RESEARCH BRIEF

Patterns of Resistance Differ in Patients with Acute Myeloid Leukemia Treated with Type I versus Type II FLT3 Inhibitors

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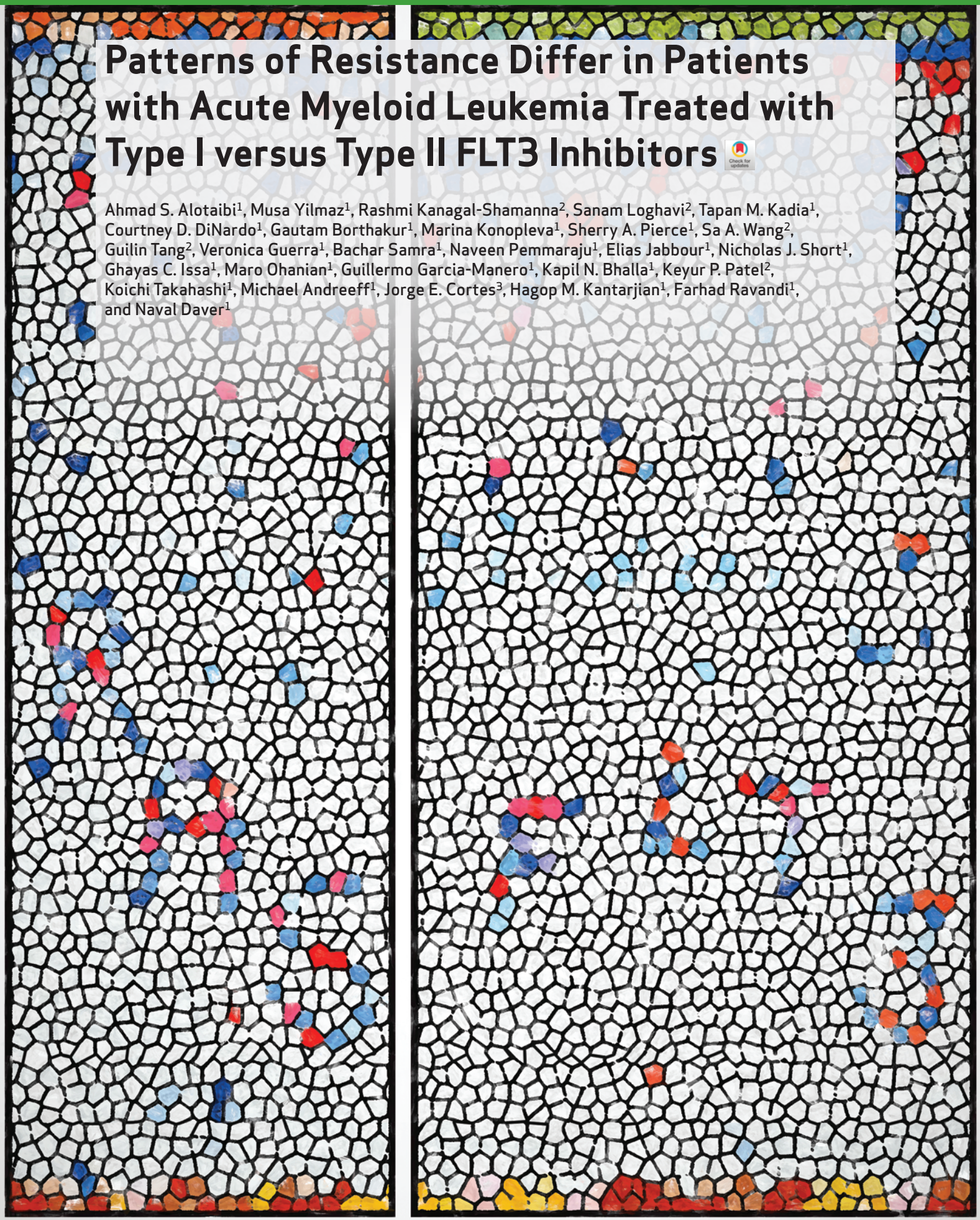

ABSTRACT Despite promising results with FLT3 inhibitors (FLT3i), response durations remain short. We studied pretreatment and relapse bone marrow samples from patients with *FLT3*-mutated acute myeloid leukemia (AML) treated with FLT3i-based therapies (secondary resistance cohort), and pretreatment bone marrow samples from patients with no response to FLT3i-based therapies (primary resistance cohort). Targeted next-generation sequencing (NGS) at relapse identified emergent mutations involving on-target *FLT3*, epigenetic modifiers, *RAS/MAPK* pathway, and less frequently *WT1* and *TP53*. *RAS/MAPK* and *FLT3*-D835 mutations emerged most commonly following type I and II FLT3i-based therapies, respectively. Patients with emergent mutations at relapse had inferior overall survival compared with those without emergent mutations. Among pretreatment *RAS*-mutated patients, pretreatment cohort-level variant allelic frequencies for *RAS* were higher in nonresponders, particularly with type I FLT3i-based therapies, suggesting a potential role in primary resistance as well. These data demonstrate distinct pathways of resistance in *FLT3*-mutated AML treated with type I versus II FLT3i.

SIGNIFICANCE: Sequential NGS-based mutational analysis at relapse after FLT3i-based therapies showed distinct pathways of secondary resistance between type I and II FLT3i. *FLT3* mutations may be lost at relapse after FLT3i-based therapies. Pretreatment *RAS/MAPK* mutations may also be associated with primary resistance in patients treated with type I FLT3i.

See related commentary by Shastri et al., p. 113.

