

Functional Consequence and Therapeutic Targeting of Cryptic ALK Fusions (ALK fus) in Monosomy7 AML

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Abstract

Acute Myeloid Leukemia (AML) patients have a wide array of cytogenetic and molecular aberrations which can influence response to therapy. Monosomy7 (Mono7) is a rare subset within pediatric AML (prevalence of 4-5%), that is highly associated with poor outcomes. Fusions involving the *ALK* gene (14.3%) were exclusively identified within this high-risk cohort while absent across all other AML. Given the dismal outcomes of Mono7, we evaluated the use of crizotinib, an FDA-approved tyrosine kinase inhibitor, used to treat patients with ALK fusions. Our findings suggest that crizotinib may serve as a novel therapy for these patients.

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Abbreviation:	Phrase:
Mono7	Monosomy 7
AML	Acute Myeloid Leukemia
TpAML	Target Pediatric AML
ALK	Anaplastic Tyrosine Kinase
ALK ^{fus}	ALK fusion(s)
NSCLC	Non-Small Cell Lung Cancer
ALCL	Anaplastic Large-Cell Lymphoma
COG	Children's Oncology Group

Abstract:

Acute Myeloid Leukemia (AML) patients have a wide array of cytogenetic and molecular aberrations which can influence response to therapy. Monosomy7 (Mono7) is a rare subset within pediatric AML (prevalence of 4-5%), that is highly associated with poor outcomes. Fusions involving the *ALK* gene (14.3%) were exclusively identified within this high-risk cohort while absent across all other AML. Given the dismal outcomes of Mono7, we evaluated the use of crizotinib, an FDA-approved tyrosine kinase inhibitor, used to treat patients with ALK fusions. Our findings suggest that crizotinib may serve as a novel therapy for these patients.

1 - Introduction:

Acute Myeloid Leukemia (AML) is characterized by a malignancy of cells in the myeloid lineage and caused by diverse genomic alterations, resulting in a heterogenous disease with varied response to conventional therapies¹. Among the heterogenous makeup of AML, Monosomy7 (Mono7) is one of the most established poor prognostic markers in AML with over four decades of clinical outcome data from a variety of clinical trials and other studies^{2, 3, 4, 5}. Mono7 is a rare event in pediatric AML resulting from the somatic copy number loss of chromosome 7; despite intensive and myeloablative therapies, outcomes for this subtype of AML remain dismal with no meaningful advances in therapy.

We recently reported the discovery of fusions involving the Anaplastic Tyrosine Kinase (*ALK*) gene and its unique occurrence in Mono7 pediatric AML⁶. The *ALK* gene encodes a receptor tyrosine kinase, belonging to a family of protein kinases linked to unregulated cell growth, that is a key component in CNS development⁷. *ALK* is located on chromosome 2 (2p23) and chimeric ALK fusions have been frequently observed in cases of inversion 2, as described in Non-Small Cell Lung Cancer (NSCLC), Anaplastic Large-Cell Lymphoma (ALCL), and Neuroblastoma^{8, 9}. Additionally, *ALK* is integral to various molecular pathways involving proliferation and differentiation in cells leading to a unique connection between *ALK* and tumorigenesis¹⁰. The genetic alterations described here involve Spectrin Beta Non-Erythrocytic 1 (*SPTBN1*), and RAN Binding Protein 2 (*RANBP2*), both of which are located on chromosome 2. While previously reported in adult AML and myelomonocytic leukemia, this report describes the first instance of *SPTBN1-ALK* and *RANBP2-ALK* in pediatric AML^{11, 12, 13}.

ALK alterations in a variety of cancers have been observed to be susceptible to targeted therapy with various kinase inhibitors^{14, 15, 16}. *ALK* positive relapsed/refractory ALCL, Neuroblastoma, and other solid tumors in children and young adults have been successfully treated with crizotinib: an FDA-approved kinase inhibitor^{17, 18, 19}. Here we provide studies defining the functional consequence of these ALK fusions (*ALK^{fus}*) demonstrating that these fusions are transforming alterations and cells harboring these fusions are sensitive to the ALK inhibitor crizotinib in a hematologic environment. These data support the potential role of crizotinib to therapeutically target this highly refractory cohort of Mono7 patients.

