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The FLT3^{N701K} mutation causes clinical AML resistance to gilteritinib and triggers TKI sensitivity switch to quizartinib

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TO THE EDITOR:

Driver mutations in the tyrosine kinase receptor FLT3, including internal tandem duplications (*FLT3-ITD*) and tyrosine kinase domain (*FLT3-TKD*) mutations, occur in approximately 30% of newly diagnosed cases of acute myeloid leukemia (AML) and enable constitutive receptor activation, leading to poor prognosis. Multiple tyrosine kinase inhibitors have shown clinical activity in patients and have improved overall treatment outcomes[1, 2]. These inhibitors are generally classified as type I or type II inhibitors, depending on whether they bind the active or inactive conformation of FLT3[3], respectively. Type I inhibitors include midostaurin, crenolanib, and gilteritinib, while type II inhibitors include quizartinib, sorafenib, and ponatinib. Despite their initial efficacy, the long-term durability of these inhibitors is curtailed by the development of drug resistance. To date, several modes of FLT3 inhibitor resistance have been identified[3]. Secondary mutations in the TKD, particularly at the D835 residue, are commonly reported as a mechanism of resistance to type II FLT3 inhibitors[4-6]. These mutations destabilize the inactive conformation of FLT3 required for the binding of type II inhibitors quizartinib[4] and sorafenib[5]. Newer type I inhibitors, including crenolanib and gilteritinib, retain efficacy against most TKD mutations, and resistance commonly occurs through mutations in the RAS or MAPK signaling pathway[2, 7]. Resistance to both type I and II inhibitors can also occur through the *FLT3*^{F691L} gatekeeper mutation[2, 4, 6-8], although clinically this is less common than *FLT3-TKD* and *RAS* mutations.

We recently characterized *in vitro* gilteritinib resistance and found that resistance emerges in a dynamic, stepwise manner where microenvironmental support led to an early phase of resistance characterized by changes in cell cycle and metabolism, that later evolved to a late resistance phase concomitant with the notable expansion of *NRAS* mutations[9], consistent with clinical data[2]. In addition to *NRAS* mutations, we also identified a novel *FLT3*^{N701K} mutation in three resistant cultures that promoted gilteritinib resistance[10]. We demonstrated that *FLT3*^{N701K} effectively acts as a gatekeeper mutation, blocking gilteritinib from binding to FLT3. Notably, *FLT3*^{N701K} did not block quizartinib binding, indicating that *FLT3*^{N701K} mutations are more specific for type I FLT3 inhibitors and, thereby serve as noncanonical gatekeepers unlike canonical gatekeepers, such as *FLT3*^{F691L}, which block binding of type I and II inhibitors[4, 6]. Herein, we report two AML patients who developed a *FLT3*$^{ITD+N701K}$ mutation while on the combination of gilteritinib and venetoclax. We show that primary cells from one patient **(patient #1,** Figure 1A) with the *FLT3*$^{ITD+N701K}$ mutation were resistant to gilteritinib *ex vivo*, but remained exquisitely sensitive to quizartinib, highlighting the potential utility of switching type I to type II FLT3 inhibitors to restore clinical response.

The first patient initially presented with AML with *NPM1*, *DNMT3A* and *FLT3-ITD* mutations and received induction with 7+3 with midostaurin and gemtuzumab ozogomacin (GO) on study. He achieved a remission, but had difficulty tolerating consolidation and maintenance therapies and relapsed 6 months later (Figure 1A). He then received salvage with FLAG + gilteritinib 120 mg daily starting on day 8, but gilteritinib was held after chemotherapy due to prolonged neutropenia and infectious complications. A later marrow demonstrated persistence of *FLT3-ITD*, so gilteritinib monotherapy at 120 mg daily was initiated, with periodic interruptions for infections and neutropenia. Gilteritinib effectively