INTRODUCTION

Multiple tyrosine kinase inhibitors (TKI) have demonstrated clinical activity in patients with *FLT3*-mutated acute myeloid leukemia (AML), including midostaurin, sorafenib, gilteritinib, quizartinib, and crenolanib, and have improved the outcome of patients with *FLT3*-mutated AML (1, 2). Midostaurin, a multikinase FLT3 inhibitor (FLT3i), was approved in many countries, in combination with anthracycline and cytarabine-based induction, for the treatment of adult patients with newly diagnosed *FLT3*-mutated AML based on improved overall survival noted in the phase III RATIFY trial (3). Second-generation FLT3i such as gilteritinib and quizartinib have demonstrated single-agent composite complete remission (CRc) rates [CRc = CR + CR with incomplete platelet recovery (CRp) + CR with incomplete neutrophil recovery (CRi)] of 45% to 55% in patients with relapsed or refractory (R/R) *FLT3*-mutated AML (4–7).

FLT3 TKIs are classified as type I, in which the FLT3i binds to the active receptor conformation (gilteritinib, midostaurin, and crenolanib), or type II wherein the FLT3i binds to the inactive conformation (quizartinib, sorafenib, and ponatinib) of the FLT3 receptor (1). Type I inhibitors inhibit FLT3 signaling in AML cells with ITD and/or TKD mutations, whereas type II inhibitors have no known preclinical or clinical activity in *FLT3*-TKD–mutated AML (8).

Despite promising responses achieved with FLT3i in AML, response durations remain short (4–14 months; refs. 6, 7), frequently driven by the emergence (acquisition or clonal expansion) of mutations that drive secondary resistance (1, 9, 10). These include secondary mutations involving the activating loop or gatekeeper residues of *FLT3*, or emergent mutations in genes involved in parallel prosurvival signaling pathways such as PI3K/AKT and RAS/MEK/MAPK (8, 9, 11). Understanding the profile of secondary mutations in patients treated with type I versus type II FLT3i-based therapies may help design strategies to abrogate resistance. Furthermore, assessing mutational profiles and variant allelic frequencies (VAF) of mutations pretherapy among patients who are nonresponders (primary resistant) to FLT3i-based therapies, and comparing mutational profiles and VAFs among primary-resistant patients versus patients who achieved initial response followed by relapse, may help improve our understanding of FLT3i failure and help identify patients most likely to need combination approaches. We used a next-generation sequencing (NGS)–based myeloid panel to compare bone marrow mutational profiles pre- and post-FLT3i–based therapy, to identify emergent mutations at relapse, in patients with *FLT3*-mutated AML with primary and secondary resistance to FLT3i-based therapies at our institution.

RESULTS

Patient Characteristics

Among 946 *FLT3*-mutated patients in our database (between 2012 and 2019), we identified 67 patients who

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achieved CRc followed by relapse (secondary resistance cohort), who had available *FLT3* analysis and NGS profiling on bone marrow (BM) samples, pre- and post-FLT3i–based therapy (Supplementary Fig. S1, CONSORT). We also identified 106 patients who had no response to a FLT3i-based therapy (primary resistance cohort). Baseline clinical characteristics and treatment outcomes of the patients in the secondary resistance cohort ($n = 67$) are summarized in Table 1. Of the 106 patients in the primary resistance cohort, most patients (92%) were R/R with median three prior therapies (range, 1–10), and only nine patients (8%) were newly diagnosed *FLT3*-mutated AML.

Among the secondary resistance cohort, at baseline, all patients had detectable *FLT3* mutations: 60 (90%) patients had a *FLT3*-ITD mutation, 11 (16%) a D835 mutation, and 4 (6%) both ITD and D835 mutations. Other comutations in this cohort are shown in Fig. 1A. Of the 106 patients in the primary resistance cohort, all had detectable *FLT3* mutations: 90 (85%) had *FLT3*-ITD, 27 (25%) *FLT3*-D835, and 11 (10%) both ITD and D835 mutations. *DNMT3A* and *NPM1* were the most common co-occurring mutations (Supplementary Fig. S2).

Treatment and Outcomes

Secondary Resistance Cohort (N = 67)

Forty-six (69%) patients received type II FLT3i-based therapies (sorafenib in 39 and quizartinib in 7), and 21 (31%) patients received type I FLT3i-based therapies (midostaurin in 7, gilteritinib in 12, and crenolanib in 2; Tables 1 and 2). Details of the clinical trials and therapies received are shown in Supplementary Tables S1 and S2. Sixty-five (97%) patients received FLT3i in combination with either low-intensity therapy (LIT; 64%) or conventional cytotoxic therapy (CCT; 33%). Only two patients received single-agent FLT3i therapy—both with gilteritinib in the R/R setting.

Twenty-four (36%), 17 (25%), and 26 (39%) patients achieved a CR, CRp, or CRi, respectively, for a CRc rate of 100% (only CRc patients eligible for secondary resistance cohort; Table 1). Twenty-one (31%) patients eventually underwent allogeneic stem cell transplant (ASCT) in remission on the current analysis. The median CRc duration and median overall survival (OS) for the cohort were 4.7 months [95% confidence interval (CI), 3.6–6.1] and 14.1 months (95% CI, 10.5–16.3 months), respectively.

Primary Resistance Cohort (N = 106)

Fifty-seven (54%) patients were treated with type II FLT3i-based therapies (sorafenib in 45 and quizartinib in 12) and 49 (46%) type I FLT3i-based therapies (crenolanib in 31, midostaurin in 13, and gilteritinib in 5). Seventy-eight of 106 (74%) patients received FLT3i in combination with LIT ($n = 57$) or CCT ($n = 21$), and 28 patients (26%) received single-agent FLT3i—25 crenolanib and 3 gilteritinib (all in R/R setting; Supplementary Table S3).

