Leukemic presentation of ALK-positive anaplastic large cell lymphoma with a novel partner, poly(A) binding protein cytoplasmic 1 (PABPC1), responding to single-agent crizotinib

Anaplastic large cell lymphoma (ALCL) is a subtype of non-Hodgkin lymphoma (NHL) characterized by T or null phenotype pleomorphic cells that express CD30. Systemic ALCL is sub-classified into two distinct entities, ALK-positive (ALK$^+$ ALCL) and ALK-negative (ALK$^-$ ALCL), according to whether it expresses the fusion protein anaplastic lymphoma kinase (ALK) due to a translocation in the ALK gene at the 2p23 locus.[1] In ALK$^+$ ALCL, the major fusion partner is nucleophosmin 1 (NPM1) due to the translocation t(2;5)(p23;q35) that results in the aberrant production of the 80-kDa fusion protein NPM1-ALK.[2] While most ALK$^+$ ALCLs are associated with the t(2;5) translocation, other translocations have been described.[3,4] In most cases that have the NPM1-ALK fusion, immunohistochemical detection of the ALK protein shows both nuclear and cytoplasmic localization due to the heterodimerization of NPM1-ALK and normal NPM1, a nucleolar phosphoprotein that is ubiquitously expressed and shuttles between the cytoplasm and the nucleus. Approximately 15% of ALK$^+$ ALCL cases lack the nuclear staining pattern, indicating that aberrant ALK expression is due to a partner gene other than *NPM1*.

Recent studies of patients with relapsed or recurrent ALK$^+$ ALCL have shown promising results following treatment with single agent oral crizotinib, an ALK inhibitor.[5] Thus far, these studies have been in patients with no curative treatment options and specifically with NPM1-ALK fusions. Here, we present a child with leukemic presentation of ALK$^+$ ALCL, exhibiting cytoplasmic-only ALK localization, due to a novel fusion partner, the poly(A) binding protein cytoplasmic 1 (PABPC1), who was treated with a single agent crizotinib after poor initial response to chemotherapy.

