TREATMENT WITH FLT3 INHIBITOR IN PATIENTS WITH *FLT3*-MUTATED AML IS ASSOCIATED WITH DEVELOPMENT OF SECONDARY *FLT3*-TKD MUTATIONS

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Abstract

Background—*FLT3*-internal tandem duplication (ITD) mutations are found in approximately 30% of AML patients. FLT3 inhibitors have shown clinical activity in AML with *FLT3*-ITD but responses are usually short lived.

Methods—We reviewed 69 *FLT3* mutated AML patients, treated with different FLT3 inhibitors to analyze emergence of new mutations.

Results—At baseline 87% of patients had an ITD mutation, 7% had a D835/I836 mutation and 6% had combined ITD and D835/I836 mutations. Responses occurred in 32% of patients, all with *FLT3*-ITD; none of the patients with D835/I836 or ITD+D835/I836 responded. Mutational assessment at the time FLT3 inhibitor discontinuation showed that 68% of patients were unchanged, 10% had become undetectable, and 22% of patients progressed from a single ITD to have combined ITD+D835/I836 mutations. In those patients with unchanged *FLT3* mutation at progression the median survival was 5 months, while in those with undetectable and with combined ITD-D835/I836 mutations the median survival was 7 months respectively.

Conclusion—This data confirm in vitro observations that a secondary TKD mutation may arise after the use of FLT3 inhibitors in patients with single *FLT3*-ITD mutated AML, a phenomenon that is associated with resistance and a poor prognosis.

Keywords

FLT3 inhibitors; FLT3-ITD; FLT3-TKD; secondary FLT3 mutations; AML

Introduction

Acute myeloid leukemia (AML), the most common acute leukemia in the United States,[1] is a heterogeneous disease that carries a variable prognosis based on pretreatment characteristics such as age, performance status, comorbid conditions, prior cancer treatments, antecedent hematologic disorders, and cytogenetic and molecular abnormalities.[2-5] The *FLT3* gene is located on chromosome 13q12 and encodes the FLT3 tyrosine kinase receptor. FLT3 has 993 amino acids in length, contains five extracellular immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain and two intracellular tyrosine kinase domains linked by a kinase-insert domain. [6-9] Under normal circumstances, cytoplasmic FLT3 undergoes glycosylation, which promotes localization of the receptor to the membrane. Binding to FLT3-ligand promotes receptor conformational changes and receptor homodimerization which in turn promotes phosphorylation of the tyrosine kinase domains and activation of downstream effectors such as the phosphatidylinositol 3-kinase (PI3K/AKT), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and signal transducer and activator of transcription 5 (STAT5) pathways.[8] Activating mutations of *FLT3* have been identified in patients with acute myeloid leukemia (AML) including internal-tandem duplications (ITDs) of the juxtamembrane region (head to tail duplication of 3-400 base pairs in exons 14 or 15), tyrosine kinase domain 1, and mutations involving the D835/I836 residues and others of the tyrosine kinase domain (TKD).[8,10-12] They occur in approximately 30% and 7% of AML patients respectively, and lead to constitutive activation of the tyrosine kinase domain.[10,11,13,14]

Patients with AML with *FLT3*-ITD mutation typically present with a normal karyotype, elevated white blood cell count, and higher percentages of blasts in peripheral blood and bone marrow. The presence of *FLT3* mutations has been associated with a poor outcome, with a greater probability of relapse and shorter overall survival.[15-19] Several FLT3 inhibitors have been developed in an attempt to overcome this aggressive outcome of FLT3-ITD AML.[20] Clinical responses have been observed with agents such as sorafenib,[21] quizartinib,[22] midostaurin[23] and others. Responses are frequently characterized by a rapid reduction in peripheral blood and/or bone marrow blasts, but they are usually transient with most patients eventually progressing. Recently, it has been reported that point mutations may confer in vitro resistance to FLT3 inhibitors.[24,25]

The frequency with which these mutations occur in the clinic among patients treated with FLT3 inhibitors and their clinical significance has not been fully described. We thus analyzed our experience among patients with AML with *FLT3* mutations treated with various FLT3 inhibitors to define the frequency and clinical significance of this phenomenon.

Materials and Methods

Patients

We analyzed the records of 69 consecutive patients with AML with *FLT3* mutations treated at our institution in clinical trials with different FLT3 inhibitors used as single agent from

October 2002 to August 2011 and in whom we obtained mutational assessment before and after treatment. Patients were enrolled in studies 2009-0560 and 2006-0850 (AC-220, quizartinib), 2004-0702 (sorafenib), 2003-0719 and ID02-274 (lestaurtinib, CEP-701), and 2006-0275 (KW-2449). Studies were approved by the institutional review board and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before study entry. Patients were also included in a retrospective chart review approved by the IRB.