Emergent Mutations at Relapse in the Secondary Resistance Cohort

Emergent mutations are defined as mutations that were not identified on NGS prior to FLT3i-based therapy but were identified at relapse (likely due to acquisition and/or expansion of a previously undetected clone). The majority of patients (55%, 37 of 67) had an at least one emergent mutation at relapse, including 30 of 46 (65%) who received type II FLT3i-based therapies and 7 of 21 (33%) who received type I FLT3i-based therapies ($P = 0.02$), respectively. Emergent mutations were noted in 14 of 28 (50%) patients who relapsed after receiving FLT3i-based first-line therapies, and 23 of 39 (59%) patients who relapsed after receiving FLT3i-based therapies in an R/R setting ($P = 0.63$), respectively. Emergent mutations were noted in 10 of 22 (45%) and 25 of 43 (58%) patients who received CCT + FLT3i– and LIT + FLT3i–based therapies ($P = 0.43$), respectively. Only two patients received single-agent FLT3i therapy with gilteritinib, and both had emergent mutations at relapse.

The most frequent emergent mutations across all 67 patients were *FLT3*-D835 in 21%, RAS/MAPK pathway mutations (including *NRAS*, *PTPN11*, and *CBL*) in 13%, *IDH1*/*IDH2* in 9%, *WT1* in 7%, and *TP53* in 7% (Table 2; Supplementary Fig. S3).

Emergent Mutations after Type II FLT3i-Based Therapies (n = 46)

The most common emergent mutations in patients who achieved a CRc and relapsed after type II FLT3i-based therapies ($n = 46$) were *FLT3*-D835 in 14 (30%), *IDH1*/*IDH2* in 5 (10%), *TP53* in 5 (10%), and *WT1* in 5 (10%; Table 2; Supplementary Fig. S3). In addition to FLT3-D835–emergent mutations, FLT3-N676K and FLT3-N841K were identified in one patient each. Mutations in the RAS/MAPK pathway were noted in a small proportion, three (6%), of patients treated with type II FLT3i-based therapies.

The most common emergent mutations in patients treated with CCT + type II FLT3i–based therapies ($n = 17$) were *TP53* in three (18%), *WT1* in three (18%), *DNMT3A* in two (12%), and *FLT3*-D835 in one (6%). The most common emergent mutations in patients treated with LIT + type II FLT3i–based therapies ($n = 29$) were *FLT3*-D835 in 13 (45%), *IDH1*/*IDH2* in 5 (17%), and *NRAS, TP53*, and *WT1* in 2 (7%) each (Supplementary Table S4). Cytogenetic evolution analysis is shown in Supplementary Tables S5 and S6.

Emergent Mutations after Type I FLT3i-based Therapies (n = 21)

Pretherapy *FLT3*-D835 mutations were more common in patients treated with type I versus type II FLT3i (38% vs. 6%), suggesting that underlying *FLT3*-D835 mutations may have directed choice of therapy to some extent. None of the patients who achieved CRc and relapsed after type I FLT3i-based therapies had emergent *FLT3*-D835 mutations. However, of the eight baseline FLT3-D835–mutated patients, four (50%) had persistent mutation at the time of relapse (Fig. 1B). One patient had an emergent noncanonical *FLT3*-N676K mutation, and another patient with baseline *FLT3*-D835 alone had an emergent *FLT3*-ITD at relapse after gilteritinib-based therapy. The most common emergent mutations in patients who achieved a CRc and relapsed after type I FLT3i-based therapies ($n = 21$) were in the RAS/MAPK pathway in six (29%), including *NRAS* in four, and *PTPN11* and *CBL* in one each (Table 2). RAS/MAPK-emergent mutations were noted in 4 of 14 (29%) patients treated with LIT + type I FLT3i and none of patients treated with CCT + type I FLT3i ($n = 5$).

Table 1. Pre-FLT3i–based therapy clinical characteristics and treatment outcomes in patients with secondary resistance (*N* = 67)

Characteristics	Total *N* = 67 *N* (%) [range]	First-line *n* = 28 *N* (%) [range]	Relapse/refractory *n* = 39 *N* (%) [range]
Median age, years	62 [19–85]	64 [27–83]	62 [19–85]
Male gender	32 (48)	12 (43)	20 (51)
Type of AML			
De novo	52 (78)	22 (79)	30 (77)
Post-MDS, MPN, MDS/MPN	12 (18)	4 (14)	8 (20)
Therapy related	3 (4)	2 (7)	1 (3)
WBC, $\times 10^9$/L	9 [0.1–208]	37.45 [0.50–208]	4.70 [0.1–123.3]
Hemoglobin, g/dl	9.2 [6.0–15.5]	8.85 [6.90–11.1]	9.40 [6.0–15.5]
Platelets, $\times 10^9$/L	47 [3–316]	41.50 [11–316]	52.0 [7–223]
Bone marrow blasts, %	60 [1–95]	65.50 [10.0–95.0[a]]	64.0 [12.0–92.0]
Cytogenetics			
Diploid karyotype	43 (64)	21 (75)	22 (56)
Adverse	14 (21)	4 (14)	10 (26)
Others	10 (15)	3 (11)	7 (18)
Number of mutations at baseline	4 [1–9]	4 [1–8]	4 [1–9]
Number of prior therapies		N/A	2 [1–5]
Prior therapies			
Low-intensity chemotherapy/HMA		N/A	10 (24)
Intensive chemotherapy		N/A	31 (76)
ASCT		N/A	7 (18)
FLT3i		N/A	18 (46)
Treatment			
Single-agent FLT3i	2 (3)	0	2 (5)
FLT3i + LIT	43 (64)	14 (50)	29 (74)
FLT3i + CCT	22 (33)	14 (50)	8 (21)
Type of FLT3i			
Type II	46 (69%)	21 (75)	25 (64)
Sorafenib	39 (58)	19 (68)	20 (51)
Quizartinib	7 (11)	2 (7)	5 (13)
Type I	21 (31%)	7 (25)	14 (36)
Midostaurin	7 (10)	4 (14)	3 (8)
Gilteritinib	12 (18)	3 (11)	9 (23)
Crenolanib	2 (3)	0	2 (5)
Treatment outcome			
CR	24 (36)	15 (54)	9 (23)
CRp	17 (25)	8 (28)	9 (23)
CRi	26 (39)	5 (18)	21 (54)
Median duration of CRc, months	4.7 [3.6–6.1]	8.1 [5.6–9.6]	3.6 [2.3–4.3]
Median OS, months	14.1 [10.5–16.3]	16.9 [14.7–25.6]	8.4 [7.8–12.7]
ASCT in remission	21 (31)	12 (43)	9 (23)

