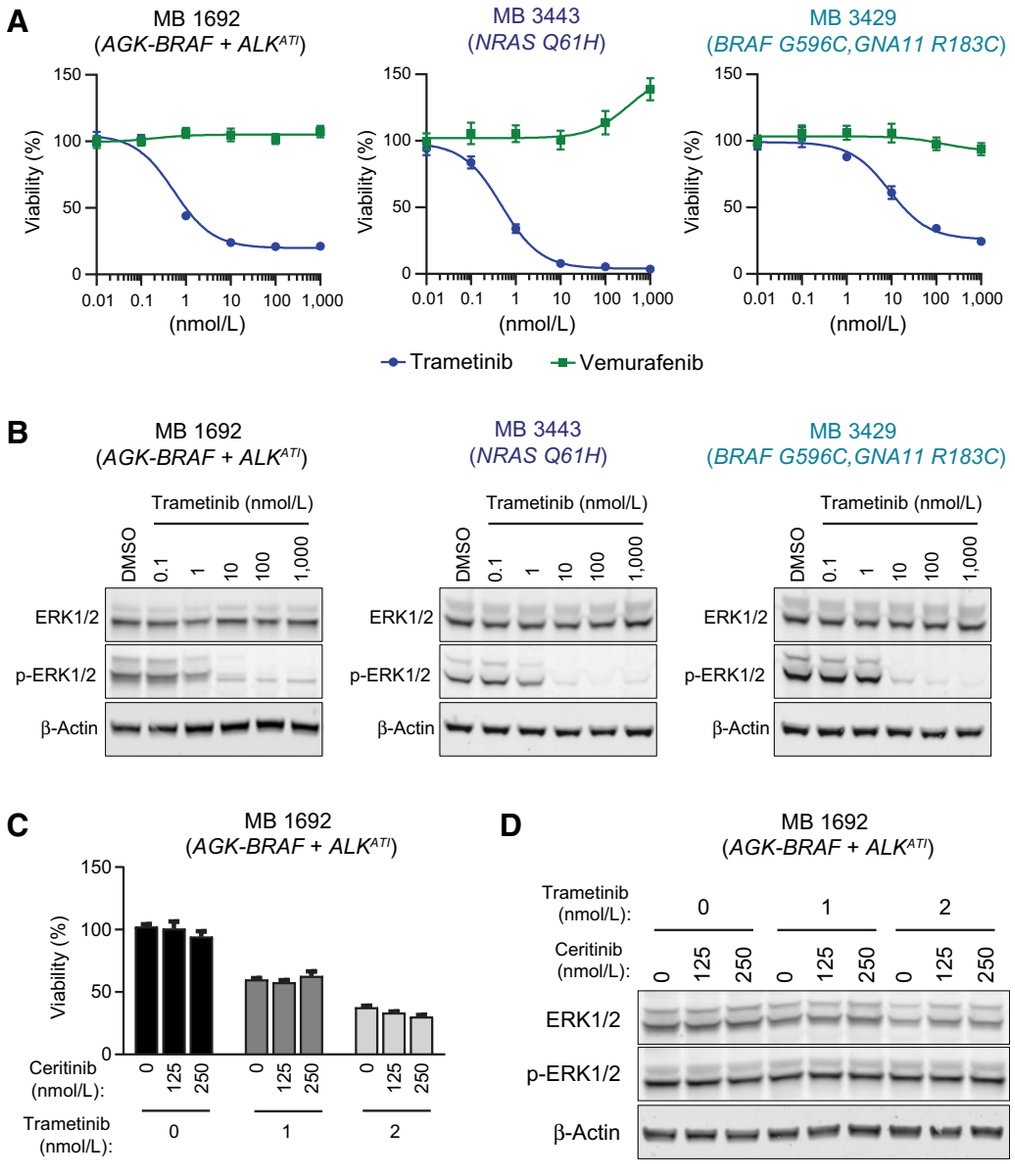

Figure 3.