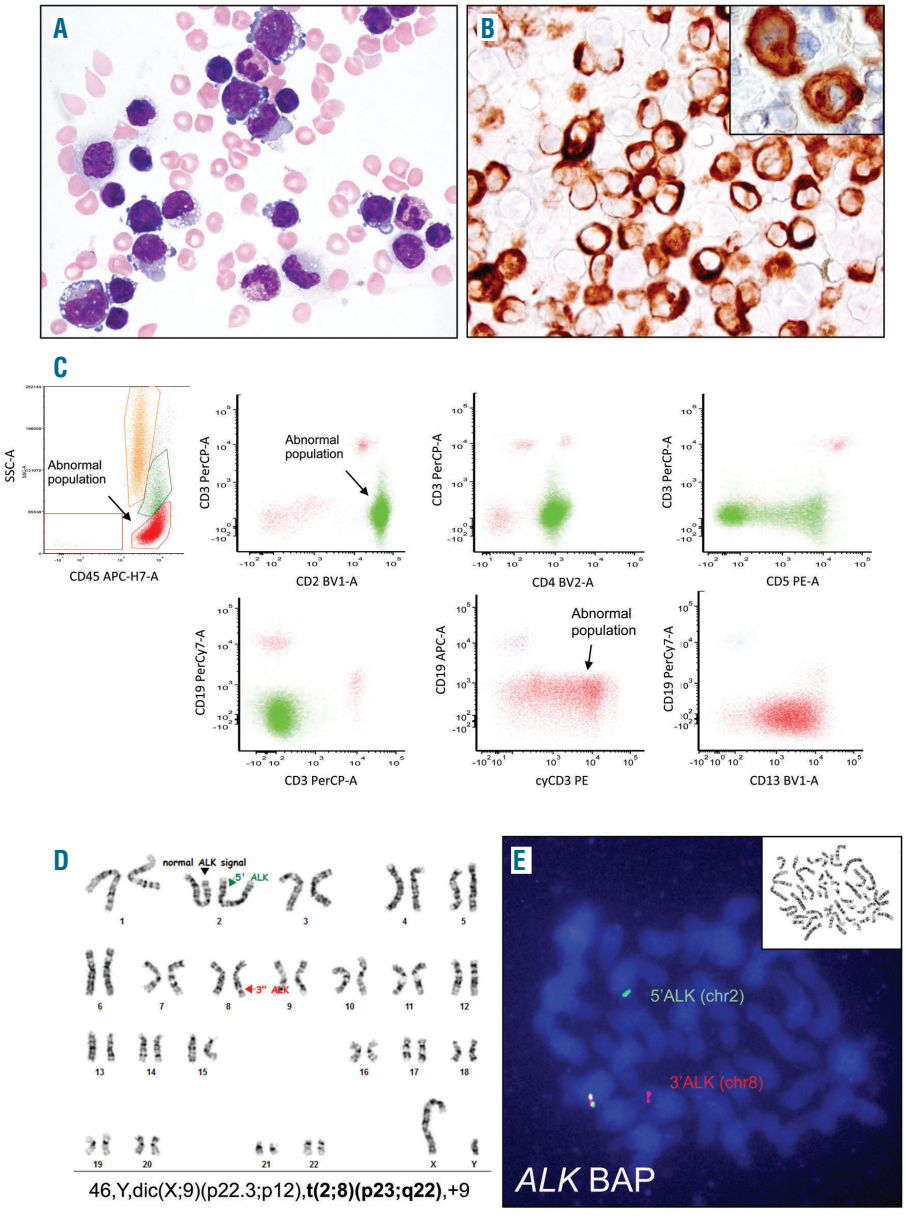