suppressed his disease, but did not clear the *FLT3-ITD* clone and blasts slowly increased in his marrow (Figure 1A, **Progression 1)**. Venetoclax 400 mg daily was added to improve response, but there were frequent interruptions and dose reductions of both gilteritinib and venetoclax due to neutropenia. The combination of gilteritinib + venetoclax was continued for 5 months until peripheral blasts increased rapidly. The $FLT3^{N701K}$ mutation was detected at high variant allele frequency (VAF) of 35% at this point (Figure 1A, **Progression 2)**, suggesting that it was likely propelled by the selective pressure of gilteritinib and venetoclax treatment. We confirmed the presence of $FLT3^{N701K}$ in genomic DNA via Sanger sequencing at Progression 2 (Supplemental Figure 1). $KRAS^{G13D}$ and $NRAS^{Q61K}$ mutations were also detected at lower VAFs at Progression 2 (Supplemental Table 1).
To reconstruct the clonal architecture and evolution of this patient's AML, we performed single-cell DNA sequencing on bone marrow mononuclear cells from Progression 1 and Progression 2 timepoints. Single-cell DNA analysis revealed *de novo* development of a $FLT3^{N701K}$ population which was co-mutated with *FLT3-ITD* in the same cells. Surpisingly, the majority of cells with $FLT3^{N701K}$ mutations were homozygous, while only a minority were heterozygous (Figure 1B). Single cell and clonal analysis also revealed that mutations in *KRAS* and *NRAS* sublones were not co-mutated with *FLT3-ITD* or $FLT3^{N701K}$ (Figure 1B).

To evaluate the relative sensitivity of the $FLT3^{N701K}$ mutation to FLT3 inhibitors, Ba/F3 cells harboring *FLT3-ITD* and a concomitant *N701K* mutation (i.e., $FLT3^{ITD+N701K}$) were exposed to titrations of gilteritinib or quizartinib, with IC_{50} values of 82.86 and 2.906 nM, respectively (Supplemental Figure 2A-B), indicating that the $FLT3^{N701K}$ mutation remains sensitive to quizartinib in this model system. To evaluate the sensitivity in primary cells, frozen viable cells from patient 1 at Progression 1 and Progression 2 were thawed and enriched for blasts via $CD33^{+}/CD34^{+}$ bead selection and then treated with gilteritinib and quizartinib *ex vivo*. Prior to the $FLT3^{N701K}$ mutation, the patient's blasts responded to gilteritinib with IC_{50} of 24.38 nM and quizartinib with IC_{50} of 1.733 nM, reflecting the higher potency of quizartinib (Figure 1C). Although the patient had already been treated with gilteritinib at this timepoint, the *ex vivo* response to FLT3 inhibition does not account for the contribution of the marrow microenvironment, which we and others have shown can impact clinical response[9, 11, 12]. However, after expansion of the $FLT3^{N701K}$ mutation, the blasts developed increased resistance to gilteritinib *ex vivo*, with an IC_{50} of >10000 nM (>400 fold increase). Despite this resistance to gilteritinib, they remained exquisitely sensitive to quizartinib with a nearly unchanged IC_{50} of 1.771 nM (Figure 1D). Primary AML blasts harboring $FLT3^{N701K}$ also retained sensitivity to the type II inhibitor sorafenib, demonstrating the efficiacy of type II inhibitors more broadly in the setting of $FLT3^{N701K}$ (Figure 1E). In contrast, primary cells with $FLT3^{N701K}$ were resistant to both type I inhibitors, crenolanib and midostaurin (Figure 1F). In parallel, we also tested the activity of the allosteric FLT3 inhibitor FF10101, which overcomes *FLT3* gatekeeper mutations by binding at a site outside of the gatekeeper region in FLT3[13]. Similar to quizartinib, FF10101 remained very potent in primary AML cells harboring the $FLT3^{ITD+N701K}$ mutation with an approximate 150-fold greater potency by IC_{50} compared to gilteritinib (Figure 1G). Ba/F3 cells with $FLT3^{ITD+N701K}$ exhibited a similar response with a 19-fold increase in potency relative to gilteritinib (Supplemental Figure 2C).