Melanoma cells respond to targeted MEK inhibition against other oncogenic drivers. **A,** MB 1692, MB 3443, and MB 3429 cells were treated with increasing concentrations of a MEK inhibitor (trametinib) or mutant BRAF specific inhibitor (vemurafenib). Viability was analyzed after 72 hours and normalized to DMSO-treated controls. **B,** Cells were treated with increasing concentrations of trametinib for 2 hours and analyzed by immunoblotting. **C,** MB 1692 cells were treated with increasing concentrations of ceritinib alone or in combination with 1 nmol/L or 2 nmol/L trametinib. Viability was analyzed after 72 hours and normalized to nontreated controls. **D,** MB 1692 cells were treated with the indicated concentrations of trametinib and/or ceritinib for 2 hours and analyzed by immunoblotting. For all viability results, error bars represent SEM of three technical triplicates.

patient experienced rapid clinical progression with worsening pain and dyspnea, and radiologic progression of multiple target lesions and nontarget lesions (20% increase by RECIST 1.1 criteria, Fig. 5B). Treatment was discontinued after 14 days.

Discussion

ALK fusions and response to targeted therapy have not been previously reported in malignant melanomas. Here, we report the first incidence of an *ALK* fusion in malignant melanoma and demonstrate it is highly sensitive to ALK inhibitors *in vitro* and *in vivo*. We identified novel *EML4-ALK* fusion variants (including exon 17 of *ALK*) in a mucosal melanoma lacking common melanoma driver mutations. Mucosal melanomas are a less common (1%–2%) subtype of malignant melanoma that arise on mucosa sites (such as sinonasal, oral, anorectoral, and vulvovaginal) and are not related to sun exposure (29). Recently, we reported the first incidence of a *ROS1* fusion in an acral lentiginous melanoma, another subtype of non-sun–exposed melanoma (30). Studies on sun exposure–related melanomas have

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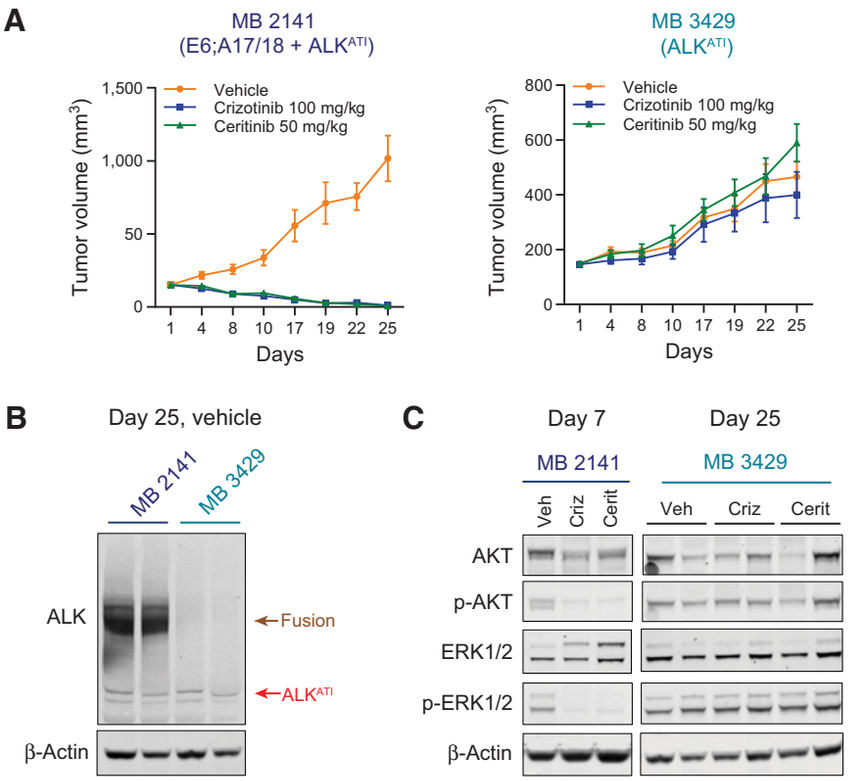

Figure 4.