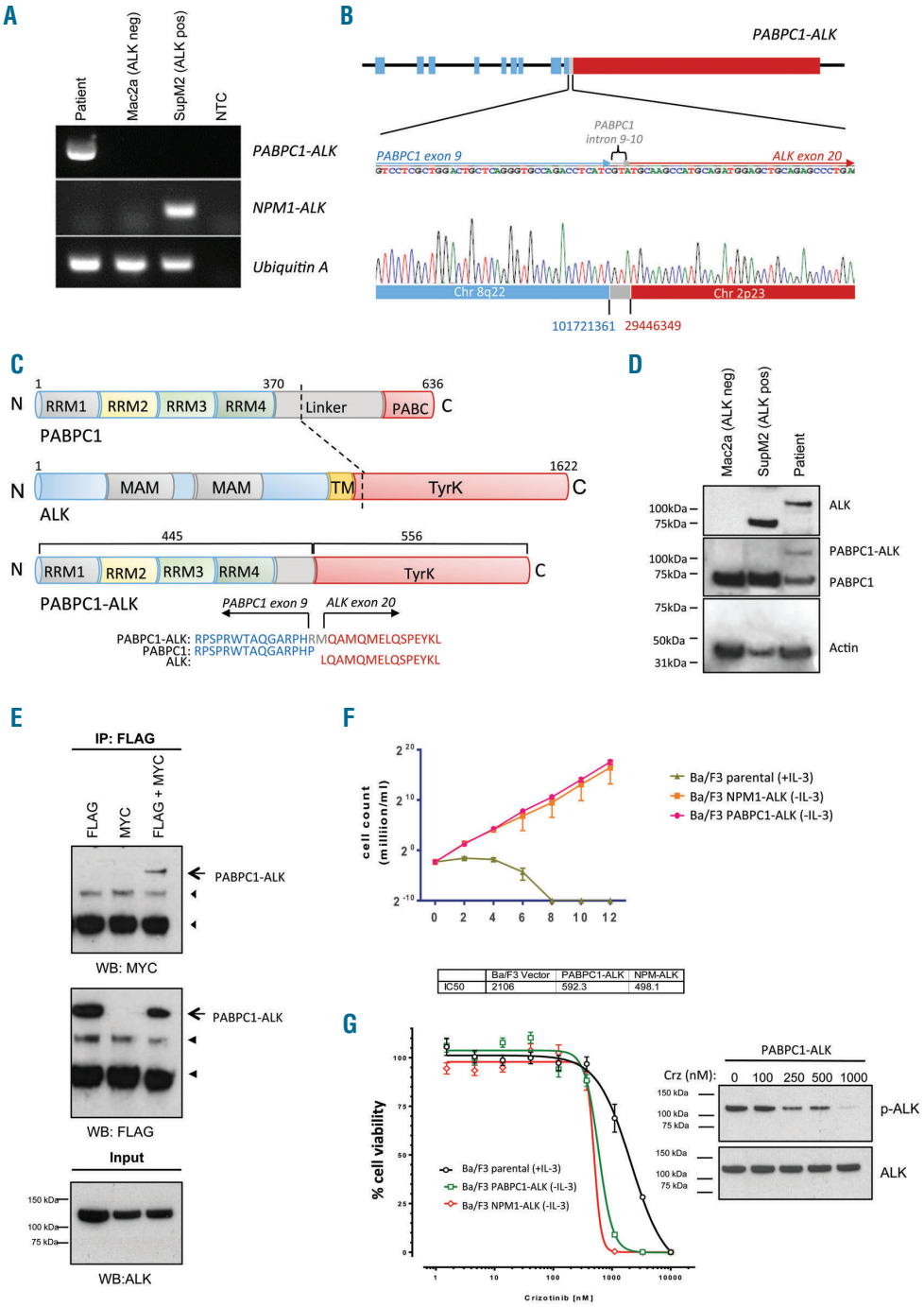


