Targetable fusions of the FRK tyrosine kinase in ALK-negative anaplastic large cell lymphoma

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Anaplastic large cell lymphomas (ALCLs) constitute a group of peripheral (i.e. post-thymic) T-cell non-Hodgkin lymphomas (PTCLs) with overlapping pathologic characteristics, but varying clinical and molecular features. Specifically, ALCLs share cytological and immunophenotypic features, including consistent expression of the lymphocyte activation marker, CD30.1 The World Health Organization (WHO) classifies ALCLs by their clinical presentation (systemic or cutaneous) and whether or not they bear rearrangements of the anaplastic lymphoma kinase gene, ALK (ALK-positive ALCL and ALK-negative ALCL, respectively).2

ALK-positive ALCL is characterized by a unique gene expression signature that distinguishes it from ALK-negative ALCL.3–5 ALK rearrangements have a broad spectrum
of functional consequences, prominent among which is activation of the signal transduction protein STAT3.\textsuperscript{6} Analogously, STAT3 may be activated in ALK-negative ALCLs by somatic events involving non-\textit{ALK} tyrosine kinase genes, including rearrangements of the \textit{TYK2} or \textit{ROS1} tyrosine kinase genes as well as mutations in \textit{JAK1} or \textit{STAT3} itself.\textsuperscript{7,8} However, the full spectrum of tyrosine kinases involved in ALK-negative ALCL pathogenesis and growth remains incompletely understood, as does the similarity of these events to the molecular signature identified in ALK-positive ALCLs. For example, a subclass of ALK-negative ALCL expressing aberrant transcripts of the \textit{ERBB4} tyrosine kinase gene had a gene expression signature distinct from ALK-positive ALCLs.\textsuperscript{9}

To evaluate the relationship between the gene expression profiles of ALK-positive and ALK-negative ALCLs, we performed expression profiling on 31 frozen ALCL tissue samples (Supplementary Table 1) using Affymetrix arrays and derived an ALK signature from our dataset comprising the 29 probes most differentially expressed between these 2 groups (see Supplementary Methods for details). Clustering using this ALK signature identified a single ALK-negative case, ALCL11, which clustered with ALK-positive ALCLs (Figure 1A.i; \textit{P}=0.02, Kolmogorov-Smirnov test). The validity of the ALK signature we derived was supported by the presence of multiple genes in common with previously published signatures, including \textit{ALK}, \textit{ARHGEF10}, \textit{ANXA3}, \textit{GALNT2}, \textit{HTRA3}, \textit{IL1RAP}, \textit{MC1R} and \textit{PDE4DIP} (Supplementary Table 2). Furthermore, clustering analysis using previously published ALK-positive ALCL signature genes replicated the unique clustering pattern of ALCL11, but not other ALK-negative ALCLs, with ALK-positive ALCLs (Supplementary Figure 1).

We next used outlier analysis to identify possible non-\textit{ALK} kinase gene overexpression underlying the ALK-like signature in ALCL11. The top kinase genes found to be outliers in this case were \textit{fyn} related Src family tyrosine kinase (\textit{FRK}) and neurotrophic receptor tyrosine kinase 1 (\textit{NTRK1}; Figure 1A.ii, Supplementary Table 3). \textit{FRK} was expressed exclusively in ALCL11. \textit{NTRK1} was also expressed in 2 cases of ALK-positive ALCL, a disease in which its protein product, TrkA, was recently shown to interact functionally with ALK fusion proteins.\textsuperscript{10} To confirm expression of these genes and evaluate this case for potential gene fusions, we performed RNA sequencing in ALCL11. The SnowShoes fusion detection algorithm revealed a novel \textit{CAPRIN1-FRK} fusion transcript (Figure 1B, Supplementary Table 4). This fusion was validated by RT-PCR and Sanger sequencing (Supplementary Figure 2A), and the resultant fusion protein was detected by Western blotting in a frozen tissue lysate from ALCL11 (Supplementary Figure 2B).