While revising this manuscript, we became aware of a second patient (patient #2) with a *FLT3*^{N701K} mutation. This patient initially presented with AML with a normal karyotype and mutations in *RUNX1, SETBP1, WT1*, and *FLT3-ITD*. He achieved complete remission after induction with 7+3 and midostaurin and subsequently received two cycles of high dose cytarabine and midostaurin followed by single-agent gilteritinib at 120 mg daily until admission for a myeloablative allogeneic transplantation from his brother. After engraftment, he restarted gilteritinib as post-transplant maintenance. However, the *FLT3-ITD* mutation reappeared on his day 100 bone marrow biopsy by PCR, and morphologic relapse followed five weeks later. NGS panel at first relapse showed the same mutations from diagnosis. Salvage therapy with venetoclax and gilteritinib was attempted but not tolerated due to prolonged cytopenias after cycle 2 and the marrow showed persistent low level marrow blasts (5-10%). After three cycles of azacitidine plus gilteritinib, circulating blasts and leukocytosis reappeared. NGS of the peripheral blood showed all prior mutations from diagnosis, but also a new *FLT3*^{N701K} mutation at high VAF (41%).

Resistance to FLT3 inhibitors via acquisition or clonal expansion of secondary mutations is a well-appreciated phenomenon in AML[2, 4, 7]. Multiple studies have shown that mutations in the activation loop of *FLT3* (most commonly D835) perturb the kinase to adopt an active, type I conformation, rendering them insensitive to type II FLT3 inhibitors, such as quizartinib or sorafenib, but these mutations remain sensitive to type I inhibitors, such as gilteritinib[2, 14]. However, secondary mutations in *FLT3* that trigger a sensitivity switch in the reverse direction, from type I to type II inhibitors, is largely an underappreciated mechanism of resistance, although retained sensitivity to quizartinib has been described in midostaurin resistant cell lines harboring *RAS* mutations[15]. To our knowledge we are the first to report this observation in a patient sample with FLT3-AML. Our data has important clinical implications as the use of FLT3 inhibitors continues to expand in the clinic, and undoubtedly, the number of resistance mutations will increase as well. Functional validation of mutations is imperative to predict the efficacy of TKI-switching, similar to what has been described for other oncogenic kinases such as BCR-ABL1[16], TRK[17], ROS1[18], EGFR[19], MET[20], and KIT[21].

FLT3 gatekeeper mutations, most commonly the F691L mutation, have also been reported in patients treated with both type I and type II FLT3 inhibitors[2, 4, 7]. In the ADMIRAL trial, F691L mutations were the most common FLT3 mutation associated with resistance, although less frequent than activating mutations in the RAS pathway[22]. Although *FLT3*^{N701K} mutations act as a gatekeeper for gilteritinib, the mutation retains a surprising sensitivity to quizartinib and other type II inhibitors[10]. Although we initially found this mutation in gilteritinib-resistant cell lines, we now report a patient with the *FLT3*$^{ITD+N701K}$ mutation with *ex vivo* sensitivity that mirrors our cell lines. *In silico* modeling suggests that the N701K mutation is predicted to sterically disrupt the binding of gilteritinib, and trigger a conformational switch that favors adoption of the inactive kinase conformation[10]. *In vitro* mutagenesis screening has also revealed a few other FLT3 mutations near F691 that confer moderate gilteritinib resistance[23]. An alternative approach is to use an allosteric inhibitor such as FF10101, that retains activity against F691L[24] and other gatekeeper mutations. Indeed, we found that *FLT3*$^{ITD+N701K}$ mutation remained sensitive to this inhibitor, however

further clinical testing is required to determine the efficacy and tolerability of FF10101 in patients[25].