Abbreviations: ASCT, allogeneic stem cell transplant; HMA, hypomethylating agents; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; N/A, not applicable; WBC, white blood count.

[a]One patient was newly diagnosed with FLT3-mutated AML in another hospital with initial WBC >200 × 10^9/L, refused chemotherapy initially, and came to us after >1 month on hydroxyurea. The patient's initial BM at our institution shows 10% blast, but outside hospital peripheral blood analysis confirmed AML with >20% circulating blasts, and the patient was treated on an AML first-line clinical trial.

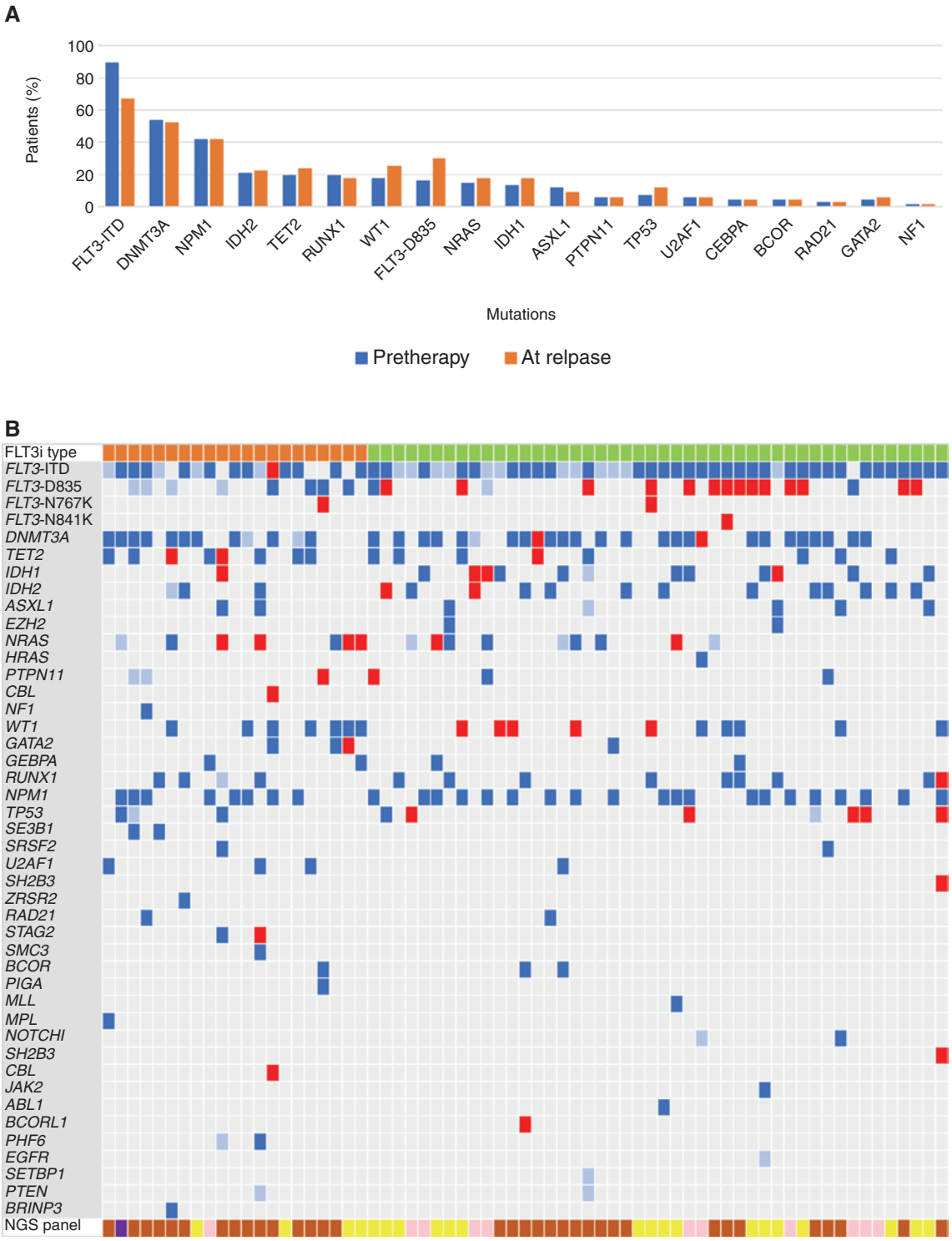

Figure 1. Frequency (**A**) and landscape (**B**) of somatic mutations pretherapy and at relapse after FLT3i-based therapies (secondary resistance cohort, *N* = 67). **A,** The blue bars represent the frequency of FLT3 and other somatic mutations (pretherapy) detected by NGS in patients with secondary resistance. The orange bars show the mutations identified at the time of relapse. **B,** The first row indicates individual patients by type of therapy received; green indicates type II and orange type I FLT3i-based therapy. The first column represents the list of mutations detected at either pretherapy or relapse. Blue color indicates persistent mutations detected both at pretherapy and at the time of relapse after FLT3i-based therapy. Light blue indicates mutations that were detected pretherapy but not at relapse after FLT3i-based therapy. Red indicates emergent mutations detected at relapse after FLT3i-based therapy that were not detected pretherapy. The last row indicates type of NGS panel applied at our institution in that time frame; brown indicates 81-gene panel before and after FLT3i-based therapy, yellow indicates 28-gene panel before and after FLT3i-based therapy, purple indicates 53-gene panel before and after FLT3i-based therapy, and pink indicates a different NGS panel in pre- and post-FLT3i analysis wherein we included mutations that were tested on both settings.

Table 2. Emergent mutations on *FLT3* analysis and myeloid NGS profile, at relapse after FLT3i-based therapies (*N* = 67)

Acquired/expanded somatic mutations	Total patients *N* = 67 (%)	Type I FLT3i *n* = 21 (%)	Type II FLT3i *n* = 46 (%)
FLT3 mutations	18 (26)	2 (10)	16 (34)
FLT3-D835	14 (21)	0	14 (30)
FLT3-ITD	1 (1)	1[a] (5)	0
FLT3-N676K	2 (3)	1 (5)	1 (2)
FLT3-N841K	1 (1)	0	1 (2)
Epigenetic modifiers	11 (16)	3 (14)	8 (17)
IDH1	4 (6)	1 (5)	3 (6)
DNMT3A	2 (3)	0	2 (4)
TET2	3 (4)	2 (9)	1 (2)
IDH2	2 (3)	0	2 (4)
RAS/MAPK pathway	9 (13)	6 (29)	3 (6)
NRAS	6 (9)	4 (19)	2 (4)
PTPN11	2 (3)	1 (5)	1 (2)
CBL	1 (1)	1 (5)	0
Transcription factors	6 (8)	1 (5)	5 (10)
WT1	5 (7)	0	5 (10)
GATA2	1 (1)	1 (5)	0
Others			
TP53	5 (7)	0	5 (10)
STAG2	1 (1)	1 (5)	0
