FLT3 Mutations in Childhood Acute Lymphoblastic Leukemia

Scott A. Armstrong, Meghann E. Mabon, Lewis B. Silverman, Aihong Li, John G. Gribben, Edward A. Fox, Stephen E. Sallan, and Stanley J. Korsmeyer

From the Departments of Pediatric Oncology, Adult Oncology and Cancer Immunology and AIDS, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts; The Division of Hematology/Oncology, Children’s Hospital, Boston, Massachusetts; and the Howard Hughes Medical Institute.

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Corresponding Author: Stanley J. Korsmeyer, M.D.
Dana Farber Cancer Institute
One Jimmy Fund Way SM756
Boston, MA 02115
Phone: (617) 632-6402
Fax: (617) 632-6401

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Abstract:

Activating mutations of the FLT3 receptor tyrosine kinase are common in acute myelogenous leukemia (AML), but are rare in adult acute lymphoblastic leukemia (ALL). We have recently shown that FLT3 is highly expressed and often mutated in acute lymphoblastic leukemias with rearrangement of the Mixed Lineage Leukemia gene on chromosome 11q23. As hyperdiploid ALL samples also show high-level expression of FLT3, we searched for the presence of FLT3 mutations in leukemic blasts from 71 patients with ALL. The data show that approximately 25% (6/25) of hyperdiploid ALL samples possess FLT3 mutations while only 1/29 TEL/AML1 rearranged samples harbored mutations (p=0.04, fisher’s exact test). Three mutations are novel in-frame deletions within a seven amino acid region of the receptor juxta-membrane domain. Finally, three samples from patients whose disease would relapse harbored FLT3 mutations. These data suggest that patients with hyperdiploid or relapsed ALL might be considered candidates for therapy with newly described small molecule FLT3 inhibitors.
Introduction:

The FMS-related tyrosine kinase-3 (FLT3) is a receptor tyrosine kinase expressed in early hematopoietic progenitors that plays an important role in hematopoietic development\textsuperscript{1,2}. Multiple studies have shown that activating mutations of FLT3 are common in blasts from patients diagnosed with acute myelogenous leukemia (AML) while they are rarely found in adult patients diagnosed with acute lymphoblastic leukemia (ALL)\textsuperscript{2-4}. We have recently shown that FLT3 is consistently highly expressed in \textit{MLL} rearranged acute lymphoblastic leukemias (MLL)\textsuperscript{5}. This prompted analysis for FLT3 mutations in MLL where we found approximately 15\% contain activating mutations in the receptor activation loop\textsuperscript{6}. A large gene expression analysis of childhood ALL samples has shown that high level FLT3 expression is also found in ALL samples containing greater than 50 chromosomes (hyperdiploid ALL)\textsuperscript{7}. Here we show that activating FLT3 mutations are found in diagnostic specimens from 6/25 patients with hyperdiploid ALL, but only 1/29 patients with TEL/AML1 rearranged ALL. Also, 3/16 leukemias that would ultimately relapse harbored FLT3 mutations. Most of the mutations are previously described activation loop mutations, but four are new mutations consisting of either small deletions or insertions in the juxtamembrane region of the receptor. These data suggest that patients with hyperdiploid or relapsed ALL should be considered possible candidates for therapy with recently described small molecule FLT3 inhibitors\textsuperscript{8-11}. 
Study Design:

Patient samples:

Leukemia samples were obtained from either bone marrow or peripheral blood at diagnosis from patients diagnosed with ALL. The peripheral blood samples all had >10% blasts at diagnosis. Patients were treated on Dana Farber Cancer Institute Protocols 91-001, 95-001, and 00-001. Informed consent was obtained in all cases after approval of the Institutional Review Board. Standard cytogenetic analysis was performed on all samples. TEL-AML1 translocations were determined by fluorescence in situ hybridization (FISH) or PCR as previously described12.