\textit{FRK}, also called protein tyrosine kinase-5 (\textit{PTK5}), is a member of the \textit{BRK} family of tyrosine kinases that are related to Src family kinases and similarly possess SH3, SH2, and kinase domains.\textsuperscript{11} Of note, the \textit{FRK} and ALK regulatory networks share a number of proteins in common, including \textit{STAT3} (Supplementary Table 5). While \textit{FRK} was originally described as a tumor suppressor, recent studies in multiple cancer types have revealed oncogenic genetic events involving \textit{FRK}, including activating mutations in hepatocellular neoplasms and an \textit{ETV6-FRK} fusion in an isolated case of acute myelogenous leukemia (AML).\textsuperscript{11–13} Consistent with exon-level \textit{FRK} expression data from RNA sequencing (Supplementary Figure 2C), the sequenced \textit{CAPRIN1-FRK} fusion transcript encodes exons
3–8 of FRK, with a breakpoint at amino acid 156 in the FRK SH2 domain that leaves the N-terminal kinase domain intact in the resultant fusion protein (Figure 1B). Caprin-1 (CAPR1) is a cell cycle-associated protein that is constitutively expressed in lymphocytes,\textsuperscript{14} and widely expressed in ALCLs based on our gene expression data (Supplementary Figure 2D). Thus, similar to NPM1-ALK and other kinase fusions in ALCL, CAPRIN1-FRK leads to expression of the tyrosine kinase domain of an otherwise unexpressed protein under control of the active promoter of a constitutively expressed partner gene.

We next developed a novel breakapart fluorescence in situ hybridization (BAP-FISH) probe for the FRK locus to investigate the frequency and subtype distribution of FRK rearrangements in ALCL and other PTCLs (see Supplementary Methods for probe details). BAP-FISH for FRK rearrangements was performed on 225 PTCLs and lymphoproliferative disorders, including the original FRK fusion case, ALCL11. The original FRK rearrangement was validated and additional rearrangements were identified exclusively in ALK-negative ALCLs, with a frequency in this group of 5.4% (\(P = 0.013\); Figure 1C; Supplementary Table 6). The ALCLs with FRK rearrangements included 5 systemic cases and 1 primary cutaneous case. All were of the so-called “triple-negative” genetic subtype, i.e., in addition to being ALK-negative they lacked rearrangements of DUSP22 and TP63.\textsuperscript{15}

To assess FRK fusion partners further, we then performed dual-fusion FISH for CAPRIN1-FRK in 5 ALCLs with FRK rearrangements and found this fusion only in ALCL11 (Supplementary Table 7). Therefore, we attempted RNA sequencing from paraffin material in these cases; while this approach either failed or was suboptimal in most of the cases, we did identify one case with a PABPC1-FRK fusion and another with a possible MAPK9-FRK fusion. For both of these events, FRK expression began at exon 3, identical to the fusion site observed in CAPRIN1-FRK (Supplementary Table 7). Because ALK-positive ALCL is associated with favorable outcome,\textsuperscript{1} we examined outcomes in ALCLs with FRK rearrangements. Indeed, although our series is small, the index case was alive 17 years after diagnosis and 3 of the 4 remaining patients with systemic disease survived at least 5 years (Supplementary Table 8).

To assess the function of CAPRIN1-FRK, the fusion transcript was cloned from ALCL11 into the pLEX lentiviral expression vector (see Supplementary Materials). We were unable to achieve stable overexpression in ALCL cells, other T-cell lymphoma cell lines, or normal T cells, despite multiple attempts and testing additional expression vectors; this may have been due in part to the large insert size of the CAPRIN1-FRK fusion transcript. However, we were able to achieve overexpression of CAPRIN1-FRK in HEK-293T cells and IL3-dependent Ba/F3 cells. The Caprin-1-FRK fusion protein localized primarily to the cytoplasm in HEK-293T cells (Supplementary Figure 3A), consistent with the pattern of FRK immunohistochemical staining in ALCL11 (Figure 1C). In HEK-293T cells, CAPRIN1-FRK promoted colony formation 2.7-fold over control vector (\(P < 0.001\); Figure 2A). Correspondingly, CAPRIN1-FRK induced STAT3 phosphorylation, which was absent in control cells despite similar levels of total STAT3 (Supplementary Figure 3B). Both effects were somewhat greater than those induced by NPM1-ALK in this model. CAPRIN1-FRK but not NPM1-ALK also induced phosphorylation of STAT1 and STAT5, suggesting that CAPRIN1-FRK may have additional targets not shared by NPM1-ALK (Supplementary Figure 3B). Previously published data by Hosoya et al that ETV6-FRK did not lead to
phosphorylation of STAT1/3/5/6\(^{13}\) may reflect differences in cellular context and/or expression of total STAT proteins. In the IL3-dependent Ba/F3 model, both CAPRIN1-FRK and NPM1-ALK, but not control vector, rescued cells from IL3 withdrawal (Figure 2B,C.i). In addition, expression of both CAPRIN1-FRK and NPM1-ALK promoted phosphorylation of STAT3 in the absence of IL3 (Figure 2C.i). We then used the Kinase Inhibitor Resource database (see Supplementary Methods) to identify dasatinib, a tyrosine kinase inhibitor with activity against Src-family and other kinases, as a candidate drug targeting FRK fusions. Indeed, dasatinib inhibited CAPRIN1-FRK- but not NPM1-ALK-driven growth following IL3 withdrawal (Figure 2C.ii), and correspondingly reversed CAPRIN1-FRK-induced STAT3 phosphorylation (Figure 2C.iii). Conversely, the ALK inhibitor crizotinib specifically inhibited growth in cells expressing NPM1-ALK with significantly less effect on CAPRIN1-FRK-expressing cells (Figure 2C.iv).