2 - Methods & Results:

2.1 – Fusion Cytogenetics

As part of our recently completed discovery efforts in childhood AML (TpAML), we interrogated the transcriptome of 1068 children and young adults treated on the COG Phase 3 clinical trial, AAML1031, via RNA sequencing. From these efforts, we identified cryptic and karyotypically identified fusions involving the *ALK* gene in 4 patients. The *ALK* fusions included *SPTBN1-ALK* (n=3) or *RANBP2-ALK* (n=1) (Fig. 1A)⁶. Notably, all four *ALK*^{fus} cases occurred exclusively in patients with Mono7, constituting 14.3% of patients with Mono7 (4 of 28); *ALK* fusions were not detected in the remaining 1064 patients (Fig. 1B). In all four cases the entire kinase domain of the *ALK* gene was retained. Importantly, overexpression of the *ALK* gene was only identified in the four *ALK*^{fus} positive cases with a mean TPM expression of 11.76 versus 0 for *ALK*^{fus} negative patients (Fig. 1B; p<0.0001), suggesting that *ALK*^{fus} upregulates *ALK* expression. The single *RANBP2-ALK* case additionally harbored chromosome inversion 2, while only one of the *SPTBN1-ALK* cases was noted to harbor inversion 2; no other cytomolecular aberration was observed in any *ALK*^{fus} cases (TABLE 1).

2.2 – Fusion Functionality

To evaluate whether the *ALK* fusion transcripts generated oncoproteins, fusion transcripts were cloned from patient samples into the pCSII lentiviral vector (AddGene) under the EF1-Alpha promoter and tagged with GFP, which was used to transduce and transfect cell lines to produce model systems.

SPTBN1-ALK or *RANBP2-ALK* fusion transcripts were transfected into HEK293T cells (ATCC), and cells were maintained in DMEM supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. IL-3 dependent Ba/F3 cells were transduced with *SPTBN1-ALK* or *RANBP2-ALK* fusion transcripts and maintained in RPMI 1640 supplemented with 10% FBS, 10 ng/mL IL3, L- glutamine, and penicillin/streptomycin. Cells were lysed with Triton X-Lysis buffer, underwent western blotting (Invitrogen iBind), stained with anti-*ALK* (Cell Signaling Technologies), and β -Actin antibodies (Cell Signaling Technologies). The presence of *ALK* protein was confirmed by western blot, depicted as bands at approximately 124kD and 175kD (*SPTBN1-ALK* and *RANBP2-ALK*, respectively) (Fig. 2A). The two protein species detected in the *RANBP2-ALK* lysates suggest that the *ALK* region of the fusion may be phosphorylated; this may suggest differential regulation of pathways involving *ALK* and would require further study.

The leukemic potential of the fusion transcripts were studied in a transformation assay. Transduced Ba/F3 cells were sorted to GFP homogeneity and growth kinetics post-IL-3 withdrawal were evaluated using Trypan blue staining and live cell counting with Countess II (Thermo Fischer Scientific).

Following IL-3 withdrawal from transduced Ba/F3 cells, cells expressing either *SPTBN1-ALK* or *RANBP2-ALK* had sustained growth and rapidly proliferated in cytokine-free media, whereas the parental line quickly died in the absence of cytokines, confirming the transformation potential of the fusion oncoprotein (Fig 2B). The presence of the *SPTBN1-ALK* led to greater cytokine-independent proliferation compared to that of *RANBP2-ALK* fusion.

2.3 – Cytotoxicity

ALK inhibitors (e.g., crizotinib) are used in clinical practice for various *ALK* expressing malignancies therefore we hypothesized that treatment with crizotinib would result in effective killing of *ALK*^{fus} cells¹³. *ALK*^{fus} + Ba/F3 cells were plated in triplicate and cultured with varying doses of Crizotinib (SelleckChem) in cytokine-free media²⁰. Cell viability and cell death were determined with the Promega Cell Titer Glow assay after 48 hours in culture on a BioTek Synergy H4 Hybrid plate reader via Gen5 2.1 Software; this was repeated for *ALK*^{fus} + cells in the presence of IL-3. Crizotinib-induced cytotoxicity in *ALK*^{fus} + Ba/F3 cells was enhanced in the absence of IL-3 compared to cells cultured in the presence of IL-3 (IC50 144nM vs. 1051nM for *SPTBN1-ALK* and 95nM vs. 1277nM for *RANBP2-ALK*) (Fig. 2C).