BCORL1	1 (1)	0	1 (2)
SH2B3	1 (1)	0	1 (2)

[a]One patient had an emergent *FLT3*-ITD at relapse. This patient received decitabine + venetoclax + midostaurin for *FLT3*-TKD only–mutated AML and at relapse, had a newly detected *FLT3*-ITD. The window for pre- and post-NGS and FLT3 sequencing was 8 weeks on each end, as long as the patient had not received intervening anti-AML therapies (except hydroxyurea) between the pre-FLT3 and NGS profiling and the start of FLT3i-based therapy, and between the time of relapse and the post-FLT3 and NGS profiling.

Two patients received single-agent gilteritinib in the R/R setting, and interestingly, both had *NRAS*-emergent mutations at relapse (Supplementary Table S4). Cytogenetic evolution analysis is shown in Supplementary Tables S5 and S6.

Loss of Detectable FLT3 Mutations at Relapse after FLT3i Therapies

Eighteen of 67 (26%) patients no longer had a detectable *FLT3* (ITD or TKD) mutation at relapse (Fig. 1B). The *FLT3* mutation was no longer detectable at relapse in 12 of 46 (26%) patients treated with type II FLT3i-based therapies, and 6 of 21 (28%) patients treated with type I FLT3i-based therapies. The *FLT3* mutation was no longer detectable at relapse in 6 of 22 (27%) patients treated with CCT + FLT3i and 12 of 43 (28%) patients treated with LIT + FLT3i therapies.

VAF Dynamics at Baseline and Relapse

We analyzed VAFs of all mutations pretherapy and at relapse for the 67 patients who achieved CRc and subsequently relapsed (secondary resistance cohort). We analyzed median cohort-level *RAS, WT1, TP53, IDH1*, and *IDH2* VAFs at baseline (annotated Pre-Rx), those that were persistently detectable at relapse for quantitative cohort-level changes in VAF from baseline to relapse (annotated Persistent), and those newly detected (annotated Emergent) at relapse (Supplementary Table S7; Supplementary Fig. S4). We identified a trend suggesting that *IDH1* (14%; Supplementary Fig. S4A), *IDH2* (5%; Supplementary Fig. S4B), and *TP53* (10%; Supplementary Fig. S4C) emerged with lower median cohort-level VAFs. However, *RAS* emerged with a higher median cohort-level VAF (32%; Supplementary Fig. S4D). The median VAF for *RAS* mutations pretherapy was only 6% in the eight patients with *RAS* mutations in this cohort who achieved CRc. On the other hand, in the six patients who did not have a *RAS* mutation pretherapy but had an emergent *RAS* mutation at relapse, the cohort-level VAF of emergent *RAS* mutations was 32%. We did not see any major cohort-level expansions by comparing cohort-level changes in the VAFs in the mutations that were noted at baseline (Pre-Rx) and persistently detected at relapse (Persistent), including RAS mutations (Supplementary Table S7; Supplementary Fig. S4).

