Functional Assessment of Novel Diagnostic FLT3 Mutations and Inhibition by Kinase Inhibitors

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BACKGROUND

Somatic mutations in FLT3 are very common diagnostic variants in acute myeloid leukemia (AML). The most common alterations are FLT3/ITD mutations in the juxtamembrane domain (JMD) and DBS35/Y599 substitutions in the activation loop of kinase domain (KD: FLT3/ITD). FLT3/ITD occurs in approximately 15% of children with AML, and increases in prevalence with age occurring in 25-30% of adult AML. FLT3/ITD is also found in 5-7% of patients. These two events lead to aberrant activation of the FLT3 receptor and enhanced kinase signaling. Sequencing of a cohort of 788 children with de novo AML treated on contemporary Children’s Oncology Group protocols detected numerous other variants, including several novel variants, in addition to the previously described FLT3 mutations (ITD and ITD). Overall, we found a cumulative FLT3 mutation prevalence of 26% in children with AML (Figure 1). These variants mostly occurred in the JMD and KD and were predicted to activate FLT3 (Figure 2), therefore increasing the number of children with AML might be amenable to FLT3-targeted therapy.

METHODS

FLT3 mutation vectors for each missense mutation were generated from E. coli plasmid and presence of the mutation was confirmed by Sanger sequencing following plasmid selection. HEK293 cells were transfected with FLT3 mutant vectors. Following transfection, lysates were collected and immunoblotting performed using anti-phospho FLT3 and STAT5 antibodies. Chemiluminescence was utilized for quantification and to determine the ratio of phosphorylated protein to total protein. The percent phosphorylation (%phos) was calculated by considering DBS5Y cells to be 100% phosphorylated, and %phos 10% compared to DBS5Y was considered aberrant phosphorylation. Mutations found to result in increased FLT3 phosphorylation underwent evaluation for response to the tyrosine kinase inhibitors (TKis): crenolanib (Arog Pharmaceuticals) and quizartinib (Selleckchem). Compounds were added to cells at selected concentrations and 60 minutes following exposure lysates were collected and immunoblotting performed to assess for inhibition of phosphorylation. The %phos inhibition at each dose was compared to the luminescence detected at 0 nM.

RESULTS

Figure 2: FLT3 Phosphorylation. Using the HEK293 cell model, FLT3 phosphorylation was assessed for all mutations. A total of 13 children with AML treated on contemporary Children’s Oncology Group protocols with enhanced kinase signaling. Sequencing of a cohort of 788 children with de novo AML treated on contemporary Children’s Oncology Group protocols detected numerous other variants, including several novel variants, in addition to the previously described FLT3 mutations (ITD and ALM). Overall, we found a cumulative FLT3 mutation prevalence of 26% in children with AML. These variants mostly occurred in the JMD and KD and were predicted to activate FLT3 (Figure 2), therefore increasing the number of children with AML might be amenable to FLT3-targeted therapy.

Figure 3: STAT5 Phosphorylation. STAT5 phosphorylation was evaluated and in general, mutations that resulted in elevated FLT3 phosphorylation were also found to result in higher levels of STAT5 phosphorylation. However, a direct correlation between the level of FLT3 and STAT5 phosphorylation was not observed.

CONCLUSIONS

- Non-ITD and ALM FLT3 variants are present in pediatric AML at the time of diagnosis
- Many FLT3 variants in pediatric AML result in aberrant phosphorylation of FLT3 and STAT5
- Exposure to the type I TKI crenolanib resulted in potent inhibition of phosphorylation of FLT3 and STAT5 with IC50 range of 1.3-13.8 nM
- Activating FLT3 variants in pediatric AML may be amenable to therapeutic targeting with FLT3 inhibitors

Figure 4: FLT3 Conformation and TKI Binding. Type I TKIs have activity both against the active and inactive conformations of FLT3. Type II TKIs (e.g. sorafenib, quizartinib) require the inactive conformation. Crenolanib is a selective type I TKI which recognizes both conformations.

Figure 5: Response of pFLT3 and pSTAT5 to Crenolanib. Crenolanib exposure resulted in potent inhibition of FLT3 (Figure 5A) and STAT5 (Figure 5B) phosphorylation for all activating mutations.