IN THE SPOTLIGHT

Polyclonal Heterogeneity: The New Norm for Secondary Clinical Resistance to Targeted Monotherapy in Relapsed Leukemia?

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Summary: In this issue, McMahon and colleagues demonstrate that secondary clinical resistance to the FLT3 inhibitor gilteritinib in relapsed acute myeloid leukemia is often polyclonal and commonly mediated by heterogeneous mutations that activate downstream RAS–MAPK pathways. These findings and recent data from others indicate that emergence of multiple clones, each with distinct mechanisms of resistance, is a common finding at secondary failure of single-agent–targeted therapies for relapsed leukemias.

See related article by McMahon et al., p. 1050 (2).

Recurrent activating mutations in the transmembrane growth factor receptor FLT3 represent one of the most frequent and prognostically important molecular abnormalities in acute myeloid leukemia (AML). The FDA approvals of small-molecule–targeted inhibitors of FLT3 kinase activity have changed practice. Midostaurin in combination with induction chemotherapy is now standard for initial therapy of FLT3-mutant AML. For relapsed or refractory FLT3-mutant AML, the second-generation FLT3 inhibitor gilteritinib was FDA-approved on November 28, 2018. Although gilteritinib monotherapy more than doubles the response rate achieved with standard chemotherapy, secondary resistance was inevitable, occurring within months despite ongoing therapy (1). In this issue of *Cancer Discovery*, McMahon and colleagues leverage single-cell technologies and make the striking observation that polyclonal resistance, not fully evident using bulk sequencing approaches, underpins tumoral adaptation to FLT3-targeted therapy in the majority of cases relapsing on gilteritinib (2, 3). These studies highlight the clinical limitations of targeted drug monotherapy and strengthen the need to rapidly accelerate development of multidrug combination approaches.

Mechanisms of resistance to type II FLT3 inhibitors (quizartinib and sorafenib), which bind to the inactive FLT3 conformation, have been reported previously (3, 4). Although polyclonal on-target mutations affecting the FLT3-D835 kinase domain or the FLT3-F691L gatekeeper residues were observed, the capacity to discover off-target convergent mechanisms of resistance was technically limited (4). Recent advances in microfluidic single-cell encapsulation and massively parallel single-cell PCR-based barcoding now enable rapid, high-throughput characterization of cooperating and subclonal molecular processes in thousands of single cells (5). McMahon and colleagues applied targeted sequencing of hotspot mutations (in *ASXL1, DNMT3A, EZH2, FLT3, GATA2, IDH1, IDH2, JAK2, KIT, KRAS, NPM1, NRAS, PTPN11, RUNX1, SF3B1, SRSF2, TP53, U2AF1*, and *WT1*) at the single-cell level to investigate secondary resistance among a cohort of 41 patients treated with the type I FLT3 inhibitor gilteritinib in two early-phase clinical trials. Gilteritinib binds to both active as well as inactive conformations of FLT3 and has an extended spectrum of activity that includes inhibition of both FLT3-D835 and FLT3-F691 variants *in vitro*.

In contrast to previous studies with other FLT3 inhibitors, the dominant observation in this study was treatment-emergent off-target mutations rather than on-target mutations in FLT3. In 15 of 41 cases, activating RAS–MAPK pathway mutations were found in branching subclones of the original FLT3-mutated clone, with multiple competing RAS-mutant subclones emerging in parallel within the same leukemic sample in all four cases subjected to detailed single-cell analysis. The authors provide functional confirmation of the ability of two *NRAS* mutations to confer resistance to high concentrations of gilteritinib in cell line model systems. Less common activating RAS/MAPK pathway mutations were also found at clinical progression in *PTPN11, CBL*, and *BRAF*. New *BCR–ABL1* fusions were found in two additional patients. In some patients, as well as multiple *RAS* subclones expanding within the polyclonal FLT3-ITD population, parallel intratumoral expansion of FLT3 wild-type clones bearing *IDH2* and *SF3B1* mutations was also evident at relapse.

In 5 of 26 cases lacking RAS–MAPK pathway mutations, gilteritinib treatment failure was associated with on-target FLT3-F691L gatekeeper mutations, suggesting this mechanism was sufficient to confer resistance to gilteritinib

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in vivo. *In vitro* studies showed that suppression of FLT3-F691L was dose-dependent, with higher concentrations being required for activity against FLT3-F691L than FLT3-ITD. They speculate that FLT3-F691L–mediated resistance may be most likely to be observed in patients receiving lower doses of gilteritinib, whereas resistance conferred by activating NRAS mutations may occur even when drug exposure is high. A further subset of patients (5/41) relapsed without evidence of the hallmark FLT3 lesion, indicating clonal emergence of non–FLT3-dependent AML clones as an alternative resistance mechanism. In these FLT3 wild-type relapse cases, activating RAS/MAPK mutations were found in every sample, but the subclonal architecture was not studied in detail. Expansion of non-FLT3 clones was also reported in a previous study utilizing a whole-exome approach to study resistance to another type II FLT3 inhibitor, crenolanib (6). In that study, rising variant allele frequencies of mutant *IDH2, BCOR, CEBPA*, and *ASXL1* emerged at relapse. The involvement of mutations in these genes which regulate metabolism and/or gene expression suggest that epigenetic mechanisms may also play a role in mediating resistance to FLT3-targeted therapies.