We also evaluated the impact of the FLT3i type on *RAS* VAF emergence. Irrespective of the type of FLT3i being used, median cohort-level VAFs of emergent *RAS* mutations were higher than the pretherapy *RAS* VAFs, especially noticeable

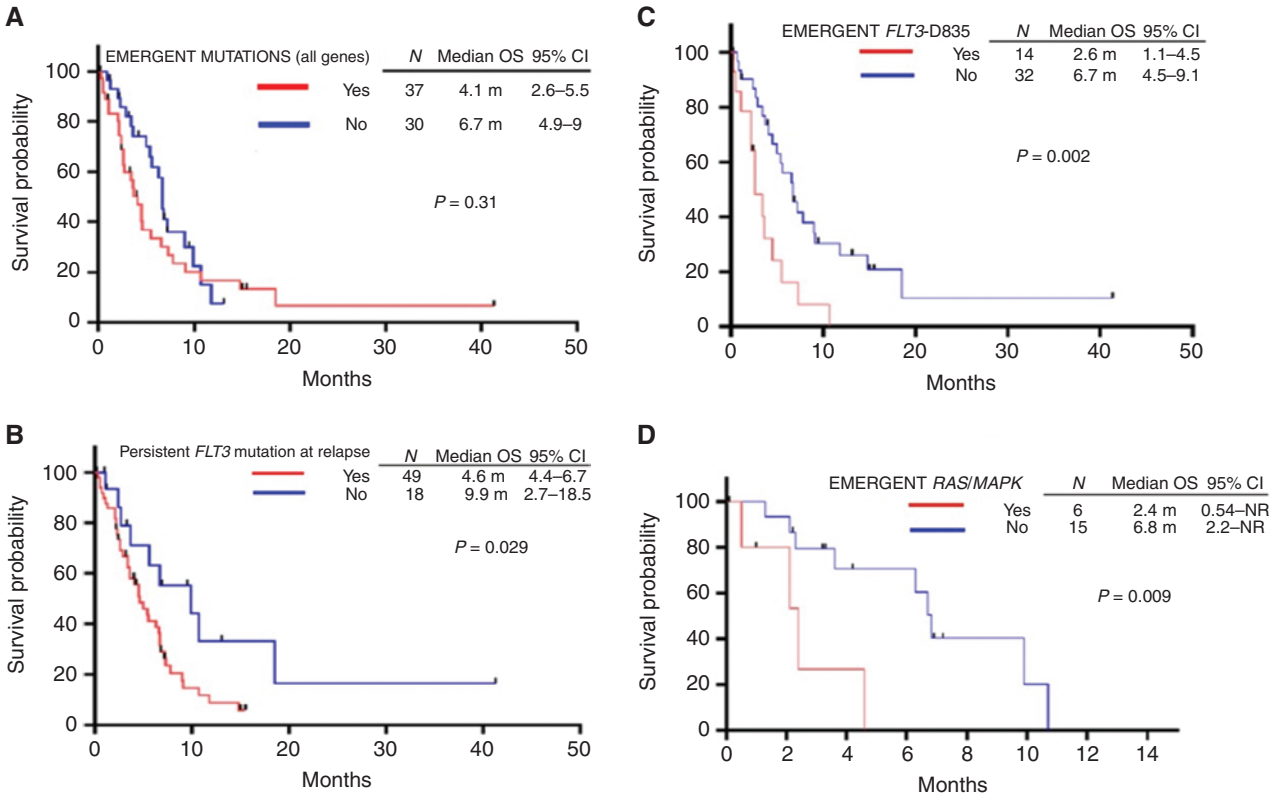

Figure 2. OS based on emergent mutations at relapse after FLT3i-based therapy. **A,** Patients with emergent mutations in any of the tested genes versus without emergent mutations after FLT3i-based therapies. **B,** Patients with no detectable *FLT3* mutation (ITD and/or TKD) at relapse versus patients with persistently detectable *FLT3* mutations at relapse after FLT3i-based therapies. **C,** Patients with versus without emergent *FLT3*-D835 mutations after type II FLT3i-based therapies. **D,** Patients with versus without emergent *RAS/MAPK* mutations at relapse after type I FLT3i-based therapies. NR, not reached.

with type I FLT3i therapies (Supplementary Table S8; Supplementary Fig. S5). These data suggest that emergent *RAS* may biologically have a different impact compared with pretherapy *RAS*.

Survival Outcomes after Relapse

After a median follow-up of 15 months [95% CI, 7.2–not reached (NR)] from the time of relapse, 18 of 67 (26%) patients are still alive. The median OS after relapse for all patients was 5.4 months (95% CI, 3.5–6.7 months), and the median OS for patients with emergent mutations (n = 37) versus those without emergent mutations (n = 30) at relapse was 4.1 months (95% CI, 2.6–5.5) versus 6.7 months (95% CI, 4.9–9.0), respectively (P = 0.31; Fig. 2A). Median OS was significantly better for patients who had an undetectable *FLT3* mutation at relapse (n = 18) compared with patients with persistent *FLT3* mutation (ITD and/or D835) at relapse (n = 49): 9.9 months (95% CI, 2.7–18.5) versus 4.6 months (95% CI, 3.4–6.7), P = 0.029 (Fig. 2B).

Among patients who relapsed after type II FLT3i-based therapy, median OS for patients with emergent mutations (n = 30) versus those without emergent mutations (n = 16) at relapse was 4.1 months (95% CI, 2.6–7.3) versus 6.7 months (95% CI, 3.4–11.8), respectively (P = 0.45). Median OS was significantly lower in patients with (n = 14) versus those without (n = 32) emergent *FLT3*-D835 mutations at relapse after type II FLT3i-based therapies [2.6 months (1.1–4.5) vs. 6.7 months (4.5–9.1), P = 0.002; Fig. 2C].

