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 D835/I836 missense mutations in the activation loop of kinase domain (KD; *FLT3*/ALM). *FLT3*/ITD occurs in approximately 15% of children with AML, and increases in prevalence with age occurring in 25-30% of adult AML. *FLT3*/ALM occurs 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 ALM). 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 an increasing the number of children with AML might be amenable to FLT3-targeted therapy.

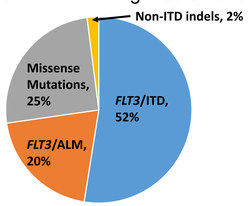
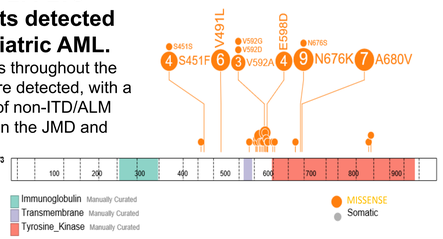


Figure 1: Prevalence of different types of *FLT3* variants detected in pediatric AML.

Figure 2: *FLT3* variants detected in pediatric AML. Mutations throughout the gene were detected, with a number of non-ITD/ALM variants in the JMD and KD.



METHODS

FLT3 mutation vectors for each missense mutation were generated from *E. coli* plasmids 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: total protein. The percent phosphorylation (%-phos) was calculated by considering D835Y cells to be 100% phosphorylated, and %-phos ≥10% compared to D835Y 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.

FLT3 PHOSPHORYLATION

Figure 2: FLT3 Phosphorylation. Using the HEK293 cell model, FLT3 phosphorylation was assessed for all mutations. A total of 13 variants, including D835, were found to result in pFLT3 > 10% (green line) and were considered to be activating. Among all *FLT3* mutations detected, those that resulted in aberrant phosphorylation were the most prevalent, therefore activating *FLT3* mutations accounted for 87% of all non-ITD mutations.

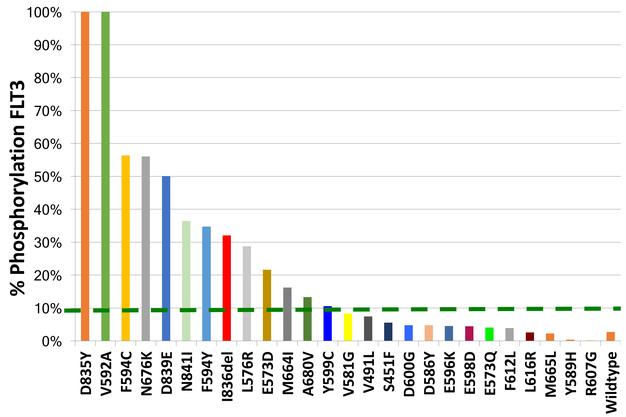
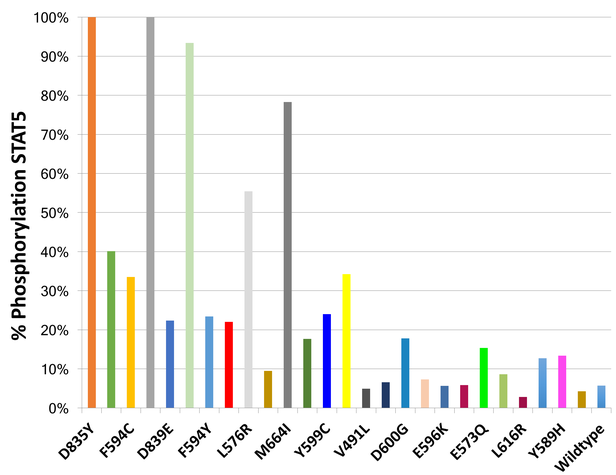


Figure 3: STAT5 Phosphorylation. STAT5 phosphorylation was evaluated and in general, mutations that resulted in elevated FLT3 phosphorylation were also found to result in higher levels of STAT5 phosphorylation. However, a direct correlation between the level of FLT3 and STAT5 phosphorylation was not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



TKI RESPONSE

FLT3 inhibitor therapy with TKIs for patients with *FLT3*/ITD mutations is now considered the standard of care, and this strategy is extending to patients with D835 mutations as well. Several TKIs, with varying activity and specifity, have or are undergoing evaluation in *FLT3* mutant AML. Importantly, not all FLT3-directed TKIs have activity against both the active and inactive conformations of FLT3.