Figure 1. Pathological findings of ALK-positive (+) anaplastic large cell lymphoma (ALCL), leukemic presentation. (A) Peripheral blood smear shows increased atypical large, medium and small lymphocytes with pronounced nuclear contour irregularities, abundant grayish cytoplasm, condensed chromatin and almost unnoticeable nucleoli (Wright-Giemsa x1000; oil). (B) Sections from the peripheral blood clot stained with ALK-1 immunohistochemical stain highlighting the lymphoma cells with cytoplasmic-only localization; insert shows the CD30 immunostain (x1000; oil). (C) Flow cytometric analysis of the diagnostic peripheral blood sample prior to treatment demonstrates a distinct population of aberrant T cells expressing cytoplasmic CD3, CD2, CD4, CD5 (subset), CD13. This population did not express surface CD3, CD8, or CD7. (D) Conventional karyotype on peripheral blood shows t(2;8)(p23;q22). (E) Fluorescence *in situ* hybridization assay of the diagnostic peripheral blood sample using the *ALK* break-apart probe (BAP) confirms the presence of *ALK* rearrangement and the diagnosis of ALK$^+$ ALCL.



	Ba/F3 Vector	PABPC1-ALK	NPM-ALK
IC50	2106	592.3	498.1

Figure 2. Identification and molecular characterization of the PABPC1–ALK fusion in anaplastic large cell lymphoma (ALCL). (A) Confirmation of the PABPC1-ALK fusion by polymerase chain reaction (PCR). PCR of cDNA prepared from the RNA tumor sample used for RNA-sequence revealed a 420-bp amplicon corresponding to the region harboring the *PABPC1-ALK* fusion site in the patient sample but not in cDNA from Mac2A (ALK$^-$) or SupM2 (containing the NPM1-ALK fusion) cell lines. Sample lacking the cDNA template was non-template control (NTC). PCR reactions were performed on the same samples used to amplify the NPM1-ALK fusion. Ubiquitin A was positive loading control. (B) DNA sequencing chromatograms show the conjoined regions at the cDNA sequence level of the *PABPC1-ALK* transcript. (C) Protein domain diagrams illustrating the organization of the PABPC1-ALK fusion kinase. The N-terminal component consists of the PABPC1 exons 1-9 encoding 445 amino acid residues, including the RRMs domains and a portion of the Linker domain. After intervening arginine and methionine residues at the PABPC1/ALK junction, the C-terminal component consists of ALK exons 20-29 encoding 556 amino acid residues and retaining the entire tyrosine kinase domain. (D) Western blot assays for ALK and PABPC1 using the tumor sample from patient and ALCL cell lines SupM2 and Mac2A. The native PABPC1 had a molecular mass of 75 kDa. In the patient sample, a discrete band with a molecular mass higher than 80 kDa as a result of the PABPC1-ALK fusion was seen (concurrently run protein standards indicated by lines). (E) Expression vectors for Flag-tagged and myc-tagged *PABPC1-ALK* were introduced into HEK293T cells together or singly. Cell lysates were immunoprecipitated with antibodies to Flag, and the precipitates were immunoblotted with anti-myc and anti-Flag antibodies. (Right) Position of PABPC1-ALK is shown (arrows). Arrow heads indicate non-specific bands. (F) PABPC1-ALK confers cytokine-independent growth to Ba/F3 cells. Stably transduced Ba/F3 cells expressing PABPC1–ALK (*Online Supplementary Figure S3B*) were assessed for growth in the absence of IL-3, along with *NPM1-ALK* and empty vector–transduced Ba/F3 cells. Viable cell counts were determined in triplicate, using trypan blue at 48-hour (h) intervals; each time point represents mean±Standard Error of Mean (SEM). (G) Cytokine-independent proliferation was inhibited by the small-molecule ALK inhibitor crizotinib. Transduced Ba/F3 cells were grown in increasing concentrations of crizotinib. Results represent mean±SEM from four experiments. Ba/F3 cells expressing PABPC1-ALK were incubated with the indicated concentrations of crizotinib for 2 h, and total cell lysates (25 μg of protein) were subjected to immunoblot analysis with antibodies to tyrosine-phosphorylated ALK (p-ALK) and total ALK.

A 16-month old male presented with lethargy, diarrhea, and decreased oral intake. On clinical examination he was pale, underweight, dehydrated, febrile, tachypneic, and tachycardic with cervical lymphadenopathy. Laboratory findings revealed leukocytosis [white blood cell (WBC) count 259x10^9/L], anemia [hemoglobin (Hb) of 8.3 g/dL], increased uric acid (10.9 mg/dL) and lactate dehydrogenase (LDH) of 439 units/L. Microscopic examination of the peripheral blood (PB) smear showed atypical lymphocytes with a predominant population of small- to medium-sized cells with irregular/folded nuclei in addition to occasional large cells with vacuolated cytoplasm (Figure 1A and *Online Supplementary Figure S1A-D*). Flow cytometric analysis using PB demonstrated aberrant T cells (57%) that expressed CD2 (bright), CD4, CD5 (subset), CD13 (dim), HLA-DR (subset), and cytoplasmic CD3 (Figure 1C). These cells did not express CD1a, surface CD3, CD7, CD8, CD16, TCR gd, TCR ab, CD34, MPO, and TdT. Immunohistochemistry performed on PB clot section showed aberrant T cells expressing CD30, cytotoxic markers (TIA-1, granzyme B), and ALK with cytoplasmic-only localization (Figure 1B and *Online Supplementary Figure S1E and F*). Karyotyping showed 46,Y,dic(X;9)(p22.3;p12),t(2;8)(p23;q22),+9 (Figure 1D), and fluorescence *in situ* hybridization (FISH) confirmed the presence of *ALK* rearrangement (Figure 1E).