Mutation Detection:

Genomic DNA was extracted from leukemia samples with Trizol (Invitrogen, Carlsbad, CA). Mutations in the juxtamembrane domain were identified by amplifying a region spanning exons 14 and 15 with primers 14F (TGTAAGACGAGGCCAGTCAATTTAGGTATGAAAAGCC) and 15R (GAGGAAACAGCTATGACCCCTTCAGCATTTTGCAGGCAACC). The upstream primer in this reaction was fluorescently labeled (6-FAM) to allow sizing of all products when electrophoresed on a sequencing apparatus (Model 377, Applied Biosystems Inc, Foster City, CA). The area under the curve of a variant product was divided by the total area under all curves to approximate the percentage of variant alleles in a sample. When variant products were determined to constitute more than 30% of the signal, the sample was directly sequenced following the initial PCR. Samples with no detectable length mutations were also directly sequenced. For a sample with a less
abundant variant (1-30%), the PCR product was run on a 1.5% agarose gel, and the variant product was cut from the gel and reamplified with the original primers prior to sequencing.

Activation loop mutations were determined by PCR amplification with primers 20F (TGTAAGACGAGGCAGTCGCGCAAGAAGCTTGCTT) and 20R (CAGGAAACAGCTATGACCAGACACCTCATTGCC), followed by EcoRV digestion. EcoRV digests wildtype but not mutant fragments at the recognition site composed by codons 835 and 836 (GATATC). A second round of PCR was performed to amplify the uncut fragments. The products of the second PCR were sequenced. In order to determine an estimate of the percentage of mutant alleles in a sample containing a FLT3 mutation, the PCR reaction was repeated in duplicate as described above. In one of the reactions the 20F primer was substituted with a TET-labeled 20F primer, and in the other reaction the 20R primer was replaced with a 6FAM labeled 20R primer. PCR amplicons were subsequently digested with EcoRV and the percentage of mutant (undigested) amplicons present were determined by dividing the area under the mutant curve by the total area under the curves from the digested and undigested products. Samples with greater than 2% mutant alleles were determined to be positive.

Results and Discussion:

We recently demonstrated that consistent high level expression of FLT3 in MLL identifies a subtype of ALL that often harbors FLT3 mutations, and have validated FLT3 as a potential therapeutic target in this leukemia. As FLT3 is also consistently expressed at high levels in hyperdiploid ALL, we searched for FLT3 mutations in 71 childhood ALL samples to determine if FLT3 mutations are more prevalent in Hyperdiploid as compared to other ALLs. We found 6 of 25 Hyperdiploid ALL samples contain mutations that are predicted to lead to constitutive activation of
the receptor while only 1/29 TEL/AML1 rearranged samples harbored a FLT3 mutation (Table 1). Also, 0/8 ALL samples with other cytogenetic characteristics, and 3/9 samples from which cytogenetics were not available possessed FLT3 mutations. Thus, 10/71 samples possessed FLT3 mutations with a significant association between FLT3 mutations and Hyperdiploid ALL (p=0.04).

Five of the mutations identified are previously described activating mutations present in the receptor activation loop\textsuperscript{4,6}. Three of the mutations are novel and result in small in-frame deletions in the juxtamembrane region of the receptor (Figure 1A, B). One of these patients (Patient 24) had a single amino acid deletion of aspartic acid 593 that was present in leukemia blasts at diagnosis and relapse but was not found in bone marrow cells taken during remission. Thus, this mutation was not an uncommon polymorphism, but was acquired in the leukemia blasts. While this type of FLT3 mutation is novel in human disease, small deletions of the 10 amino acid region of FLT3 from tyrosine 589 to tyrosine 599 have been previously shown to lead to constitutive activation\textsuperscript{13}. When we compared these mutations to those found in tyrosine kinases in other diseases, we found that similar small deletion mutations are found in the receptor tyrosine kinase c-KIT in gastrointestinal stromal tumors (GIST) (Figure 1A)\textsuperscript{14-16}. Similar deletion mutations are also found in c-kit in the murine mastocytoma cell line FMA3\textsuperscript{17}. In both cases this type of mutation leads to receptor activation. Another unique mutation is a novel 15-bp insertion in the juxta-membrane region of the receptor that maintains an open reading frame. This is reminiscent of the FLT3 internal tandem duplications (FLT3-ITDs) found in AML (Figure 1B). Finally, one TEL/AML1 rearranged sample harbored a 30-bp FLT3-ITD.