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

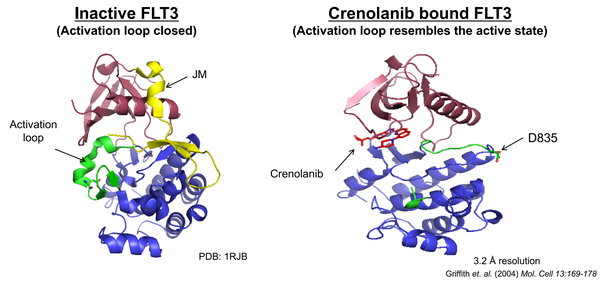


Figure 6. Response of pFLT3 and pSTAT5 to Quizartinib. Quizartinib exposure resulted in inhibition of FLT3 (**Figure 6A**) and pSTAT5 (**Figure 6B**) phosphorylation for many activating mutations. Several mutations were less sensitive to the type II TKI quizartinib compared to the type I TKI, namely PMs in the TKDs.

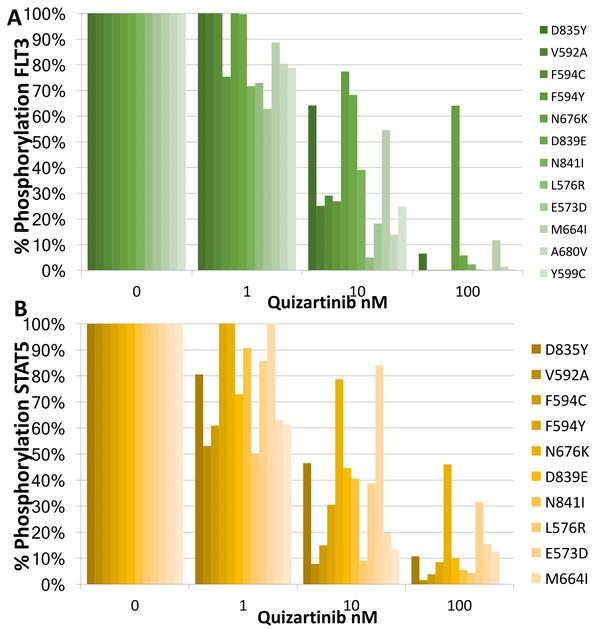
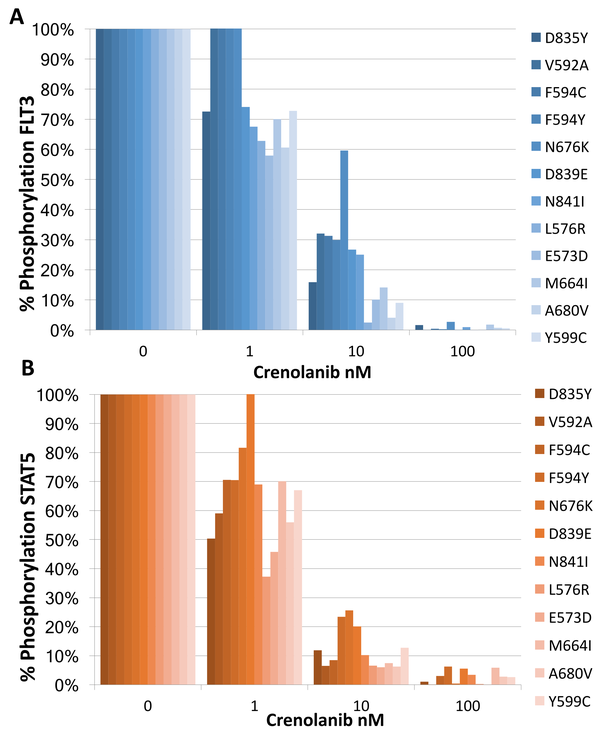


Figure 5. Response of pFLT3 and pSTAT5 to Crenolanib. Crenolanib exposure resulted in potent inhibition of FLT3 (**Figure 5A**) and STAT5 (**Figure 5B**) phosphorylation for all activating mutations.



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