The median OS for patients with emergent mutations (n = 7) versus those without emergent mutations (n = 14) at relapse after type I FLT3i-based therapies was 2.4 months (95% CI, 0.54–NR) versus 6.7 months (95% CI, 2.25–NR; P = 0.04). Median OS for patients with (n = 6) versus those without (n = 15) emergent RAS/MAPK mutations at relapse after type I FLT3i-based therapies was 2.4 months (95% CI, 0.54–NR) versus 6.8 months (95% CI, 2.2–NR; P = 0.009; Fig. 2D).

Pretherapy Mutational Profile and Cohort-Level VAFs in Patients with Primary versus Secondary Resistance

We assessed the cohort-level VAFs of *DNMT3A, NPM1, NPM1/DNMT3A, RAS, RAS/MAPK* mutations (including *N/K-RAS, PTPN11, NF1*), *IDH1, IDH2, WT1, PTNPN11*, and *TP53* in patients with who achieved CRc followed by relapse (secondary resistance; N = 67) and patients with no response (primary resistance; N = 106; Supplementary Tables S9 and S10; Supplementary Fig. S6). The pre-FLT3i frequency of *DNMT3A* and *IDH2* mutations was higher in patients who achieved CRc compared with nonresponders (54% vs. 30%; P = 0.002) and (21% vs. 7%; P = 0.005), respectively. We identified no statistically significant difference in pretreatment *RAS, PTPN11,*

IDH1, IDH2, WT1, and *TP53* (Supplementary Fig. S6A–S6E) cohort-level VAFs between responders and nonresponders.

Overall, the median cohort-level VAF for *RAS* pre-FLT3i therapy was identified as 19% for the whole cohort ($n = 173$, including 106 primary and 67 secondary resistance; Supplementary Table S9), with pre-FLT3i cohort-level VAF of 6% among patients who achieved CRc and relapsed compared with 31% among patients who were nonresponders ($P = 0.19$), suggesting a nonsignificant trend toward primary resistance to FLT3i-based therapies among patients with a higher pre-FLT3i burden of *RAS*. Establishing an arbitrary cutoff of 20% for RAS, we identified that more nonresponders had *RAS* VAF >20% pre-FLT3i ($n = 10/16$) compared with patients who achieved CRc ($n = 2/8$; 63% vs. 25%; $P = 0.083$).

Among *RAS*-mutated patients who received type I FLT3i-based therapies ($n = 9$), more nonresponders had *RAS* VAF >20% pre-FLT3i ($n = 6/7$) compared with patients who achieved CRc [$n = 0/2$ (86% vs. 0%; $P = 0.023$); Supplementary Table S9]. On the other hand, in patients who received type II FLT3i therapies, the proportion of responders (2/6) and nonresponders (4/9) who had *RAS* VAF >20% pre-FLT3i was similar (33% vs. 44%, $P = 0.67$). Although patient numbers were small, these data suggest that nonresponding patients, especially nonresponders to type I FLT3i-based therapies, were more likely to have higher burden *RAS* mutations (VAF >20%) pretherapy.

DISCUSSION

Patients with *FLT3*-mutated AML usually achieve remission with FLT3i-based therapies; however, nearly all responders eventually develop resistance to therapy and relapse, with the exception of patients bridged to ASCT. Here, we note that the majority of the patients (55%) who responded and relapsed (secondary resistance) had treatment-emergent mutations at the time of relapse, including on-target mutations in *FLT3* (26%), and off-target mutations in epigenetic modifiers (16%), RAS/MAPK pathway genes (13%), WT1 (7%), and *TP53* (7%). *FLT3*-D835 was the most common emergent mutation (30%) in patients treated with type II FLT3i-based therapies, and the emergence of *FLT3*-D835 was associated with inferior survival. Although none of the patients who received a type I FLT3i developed a *FLT3*-D835 mutation at relapse, emergent mutations involving RAS/MAPK pathway genes were observed in 29%. The emergence of RAS/MAPK mutations was associated with inferior survival in patients treated with a type II FLT3i.

Although the majority of the responding patients (55%) developed emergent mutations at relapse, *FLT3* (ITD and/or TKD) mutations persisted in 74% of the patients at relapse. This is slightly lower than the 88% *FLT3* mutation persistence reported in 41 patients with AML relapsing after single-agent gilteritinib failure (9) and may be due to the combinatorial therapies more commonly administered in our population. Another important observation was that the incidence of emergent *FLT3*-D835 mutations was less common (6% vs. 44%, $P = 0.007$) when a type II FLT3i was combined with CCT. Conversely, *TP53* emergence trended lower in patients treated with LIT + type II FLT3i compared with CCT + type II FLT3i (7% vs. 18%, $P = 0.343$). Overall, these findings suggest that improved understanding of secondary resistance patterns and strategic use of backbone chemotherapy (CCT or LIT) with FLT3i combinations may be able to further delay resistance.

Emergent mutations in the RAS/MAPK pathway were more common in patients treated with type I FLT3i than type II FLT3i (29% vs. 6%, $P = 0.014$). *NRAS* was the most commonly mutated gene. Similar findings were observed in the study by McMahon and colleagues, in which 37% of the patients developed RAS/MAPK mutations after failing single-agent gilteritinib (9). On the other hand, only three (6%) patients treated with a type II FLT3i developed an emergent RAS/MAPK mutation, suggesting that under the selective pressure of a particular FLT3i (type I vs. type II), the leukemic cells may exploit distinct yet potentially predictable secondary pathways of resistance.