3 - Discussion:

Identification of targetable lesions in AML provides clinicians with novel treatment options to improve clinical outcomes for patients who respond poorly to current conventional chemotherapy, such as patients

with Monosomy 7 AML. With ongoing discovery efforts and further interrogation of large patient repositories, we hope to identify additional novel targets, particularly for patients with rare subtypes of AML.

Here, we demonstrate that newly discovered ALK^{fus} in Mono7 AML leads to a functional ALK protein with the ability to transform cells and induce a proliferative advantage in a cytokine-independent manner. We show cells harboring the ALK^{fus} to be susceptible to the ALK inhibitor crizotinib, inducing tumor cell death in the context of AML *in vitro* modeling systems. We hypothesize treatment with newer generations of ALK-inhibitors will result in more robust tumor cell killing and warrants additional evaluation for exploration. Additionally, to evaluate the preclinical efficacy of treating ALK^{fus}AML with ALK-inhibitors, such as crizotinib, future *in vivo* experiments should be performed. The data presented here provide rationale for ongoing exploration of crizotinib as an effective therapeutic option for these high-risk patients with Monosomy7 AML.

Conflict of Interest:

The authors have no relevant conflicts of interests to disclose.

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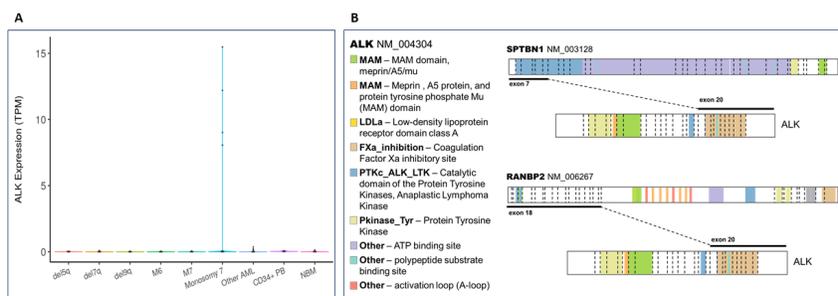


Figure 1. ALK expression and Fusions detected in AML. **A.** Distribution of ALK gene expression by cytogenetic types of AML in transcripts/million (TPM) as determined by RNA sequencing. Each dot represents 1 patient. **B.** Depiction of ALK fusions and breakpoints at each gene. Colored domains of the ALK gene are indicated on the left.

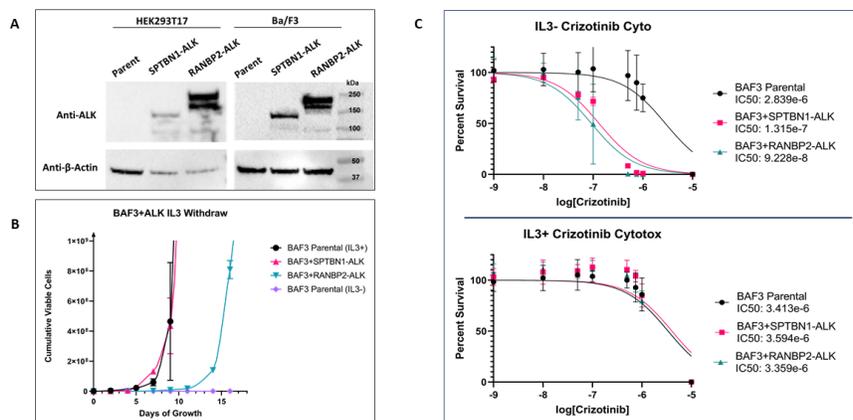


Figure 2. Confirmation of ALK fusion proteins, Transformation assay, and Evaluation of crizotinib. **A.** Expression of the resultant GFP tagged oncoproteins for SPTBN1-ALK and RANBP2-ALK at 124kD and 175kD, respectively, was confirmed by western blot of total protein lysates collected from transfected HEK293T17 and transduced Ba/F3 cells. **B.** Evaluation of the oncogenic potential of ALK fusions utilizing Ba/F3 cells with either SPTBN1-ALK or RANBP2-ALK vs. Ba/F3 parental lines with and without dependence of cytokine, IL3. **C.** Measuring sensitivity of IL3 independent ALK fusions with kinase inhibitor, crizotinib.

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TABLE 1.docx available at <https://authorea.com/users/505788/articles/584770-functional-consequence-and-therapeutic-targeting-of-cryptic-alk-fusions-alk-fus-in-monosomy7-aml>