ALK inhibitors decrease growth of melanoma xenograft tumors expressing an *EML4-ALK* fusion, but not ALK^{ATI} only. **A**, MB 2141 and MB 1692 subcutaneous xenograft tumors were generated in nude mice and treated with vehicle, crizotinib (100 mg/kg), or ceritinib (50 mg/kg). Growth was measured biweekly for 25 days, at which time all tumors were harvested. **B,** Immunoblot analysis for total ALK and β-actin in tumor lysates from vehicle tumors harvested at day 25. Lysates from two independent tumors were analyzed for each condition. **C,** Immunoblot analysis for AKT/p-AKT, ERK1/2/p-ERK/1/2, and β-actin in MB 2141 tumors harvested after 7 days of treatment and MB 3429 tumors harvested after 25 days of treatment. Lysates from two independent tumors were analyzed for each condition for MB 3429 tumors. (Veh, vehicle; Criz, crizotinib; Cerit, ceritinib).

identified *BRAF* and *RAF1* fusions, but fusions in other kinases such as *ALK* and *ROS1* have not been identified (15, 16). Both mucosal and acral lentiginous melanomas have been shown to have overall lower mutation burden but increased frequency of chromosome alterations compared with sun-exposed melanomas (31). Altogether, these data suggest certain gene rearrangements and fusion events may be specific to non-sun–exposed melanomas or have an increased frequency in these subtypes compared with sun-exposed melanomas. *ALK* and *ROS1* fusions may not occur or may be extremely rare in cutaneous melanomas, and the frequency of these fusions in non-sun–exposed melanomas such as mucosal and acral lentiginous melanomas remains unclear. Broad testing of melanomas, especially melanomas known to lack other common driver mutations, with multiplexed next-generation sequencing would diminish the need to make decisions on specific patient populations to screen. This information is clinically relevant for the treatment of patients with acral and mucosal melanomas that do not have targetable driver mutations.

Although an *EML4-ALK*–expressing melanoma was sensitive to ALK inhibitors, melanomas expressing only wt *ALK* or ALK^{ATI} were not sensitive to ALK inhibition. Previous studies have shown high level amplification and overexpression of endogenous *ALK* can be oncogenic and respond to ALK inhibition, but the levels of wt *ALK* expression in the MB 1374 tumor sample is not strongly elevated therefore the lack of sensitivity to ALK inhibitors is not unexpected (32, 33). The recent report first describing the ALK^{ATI} in melanoma did show ALK^{ATI} to be activated and responsive to ALK inhibition, but all *in vitro* and *in vivo* inhibitor studies were performed in cell lines (NIH3T3, Ba/F3, and melan-a) that exogenously expressed ALK^{ATI}, not human-derived melanoma cell lines or tumors that have endogenous expression of ALK^{ATI} (19).

In the current study, we found all melanomas that express ALK^{ATI} had a cooccurring mutation or fusion in one of the common melanoma driver genes (*BRAF*, *NRAS*, *GNA11*, or *NF1*). We hypothesized that the presence of an additional driver oncogene may prevent response to ALK inhibition, but cotreatment with MEK inhibitor in the melanoma cell line harboring an *AGK-BRAF* fusion did not improve ALK inhibitor sensitivity. Interestingly, we observed a loss of ALK^{ATI} expression in the majority of melanomas when they were cultured *in vitro*, but ALK^{ATI} was reexpressed in a corresponding xenograft model when the cells were reinjected into mice. This suggests plasticity in ALK^{ATI} expression that may be dependent on the tumor microenvironment. The loss of ALK^{ATI} expression *in vitro* and the cooccurrence with other oncogenic drivers suggest ALK^{ATI} is not an oncogenic driver in melanoma, providing a possible explanation for lack of inhibitor response in ALK^{ATI}-expressing melanomas.

In both melanoma cells and NIH3T3 cells expressing ALK^{ATI}, we observed a low level of phospho-ALK expression. In the original ALK^{ATI} study, phospho-ALK expression was not quantified in patient tumor samples and was not reported for NIH3T3 and melan-A cells expressing ALK^{ATI}. Kinase activity assays showed ALK^{ATI} had activity *in vitro*, but the experiment was performed only with NIH3T3 cells and not melanoma patient derived cells (19). Therefore, an alternative explanation for the lack of sensitivity to ALK inhibition may be low level of ALK activation in actual melanoma patient samples which express ALK^{ATI}. In addition, we cannot rule out the possibility that current ALK inhibitors are not effective at binding and/or inhibiting the ALK^{ATI} variant protein.