We noted that the pre-FLT3i therapy frequency of *DNMT3A* and *IDH2* mutations was higher in patients who achieved response compared with nonresponders. For *RAS* mutations, we noted a significantly lower pre-FLT3i cohort-level VAF among responders (6%) compared with nonresponders (31%). Although this did not reach statistical significance, likely due to the small number of patients, it suggests a potential role for RAS mutation, especially those with sizable *RAS* clones, in primary resistance to FLT3i-based therapies. The impact of pretherapy RAS mutations was most prominent in patients treated with type I FLT3i-based therapies, wherein using an arbitrary RAS VAF cutoff of 20%, we noted that fewer patients who achieved response had pretherapy RAS VAF >20% compared with patients with primary resistance. It will be interesting to see if ongoing novel combinations of type I FLT3i such as venetoclax with gilteritinib, or azacitidine with venetoclax with gilteritinib, will be able to overcome such RAS-mediated resistance to type I agents.

We note several clear limitations to our analysis. The NGS and *FLT3* mutational analyses were performed on 67 paired pre- and post-BM samples from patients treated on heterogeneous FLT3i-based combinations. These data may or may not be directly applicable to single-agent FLT3i-based therapy in R/R AML, although the frequency of RAS/MAPK-emergent mutations after type I FLT3i therapies in our analysis was very similar to that published after single-agent gilteritinib by McMahon and colleagues (9). The original clinical trial designs or standard of care did not mandate end-of-treatment mutational analysis, so our results may reflect a selection bias for patients who had mutational analysis available. The number of patients treated on specific combinations of type I or type II FLT3i with CCT or LIT are too small to make definitive conclusions regarding the impact of the specific combination partners on subsequent mutational emergence, but some of the hypotheses generated are of interest for future investigation.

The analytical sensitivity of the NGS platform used in this study is approximately 1% mutant allele in a background of wild-type allele (Supplementary Table S11). Hence, our analysis may indeed have missed small subclones, which could have expanded at relapse under the therapeutic pressure of FLT3i-based therapies and eventually have been detected as emergent mutations when NGS was performed at relapse. Future studies performed with ultra-deep sequencing platforms such as droplet digital polymerase chain reaction (PCR) pretherapy and at relapse may help us better understand "true mutational acquisition" versus "clonal expansion."

In conclusion, emergent mutations are common in *FLT3*-mutated AML relapsing after FLT3i-based therapy. Eradication of emerging and coexisting subclones will be needed for eventual cure. Our findings expand previous information regarding emergent mutations post–type II FLT3i, enhance our understanding of differential patterns of primary and secondary resistance to type I and II FLT3i, and highlight the prognostic implications of specific emergent mutations at relapse in *FLT3*-mutated AML. Rational, targeted, and dynamic combination therapies, selecting type I or type II inhibitors with the optimal combination partner to target specific scenarios, may improve response durations and hopefully improve cure rates.

METHODS

We retrospectively reviewed 810 consecutive patients with *FLT3*-mutated (ITD and/or TKD) AML who had received FLT3i-based therapy (single agent or combination) in the first-line or R/R setting at our institution between January 1, 2012, and May 1, 2019. Patients with an initial CRc response (defined as CR + CRp + CRi), and a subsequent relapse with available pre-BM *FLT3* and NGS myeloid mutation profiles pretherapy and at the time of relapse were included in the secondary resistance cohort. *FLT3* and NGS analysis had to be done at the time of relapse after FLT3i-based therapy and prior to starting the next AML therapy (i.e., no intervening therapy was allowed). ASCT in remission was allowed and not considered an independent salvage therapy.

We also identified a cohort of 201 patients (January 2012–December 2019) who received FLT3i-based therapies with no response at our institution. In 106 of the patients, a myeloid NGS panel was available prior to therapy (primary resistance cohort). We compared cohort-level mutation frequencies with χ^2 analysis. Independent samples median test was used to compare baseline VAFs of responders versus nonresponders and VAFs at pretherapy versus relapse in responders. The Kaplan–Meier method was used to estimate the probability of OS, and the log-rank test was used to compare OS between cohorts of patients. Statistical calculations were performed in SPSS (version 24).

Response was defined based on the International Working Group criteria and as reported in phase II/III FLT3i trials (6, 7, 12). A relapse was defined by >5% blasts in a BM aspirate or by the emergence of extramedullary disease.

Single-agent FLT3i, FLT3i-based combinations with CCT, and FLT3i-based combinations with LIT (hypomethylating agent or low-dose cytarabine-based combinations) were included. Most of the FLT3i-based treatments (62%) included in this analysis were administered on clinical trials. The clinical trials utilized are outlined in Supplementary Table S1.

The study was conducted in accordance with the Declaration of Helsinki. All patients had signed a written informed consent form approved by the Institutional Review Board (IRB). Data were collected under MD Anderson Cancer Center (MDACC) IRB protocols DR09-0223 and PA12-0395 for retrospective data collection in patients with *FLT3*-mutated AML.

Molecular Analysis

A multiplex fluorescent-based PCR analysis followed by capillary electrophoresis for detection of ITD and/or TKD mutations in *FLT3* was performed on DNA isolated from BM aspirate samples, as previously described by our group, with an analytical sensitivity of ~1% mutant DNA in the background of wild-type DNA (13). NGS was done using one of three clinical-grade myeloid gene panels (28-gene, 53-gene, or 81-gene) using the Illumina MiSeq (Illumina, Inc.) platform validated at the Clinical Laboratory Improvement Amendments–certified molecular diagnostic laboratory at MDACC as described previously (Supplementary Table S11; ref. 14). All three panels included coverage for *FLT3* D835. A minimum of 250× coverage with a detection sensitivity of ~5% was used for variant calling. A majority (56 of 67; 84%) had the same NGS panel before and after FLT3i-based therapy; 11 (16%) patients had a different panel, and for these 11 patients, for consistency, we only included genes that were included in both panels. All NGS data reported in this article (primary and secondary resistance cohorts) were deposited as supplementary material.

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