Patient Monitoring and Response Criteria

Patients were followed with complete blood counts at least weekly during the first 4 weeks of therapy, then every other week during the next 4-8 weeks, and then every 1-3 months based on response or clinical status. AML response criteria followed the recommendations of the International Working Group.[26,27] Briefly, complete remission (CR) was defined by the presence of <5% blasts in the BM with >1 $\times 10^9$/L neutrophils and >100 $\times 10^9$/L platelets in the peripheral blood. Morphologic complete remission with incomplete platelet recovery (CRp), was defined in patients with CR but persistent platelet count <100 $\times 10^9$/L. Morphologic complete remission with incomplete blood count recovery (CRi), was defined in patients with persistent neutrophil count <1 $\times 10^9$/L, or without platelet recovery. Patients showing a significant decrease (>50%) bone marrow blast reduction (BMBR), without peripheral blood counts recovery are described separately. A relapse was defined by >5% blasts in a bone marrow aspirate or by the presence of extramedullary disease. Induction death was defined as death that occurred within 6 weeks from start of therapy.

Molecular Analysis for FLT3 Mutations

Genomic DNA extracted from fresh BM aspiration specimens using the Autopure extractor (QIAGEN/Gentra, Valencia, CA) was used for mutation analysis. *FLT3* (ITD and D835) mutations were screened using polymerase chain reaction (PCR) followed by capillary electrophoresis on an ABI Prism 3100 or 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA), as previously described.[28,29] To facilitate the detection of PCR products by capillary electrophoresis forward primers for ITD and D835 were labeled with a fluorescent dye, 6-carboxyfluorescein (FAM). The presence of any PCR fragment larger than the WT allele was considered positive for ITD. For *FLT3* codon 835 point mutation analysis, PCR products were digested with *Eco*RV before capillary electrophoresis. The WT allele cut by this enzyme resulted in 2 fragments of 64 and 48 base pairs.[29] In contrast, mutations at D835 alter the EcoRV recognition site and result in 1 112 base-pair fragment. The sensitivity of these assays is approximately 2%, that is, 1 mutated cell in 50 total cells, as established by dilution studies.

Statistical Methods

Patient characteristics are summarized using median (range) for continuous variables and frequency (percentage) for categorical variables. Categorical and continuous variables were compared by using the Fisher exact test and the Wilcoxon test, respectively.[30] Event-free survival (EFS) was calculated from the beginning of treatment until an event, defined as relapse, resistance to induction therapy, or death. Disease free survival (DFS) was calculated

from the moment of CR until relapse or death in CR. Overall survival (OS) was calculated from the start of treatment until death. Patients who remained alive at the last follow-up were censored. The probabilities of OS and EFS were estimated using the Kaplan-Meier method,[31] and were compared among subgroups of patients using the log-rank test.[32] All statistical analyses were conducted using SAS 9.0.

Results

Patient Characteristics (Table 1)

Before the start of therapy with a FLT3 inhibitor, 60 (87%) of the 69 patients had a *FLT3* ITD mutation, 4 (6%) had a D835/I836 kinase domain mutation, and 5 (7%) had combined ITD and D835/I836 mutations. The median age for the 69 patients was 54 years (range, 18-87 years), and the median number of prior leukemia treatments was 2 (range 1-6), including 16 (23%) patients with prior stem cell transplantation (SCT). Karyotype was diploid in 24 (35%) patients, complex in 12 (17%), 5 (7%) patients had either -7, 11q, or +8, miscellaneous in 11 (16%), and an additional 17 (25%) patients had either insufficient metaphases or cytogenetics were not done at the time FLT3 inhibitor therapy was started. Concomitant *NPM1*, *RAS*, and *CEBPA* mutations were observed in 22%, 13%, and 4% of the patients, respectively.

Survival Outcomes

Patients received therapy for a median of 78 days (range 19-838). Forty-three (62%) patients did not achieve response to therapy, and 4 (6%) patients died within 42 days after the start of therapy. Four (6%) patients achieved CR, 9 (13%) patients achieved CRp, and 9 (13%) had a significant BMBR from a median of 66% (range 20-92) blasts to a median of 5% (range 2-26); of note 3 of them had documented BM blasts less than 5% (i.e., morphologic leukemia free state) but did not reach hematologic parameters for CR, CRp or CRi. All responders had *FLT3* ITD alone. None of the patients with D835/I836, whether alone or concomitant with *FLT3* ITD achieved a response. All patients eventually discontinued therapy. Among responders, nine patients received stem cell transplantation (CR=1, CRp=3 and BMBR=5), and 7 of them relapsed with a median time to relapse after transplant of 4 months. Of note only two patients, 1 in CR and 1 in CRp had a negative FLT3 ITD by PCR prior to transplant. Two patients who were transplanted in CRp achieved complete remission after SCT and are the only two survivors, without relapse at the time of this analysis (3.5 and 5 years after transplant, respectively). Ten (14%) patients in the non-response group received SCT, 6 immediately after FLT3 inhibitor (median bone marrow blast percentage at the time of SCT 38%) and 4 after another salvage combination chemotherapy (IA, FA-sorafenib, decitabine + gemtuzumab ozogamicin, and MEC, respectively). The median time to progression post-transplant for these 10 patients was 2.3 months (range, 0 to 24 months). Among patients not transplanted, 4 (1 in CR, 1 in CRp and 2 with BMBR) discontinued FLT3 inhibitor due to toxicity and eventually progressed. All other patients discontinued therapy because of progressive disease and all patients (except those two still alive) eventually progressed with same malignant clone at presentation.