It is of interest to note that 2 of the samples contained mutant FLT3 alleles at a frequency of <2% even though the blast percentage was high. While this could possibly represent contamination, we feel that this is unlikely, as we have performed over 100 negative controls in the laboratory with
no false positives. We feel that it is more likely to represent clonal evolution within the leukemias as has been demonstrated for FLT3 mutations in AML\textsuperscript{18,19}. Future study will determine the importance of FLT3 mutations in such cases.

These data show that FLT3 mutations are common in hyperdiploid acute lymphoblastic leukemia in contrast to published reports that have assessed adult ALL where FLT3 mutation is uncommon\textsuperscript{2}. We propose that this reflects an association between FLT3 mutations and hyperdiploid ALL or MLL, both of which are quite rare in adults. It is of interest to note that while we found multiple mutations of FLT3 in hyperdiploid ALL, we found no cases that possessed a FLT3-ITD, the most common mutation found in AML (Figure 1B). Further study will determine if this is due to different mechanisms of mutagenesis in developing myeloid as compared to lymphoid cells, or if the distinct FLT3 mutants have unique signaling properties.

An important question is whether or not the presence of FLT3 mutations in ALL has prognostic significance. A definitive answer to this question will require larger studies of MLL and hyperdiploid ALL samples; but it is important to note that 3 of the 16 ALLs that relapsed harbored FLT3 mutations at diagnosis. The presence of FLT3 mutations in these cases suggests that FLT3 inhibition may represent a therapeutic opportunity in at least a subset of patients with relapsed ALL.

The data presented here provide further evidence that genomic analysis of cancer cells will uncover important biologic subsets. Even though FLT3 mutations were considered rare in lymphoblastic leukemias, microarray analysis revealed high-level FLT3 expression prompting DNA sequencing which uncovered two subtypes of ALL that frequently contain FLT3 mutations. Thus there appears to be an association between high-level expression of FLT3 and the presence of activation loop mutations. This has also been demonstrated recently for AML where all samples with activation loop mutations have high-level expression\textsuperscript{20}. Further study will determine the molecular
underpinnings of this association. These data in conjunction with our previous finding in MLL provide strong support for the development of clinical trials that will test the efficacy of FLT3 inhibitors in childhood ALL. Furthermore, the presence of FLT3 mutations will prompt a more systematic analysis of tyrosine kinase mutations in childhood ALL.

**Acknowledgments:**

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References:


Table 1.

Seventy-one ALL samples were assessed for the presence of mutations in the juxtamembrane and activation loop regions of FLT3. The cytogenetic abnormalities (when available) are noted. The types of FLT3 mutations either in the juxtamembrane region (Exon 14) or in the activation loop are noted. * The 18-base pair insertion (AACTTAAGGAACCCACCA) is inserted 3’ to nucleotide 1731.

Figure 1.

FLT3 mutations are found in the juxta-membrane region and activation loop in ALL and AML. 

A. An alignment of the Juxtamembrane regions encompassing amino acids 563-610 of FLT3 and 544-589 of c-KIT is shown. The region of mutation of c-KIT in GIST is in bold as are the four FLT3 mutations found in ALL samples 12, 24, 21, and 25. B. The different FLT3 mutations found in AML and ALL are shown. Internal tandem duplications (FLT3-ITDs) in the juxta-membrane region have been described in AML. Two FLT3-ITDs are shown as examples. Deletions in the juxta-membrane region are found in ALL. Activation loop mutations are found in ALL, AML and MLL.
### Table 1