Based on morphological, immunophenotypic, and cytogenetic findings, the diagnosis of leukemic ALK$^+$ ALCL, was established favoring small cell variant histology. He commenced chemotherapy as per the International Protocol for the Treatment of Childhood Anaplastic Large Cell Lymphoma (ALCL99). After completing pre-phase and two cycles of chemotherapy (Course A and Course B per ALCL99)[6,7] flow cytometry of PB showed detectable residual disease (*Online Supplementary Figure S2*). Because of persistence of low level disease in PB by immunophenotypic testing after two courses of chemotherapy, he transitioned to single agent crizotinib at a dose of 280 mg/m^2/dose twice daily, which was shown to be tolerable and had demonstrated a 90% response rate in pediatric patients with relapsed or recurrent ALK$^+$ ALCL.[5] After two weeks of crizotinib treatment, flow cytometry of PB demonstrated 0.05% lymphoma cells, and after a month he had no detectable disease (*Online Supplementary Figure S2A*). He was monitored with monthly immunophenotyping and treated with monthly intrathecal chemotherapy given the high level of leukemia on presentation and the risk for unknown central nervous system (CNS) involvement on presentation for 10 months. Unfortunately, the patient suffered a traumatic skull fracture complicated by abscess and osteomyelitis, and, given the concern for delayed healing, the crizotinib treatment was discontinued. PB immunophenotyping was performed weekly. Five weeks after discontinuing crizotinib, he presented again with fevers and right-sided maxillary lymphadenopathy. At this time, flow cytometry on PB was positive for abnormal T cells representing 0.07% CD45$^+$ cells, which increased to 2.23% within a week. Treatment with crizotinib was started at a dose of 165 mg/m^2/dose twice daily, which has shown sustained activity in previous trials[5] and is currently being used by the Children's Oncology Group phase II study for newly diagnosed patients with ALCL. PB immunophenotyping was performed weekly after re-starting crizotinib and demonstrated decreasing disease burden (*Online Supplementary Figure S2B*). He again achieved complete remission with criztonib treatment. The patient remains in remission on crizotinib therapy without any apparent toxicity.

To identify the non-NPM1 partner of the ALK fusion in this case of leukemic ALK$^+$ ALCL with cytoplasmic-only ALK localization and the novel t(2;8) translocation, we performed whole genome sequencing (WGS) and RNA sequencing (RNA-seq). A chimeric in-frame fusion that juxtaposed exon 9 of PABPC1 to exon 20 of ALK was identified (*Online Supplementary Figure S3A*). No mutations were detected within the coding sequence of *PABPC1-ALK*. Reverse-transcription polymerase chain reaction (PCR) confirmed the presence of *PABPC1-ALK* transcripts in the tumor sample but not in the NPM1-ALK$^+$ ALCL cell line (Figure 2A). Sanger sequencing confirmed that the fusion occurred at PABPC1 NM_002568 c.1840 (exon 9) and ALK NM_004304 c.4149 (exon 20) (Figure 2B). FISH on primary tumor cells verified the presence of the *PABPC1-ALK* rearrangement (*Online Supplementary Figure S3B*). The tumor cells did not show extra copies of the rearranged *ALK* gene, as has previously been observed in cases harboring non-NPM1-ALK fusions.[8] Previous case reports have described the presence of *MYC* rearrangement in addition to the *ALK* fusion in cases of leukemic ALK$^+$ ALCL that may be associated with a more aggressive clinical course. Both FISH studies (*Online Supplementary Figure S3C*) and RNA-seq analysis showed no *MYC* rearrangement or fusion in our patient.