In summary, FRK rearrangements are recurrent in ALK-negative ALCLs, with a frequency of 5.4% and encompassing both systemic and primary cutaneous subtypes. In the index case, a CAPRIN1-FRK fusion transcript was discovered and expression of the resultant fusion protein was confirmed. Other fusion partners also exist. CAPRIN1-FRK promoted phosphorylation of STAT3 and in vitro cell growth that could be inhibited by the kinase inhibitor dasatinib. Thus, FRK rearrangements represent a novel, candidate therapeutic target in a subset of ALK-negative ALCLs.

**Supplementary Material**

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Figure 1. Discovery of FRK rearrangements in ALK-negative ALCL
A. i. Clustering of 31 ALCLs using 29 probes that most significantly differentiated ALK-positive and ALK-negative ALCL groups (see also Supplementary Table 2). A single ALK-negative ALCL clustered with the ALK-positive cases (ALCL11, arrows). ii. Kinase gene outlier analysis in ALCL11 identified FRK and NTRK1 as the top outlier genes (see also Supplementary Table 3). B. RNA sequencing and fusion detection in ALCL11 identified a chimeric CAPRIN1-FRK transcript fusing exon 18 of CAPRIN1 to exon 3 of FRK (see also Supplementary Table 4). The domain structure of the predicted Caprin-1-FRK fusion protein resulting from the identified CAPRIN1-FRK transcript is shown at right. C. FISH evaluation
of 225 PTCLs showed \textit{FRK} rearrangements in 5.4\% of ALK-negative ALCLs and not in any other PTCL subtype (see also Supplementary Table 5). Top center image, hematoxylin and eosin stain of ALCL11 (original magnification, ×1000). Top right image, immunohistochemical staining for FRK was present in ALCL11; inset: staining is absent in an ALK-positive case without an \textit{FRK} rearrangement (ALCL26; see also Supplementary Figure 2B; original magnification, ×400). Bottom center image, FISH in ALCL11 using a breakapart probe to the \textit{FRK} locus showed one normal red-green fusion signal and abnormal separation of the remaining red and green signals; inset: signal pattern in a normal cell showing two fusion signals (original magnification, ×600).
Figure 2. Function and targetability of \textit{CAPRIN1-FRK}

A. \textit{CAPRIN1-FRK} promoted colony formation 2.7-fold in HEK-293T cells compared to empty vector (pLex; \(P<0.001\)). Cells expressing \textit{NPM1-ALK} also are shown. B. Ba/F3 cells were cultured in the absence (48 h withdrawal) of IL3 followed by analysis by Western Blot. \textit{CAPRIN1-FRK} (as well as \textit{NPM1-ALK}) induced STAT3 phosphorylation at Y705 in Ba/F3 cells (V, empty vector; NA, \textit{NPM1-ALK}; CF, \textit{CAPRIN1-FRK}). C. i. \textit{CAPRIN1-FRK} and \textit{NPM1-ALK} rescued IL3-dependent Ba/F3 cells from IL3 withdrawal. ii. The tyrosine kinase inhibitor dasatinib targeted \textit{CAPRIN1-FRK} but not \textit{NPM1-ALK} in Ba/F3 cells. iii. A dose-dependent inhibition of pSTAT3 was observed in \textit{CAPRIN1-FRK}-expressing cells but
not in cells expressing NPM1-ALK. iv. The ALK inhibitor crizotinib targeted \textit{NPM1-ALK} but was relatively ineffective in inhibiting Ba/F3 cells expressing \textit{CAPRIN1-FRK}. 