<table>
<thead>
<tr>
<th>Primary ID# (%Blasts in Sample)</th>
<th>Exon 14-15</th>
<th>Activation loop</th>
<th>Remission Stat</th>
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<tbody>
<tr>
<td></td>
<td>Nucleotide positions (% mutant alleles)</td>
<td>Mutation (% mutant alleles)</td>
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<td><strong>Hyperdiploid ALL (&gt; 50 Chromosomes): (6/25)</strong></td>
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<tr>
<td>ALL-6 (88%)</td>
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<td>I836Δ(30%)</td>
<td>CCR</td>
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<tr>
<td>ALL-7 (90%)</td>
<td>-</td>
<td>D835Y(20%)</td>
<td>CCR</td>
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<tr>
<td>ALL-12 (89%)</td>
<td>Δ1770-1784 plus GA₁₇₈₇:ₐ₁₇₈₈ -&gt; GGG (30%)</td>
<td>-</td>
<td>CCR</td>
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<tr>
<td>ALL-25 (95%)</td>
<td>TTC₁₇₇₀ -&gt; TTA Δ₁₇₇₁–₁₇₈₂ (15%)</td>
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<td>CCR</td>
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<tr>
<td>ALL-34 (22%)</td>
<td>Δ1777-1779(47%)</td>
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<td>Relapse</td>
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<td>ALL-24 (95%)</td>
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<td><strong>TEL/AML1: (1/29)</strong></td>
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<td>ALL-68 (95%)</td>
<td>30bp ITD (5%)</td>
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<td>CCR</td>
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<tr>
<td><strong>Other: 1 E2A/PBX, 2 pseudodiploid, 5 diploid (0/8)</strong></td>
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<td><strong>Cytogenetics not available: (3/9)</strong></td>
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<tr>
<td>ALL-35 (35%)</td>
<td>-</td>
<td>D835E (7%)</td>
<td>CCR</td>
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<tr>
<td>ALL-19 (70%)</td>
<td>-</td>
<td>I836D (10%)</td>
<td>Relapse</td>
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<tr>
<td>ALL-21 (30%)</td>
<td>Δ1732-1734 plus 18 bp insertion*(18%)</td>
<td>-</td>
<td>Relapse</td>
</tr>
</tbody>
</table>
**FIGURE 1**

A

C-KIT         TYKYLKQPMYEYQWKKVVEI--NGNNYVYIDPTQLPYDKHWEFPRNRL  544-589
FLT-3         CHKYKKQFRIESOLQMVQVTGSSDNEYFYVDFREYELKWEFPRNENL  563-610

ALL-12        CHKYKKQFRIESOLQMVQVTGSSDNEYL---GYEYDLKWEFPRNENL  563-610
ALL-24        CHKYKKQFRIESOLQMVQVTGSSDNEYFYV-FREYELKWEFPRNENL  563-610
ALL-25        CHKYKKQFRIESOLQMVQVTGSSDNEYL---REYELKWEFPRNENL  563-610

ALL-21        CHKYKKQFRIESOLQ-VQVTGSSDNEYFYVDFREYELKWEFPRNENL  563-610

NLRNPP

B

Activation Loop Mutations
Hyperdiploid ALL / MLL / AML
D835, I836Δ

Transmembrane domain

NH₂ Extracellular domain PTK COOH

Juxtamembrane domain mutations

Hyperdiploid ALL
(NEWLY DESCRIBED DELETIONS)

WT-FLT3       MVQVTGSSDNEYFYVDFREYELKWEFPRNENL
ALL-12        MVQVTGSSDNEYFYVDFREYELKWEFPRNENL
ALL-24        MVQVTGSSDNEYFYV-FREYELKWEFPRNENL
ALL-25        MVQVTGSSDNEYFYV-FREYELKWEFPRNENL

AML
(INTERNAL TANDEM DUPLICATIONS)

WT-FLT3       MVQVTGSSDNEYFYVDFREYELKWEFPRNENLΔ

LVQVTGSSDNEYFYVDFREYEL

REYELD
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