The PABPC1 (PABP) binds to the 3' poly(A) tail of eukaryotic mRNAs and plays an important role in translation and mRNA metabolism.[9] The N-terminus of PABPC1 contains four tandemly repeated RNA recognition motifs followed by a proline-rich linker region and a C-terminus PABP domain. The poly(A)-bound PABPC1 forms multimers *via* mutual intermolecular interactions, which reportedly involve the linker region.[10,11] The *PABPC1-ALK* fusion encodes a 1001-amino-acid chimeric protein with a predicted molecular mass of 112 kDa. In the PABPC1-ALK fusion reported herein, the 5' partner of the fusion protein retained the N-terminus and the portion of the linker region of PABPC1 that has been shown to play a role in the PABPC1 multimers on poly(A) (Figure 2C). Western blot analysis for both ALK and total PABPC1 revealed a band corresponding to a molecular mass higher than that for the 80-kDa NPM1-ALK present in the ALK$^+$ ALCL cell line SupM2, which was consistent with expression of the novel fusion (Figure 2D). Ectopic expression of the full-length PABPC1-ALK fusion protein in HEK293T cells resulted in constitutive activation of ALK signaling pathway (*Online Supplementary Figure S4A*). Translocation with *NPM1* or alternative partners leads to aberrant constitutive activation of the catalytic domain of ALK kinase through homodimerization. To verify the dimerization potential of PABPC1, we performed immunoprecipitation (IP) experiment by co-transfecting HEK293T cells with Flag-PABPC1-ALK and PABPC1-ALK-MYC plasmids. FLAG IP, followed by MYC western blot confirmed heterodimerization potential of the PABPC1-ALK fusion protein (Figure 2E).

To establish the oncogenic potential of PABPC1-ALK fusion protein, *PABPC1-ALK* was cloned into the p MSCV-IRES-GFP (p MIG) expression vector, murine Ba/F3 cells were retrovirally transduced and interleukin-3 was withdrawn. PABPC1-ALK expression conferred cytokine-independent proliferation (Figure 2F) similar to that observed in Ba/F3 cells expressing the NPM1-ALK fusion protein. Importantly crizotinib reduced the cytokine-independent proliferation of transduced Ba/F3 cells (Figure 2G). Immunoblot analysis revealed that crizotinib inhibited the phosphorylation of tyrosine on PABPC1-ALK in a concentration-dependent manner in

transfected Ba/F3 cells. From these experiments we conclude that the novel PABPC1-ALK fusion exhibits a cell-transforming activity that is dependent on ALK kinase activation, similar to NPM1-ALK fusion protein.

This clinical case is consistent with previous studies that describe patients with leukemic presentation of ALK$^+$ ALCL showing significant leukocytosis and widespread organ involvement. Leukemic involvement in ALK$^+$ ALCL is most often associated with the small-cell histological variant, which is also favored in our case, and poor prognosis.[12-14] The patient in this study showed a partial response to traditional chemotherapy, but he achieved complete remission after treatment with crizotinib, based on non-detectable disease by flow cytometry. The recurrence of the disease in this case after interruption of crizotinib highlights a possible limitation of immunophenotypic studies to detect minimal residual disease in this patient with the novel *PABPC1-ALK* fusion. However, this approach may still be of clinical use due to the lack of available PCR testing in the clinical laboratory for non-NPM1-ALK fusions. This immunophenotyping method should be performed in conjunction with clinical monitoring and imaging studies. Recent case reports have also suggested that crizotinib may be used as a long-term treatment for ALK$^+$ ALCL, but that patients must be monitored closely when treatment is interrupted because ALCL may recur rapidly.[15]

In summary, this report describes a young patient presenting with leukemic ALK$^+$ ALCL who was found to have a previously undescribed novel *PABPC1-ALK* fusion, and was treated successfully with crizotinib. This case highlights the sensitivity of ALK$^+$ ALCL to crizotinib and demonstrates that while the disease is likely to relapse if the ALK kinase inhibitor is discontinued, the lymphoma cells remain sensitive to crizotinib treatment upon rechallenge. At the time of writing, three months after re-starting crizotinib, our patient has not developed resistance. In addition, our functional studies provide support for the dimerization properties of the novel PABPC1-ALK chimeric protein and constitutive activation of ALK kinase that is inhibited by crizotinib treatment.

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Acknowledgments: we are grateful to the patient and his family members in allowing us to participate in his care.

Funding: this work was supported in part by ALSAC and by the National Cancer Institute Grant CA21765 (St. Jude Children's Research Hospital Cancer Center Support Grant). We thank the staff of the Flow Cytometry and Cell Sorting Core Facility at St. Jude Children's Research Hospital.

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doi:10.3324/haematol.2018.215103

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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