In summary, we identified a mucosal melanoma which expresses novel *EML4-ALK* fusion variants and is highly sensitive

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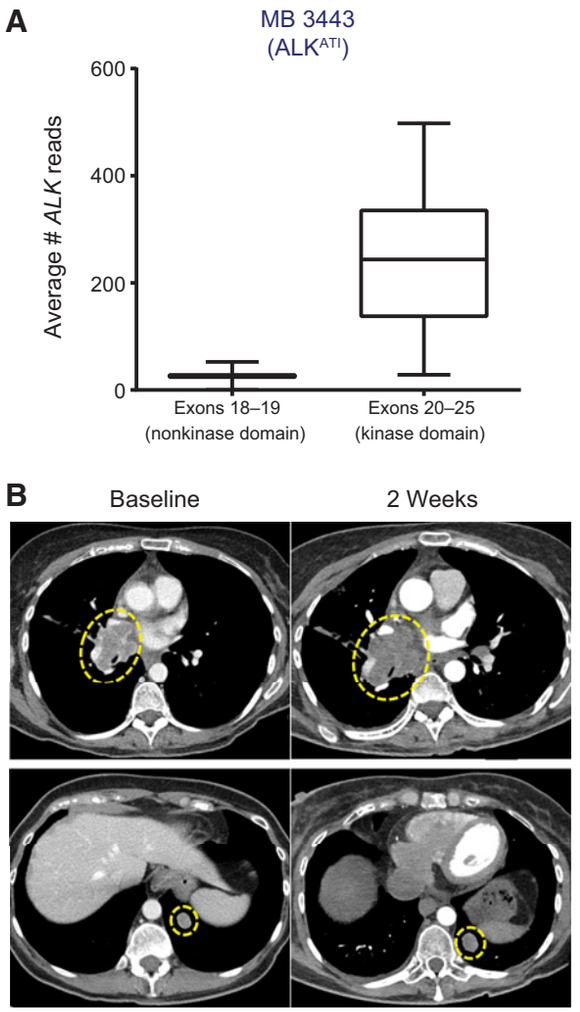

Figure 5.

A patient with melanoma expressing ALK^{ATI} does not respond to treatment with entrectinib. **A,** The patient specimen was analyzed for *ALK* gene expression using targeted RNA next-generation sequencing. The average number of sequencing reads for exons 18–19 (upstream of the kinase domain, not included in ALK^{ATI}) and exons 20–25 (kinase domain included in ALK^{ATI}) of the *ALK* gene are shown. **B,** Radiographic images from CT scans performed prior to treatment (baseline) and 2 weeks after the start of treatment are shown for a right hilar pulmonary tumor nodule (top) and a left lower lobe pulmonary tumor nodule (bottom).

to ALK inhibition. This is the first report of a characterized *ALK* fusion in a non-Spitzoid melanoma subtype and response to ALK inhibition. We also identified several melanomas which express wt *ALK* and/or ALK^{ATI}, an alternate isoform of *ALK*, but these melanomas do not respond to ALK inhibitors. Furthermore, a patient with mucosal melanoma expressing ALK^{ATI} did not respond to ALK-targeted therapy. Further studies are required to understand the discrepancy of ALK inhibitor response in melanomas with varying levels of wt *ALK* and ALK^{ATI} expression. Our data expand the current knowledge of targeted therapy response in *ALK* fusion tumors to a new histology and provide a new targeted therapy option for patients with mucosal melanomas.

Disclosure of Potential Conflicts of Interest

A. Le has ownership interest (including patents) in Abbott Molecular. P. Multani is a Chief Medical Officer at Ignyta, Inc. E. Chow-Maneval is a Vice President, clinical development, at Ignyta. R.C. Doebele reports receiving a commercial research grant from Ignyta, has received speakers bureau honoraria from Guardant Health, has ownership interest (including patents) in Abbott Molecular, and is a consultant/advisory board member for AstraZeneca, Ariad, Takeda, Spectrum Pharmaceuticals, and Ignyta. No potential conflicts of interest were disclosed by the other authors.

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