In the current study of McMahon and colleagues, polyclonal relapse was observed in all four cases subjected to detailed single-cell analysis, and could be inferred from variant allele frequencies in several others. The concurrent emergence of multiple subclones displaying independent acquisition of distinct mechanisms of resistance seems to be a common theme when small-molecule–targeted therapies are applied to leukemias failing prior chemotherapy. Similar polyclonal heterogeneity has been reported recently for IDH2-mutant relapsed AML treated with the mutant IDH2 inhibitor enasidenib (7), and for relapsed chronic lymphocytic leukemia treated with the BCL2 inhibitor venetoclax (8). It is tempting to speculate that mutagenic DNA damage inflicted by previous therapy may increase clonal complexity at initial relapse, which in turn predisposes to the parallel emergence of several competing subclones during ongoing selection pressure with targeted monotherapy.

What do these new observations mean clinically? The first conclusion is that the management of patients with relapsed FLT3-aberrant AML using FLT3 inhibitors is a lot more complicated than previously imagined, with different FLT3 inhibitors likely to be associated with distinct patterns of resistance. For sorafenib and quizartinib failures dominated by kinase domain mutations, gilteritinib or novel irreversible FLT3 inhibitors may continue to have activity. For gilteritinib or midostaurin failures characterized by *RAS* mutations, it is probable that other FLT3 inhibitors will lack clinically sustainable activity, and alternative drugs targeting the RAS–MAPK axis will be required. Furthermore, the ideal of precision medicine, matching targeted therapy very specifically to an identified mutation, may prove naïve in this setting, given the complexity of clonal architecture identified using single-cell technologies, and the insensitivity of bulk sequencing to identify rare subclones.

Future research will therefore need to determine how best to eliminate all mutant clones most likely associated with patterns of resistance associated with particular FLT3 inhibitors. Although the RAS–MAPK pathway has now emerged as an important target for patients receiving gilteritinib, it remains unclear whether the best strategy to target this pathway is either preemptively upon initiation of gilteritinib therapy, or adaptively at the time of molecular or clinical progression. Alternatively, FLT3 inhibitors in combination with intensive combination chemotherapy as initial therapy for AML may be more effective, as reflected in the approval of midostaurin for this indication. Preliminary studies of paired diagnosis–relapse samples from patients with FLT3-mutant AML treated with midostaurin chemotherapy indicate that almost half the patient population had detectable FLT3-ITD absent at relapse, in contrast to the persistence of *FLT3* mutation at progression in the vast majority of patients receiving FLT3 inhibitor monotherapy (9). Combining gilteritinib with intensive chemotherapy appears feasible and effective, with composite complete remission rates of over 90% reported in a preliminary phase Ib study (10). A randomized international trial led by HOVON/AMLSG comparing gilteritinib and midostaurin in combination with first-line chemotherapy is now under way to determine whether a more potent FLT3 inhibitor, such as gilteritinib, will lead to enhanced survival in this patient population.

The listing of two FLT3 inhibitors by the FDA for AML will ensure that this class of drugs is investigated in combination with a growing array of novel agents emerging onto the clinical stage. The recent insights into drug resistance mechanisms made possible by single-cell technologies are beginning to inform clinical trial design, and should encourage the routine incorporation of prospective collection of viable tumor cells into future protocols. Compared with light microscopy, flow cytometry or even panel-based bulk sequencing, rich insights into drug resistance mechanisms are now achievable using the perspective of a "single-cell lens." In the near future, detailed correlations between adaptive changes at the DNA, RNA, and protein level will likely reveal additional molecular pathways to polyclonal drug resistance, both genetic and epigenetic in nature. Our greater challenge as we enter this exciting era of targeted AML therapies is to develop practical strategies able to circumvent resistance and thereby translate these new insights into improved clinical outcomes.

Disclosure of Potential Conflicts of Interest

A.H. Wei reports receiving commercial research grants from Celgene, Servier, Novartis, AbbVie, and Amgen; has received speakers bureau honoraria from AbbVie and Novartis; has received income from royalties related to venetoclax through previous employment by the Walter and Eliza Hall Institute; and is a consultant/advisory board member for AbbVie, Servier, Amgen, Janssen, Novartis, Macrogenics, Astellas, and Daiichi Sankyo. A.W. Roberts has received income from royalties related to venetoclax through employment by the Walter and Eliza Hall Institute of Medical Research.

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