In summary, we describe two AML patients with *FLT3*$^{ITD+N701K}$ mutations that arose during development of clinical resistance to gilteritinib. Single-cell analysis confirmed this mutation was co-mutated with *FLT3-ITD* and was predominantly homozygous, at least in the one patient we tested. *Ex vivo* assays reveal that primary cells with *FLT3*^{N701K} were resistant to gilteritinib, but retained *ex vivo* sensitivity to quizartinib, consistent with our work in cell line models. As quizartinib[26] was recently approved by the FDA for frontline AML treatment, our data provides compelling evidence that switching to quizartinib with *FLT3*^{N701K} mutations may restore sensitivity to FLT3 inhibitors in the setting of relapsed/refractory disease, extending the duration of response to therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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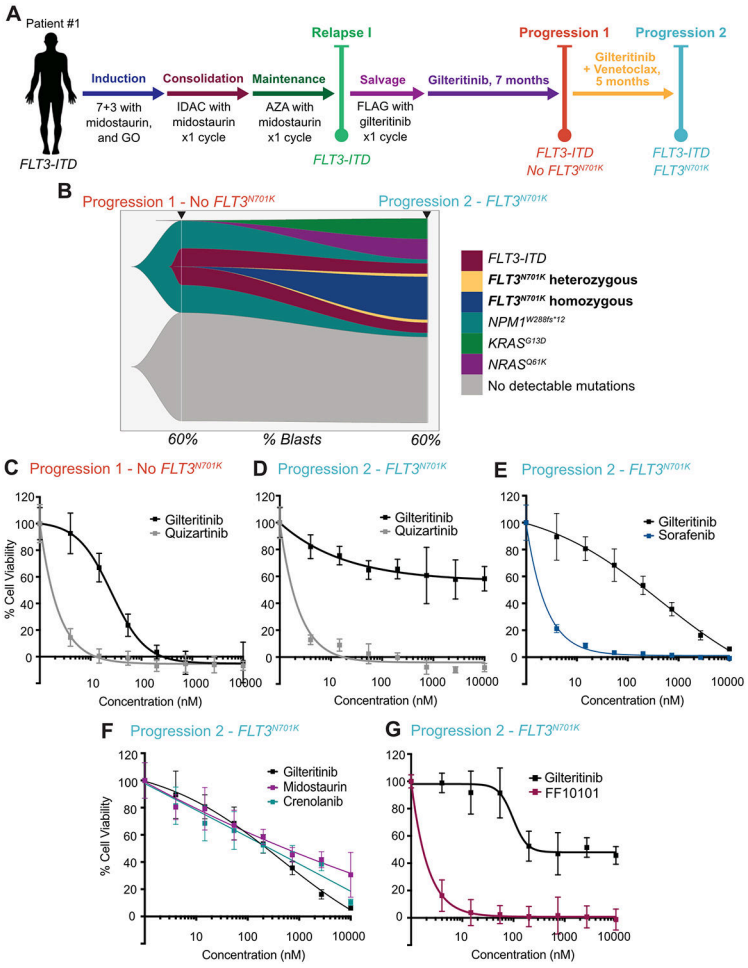

Figure 1: *FLT3*^{N701K} confers resistance to type I inhibitor, gilteritinib in primary cells but retains sensitivity to the type II inhibitor, quizartinib.

A) Treatment time course is outlined with pertinent details about *FLT3* mutation status. The *FLT3*^{N701K} mutation was detected at Progression 2 via GeneTrails (clinical mutational panel at OHSU). Complete mutational profiling data are available in Supplemental Table 1. **B)** Fishplot illustrating clonal architecture and evolution of 1,330 single cells from cryopreserved bone marrow aspirate as captured by single cell DNA sequencing prior to gilteritinib and venetoclax (Progression 1) and at time of clinical resistance (Progression 2). Top x-axis indicates clinical timepoint and bottom x-axis indicates percent blasts on corresponding clinical bone marrow biopsy report. This patient demonstrated complex on- and off-target clonal evolution with therapeutic pressure, including *de novo* development of a *FLT3*^{N701K} hetero- and homozygous mutation within the *FLT3-ITD* mutated subclone, *de novo* development of a *NRAS*^{Q61K} mutated subclone, and selection for a pre-existing *KRAS*^{G13D} mutated subclone. The gray area indicates cells that did not have detectable mutations, although the single cell mutation panel via the Tapestri platform is more limited (Supplemental Table 2) than the sequencing panel used in Supplemental Table 1, so these cells may still have mutations. Primary AML cells at **C)** Progression 1 and **D-G)** Progression 2 were enriched with CD33^{+}/CD34^{+} selection and treated with gilteritinib, quizartinib, sorafenib, midostaurin, crenolanib, or F10101 as single agents (0 – 10000 nM).

Cell viability was measured after 72 hours using a tetrazolamine-based viability assay and normalized to untreated cells. Mean of triplicates ± standard deviation are shown for all viability results.