All patients had assessment of their *FLT3* mutation status at the time FLT3 inhibitor was discontinued. In 15 (22%) patients where a single *FLT3* ITD mutation had been identified at baseline, both *FLT3* ITD and D835/I836 mutations were identified at the end of therapy. In 7 (10%) patients (6 with *FLT3* ITD at baseline and 1 with D835/I836) no *FLT3* mutation was identified at the time treatment was discontinued. In all other 47 (68%) patients the mutations status was unchanged.

Patient characteristics of 15 patients developing a secondary *FLT3* mutation are summarized in table 2, their median age was 43, and median number of prior treatments was 2, including 4 patients with prior SCT (one of them with 2 prior transplants). The median duration of treatment with FLT3 inhibitors was 126 days (range 77-570 days). Best response to therapy included NR in 7, significant BMBR in 3, CRp in 3 and CR in 2. In this group we include 2 patients that received a SCT after FLT3 inhibitor, one with no response but having low burden disease (patient number 9) and the other (patient number 12) in whom BM blast had decreased to 4 %. In these two cases, the D835/I836 mutation was detected at disease progression 2 and 3 months post-transplant, respectively.

Second line FLT3 inhibitor treatment was attempted in 7 out of 15 patients with both *FLT3* ITD and D835/I836 mutations. None of the patients responded to further therapy. In patients in whom *FLT3* mutation was unchanged or became negative, the median white blood cell count (WBC), peripheral blood blasts (PBB) and bone marrow blasts (BMB) at baseline were 5.5×10^9/L, 27%, 68.5%, and at the time of disease progression 2.6×10^9/L, 7.8%, 70%, respectively. In those who developed both mutations they were 7.8, 51%, 66%, and 20.5, 80% and 74% respectively.

At the time of this analysis 67 (97%) patients had died. The median survival in the entire group from the time the FLT3 inhibitor was started was 6 months. In the subgroup analysis, those patients in whom the *FLT3* mutation became negative the median survival was 7 months, compared to 5 months for those with in no change in mutation status and 7 months in those with ITD-D835/I836 mutations. The median survival for transplanted patients was 7 months and the median survival from time of treatment discontinuation in the entire group was 3 months (Figure 1).

Discussion

It has been established that *FLT3* ITD mutated, normal karyotype AML carries a poor prognosis. Although several multivariate analyses have failed to establish a direct impact in the ability to achieve a complete remission, they have proved a strong correlation with an increased risk of relapsed and poor overall survival.[10,11,13-17,19,33] Therefore it is generally accepted that these patients should undergo hematopoietic cell transplantation in first remission if feasible. However the outcome of these patients is also inferior after allogeneic stem cell transplantation compared to patients comparable patients with no *FLT3* mutation. In one recent report of patients transplanted in first remission, the 2-year relapse rate was 30% vs. 16% for those with no *FLT3* ITD, and the leukemia-free survival probability lower (58% vs. 71%).[34] Thus there is a need to investigate new treatment options for these

patients. Among them, FLT3 inhibitors have emerged in recent years as promising additions to the treatment armamentarium that may improve the outcome of these patients.[20]

Several inhibitors of FLT3 kinase activity have entered the clinical development arena. Sorafenib has shown inhibition of the phosphorylation of tyrosine residues in ITD mutant in vitro using primary human AML cells and in an animal model.[35] A phase I study conducted in patients with MDS and relapsed or refractory AML, showed CR or CRp in 10% of patients; significant reduction in bone marrow and or peripheral blood blasts was seen in an additional 34% patients (all responses in patients with *FLT3*-ITD mutation).[21] Activity of midostaurin (PKC412) was reported in a phase IIB trial with 95 patients with AML or MDS, with either wild-type (n=60) or mutated (n=35) FLT3; one patient achieved PR and reduction in peripheral blood or bone marrow blasts of at least 50% was observed in 71% of patients with *FLT3*-mutant and 42% of patients with *FLT3* wild-type.[23] Quizartinib (AC220) is a second-generation FLT3 inhibitor with greater kinase selectivity.[36] In a phase 1 study, 76 patients with relapsed refractory AML, irrespective of *FLT3*-ITD mutation status, received quizartinib at escalating doses. Of 17 patients with *FLT3*-ITD mutation 53% responded, and among 37 patients without the mutation 14% responded. There were 22 patients with *FLT3*-ITD indeterminate or not tested status, and among them, 41% responded. The median duration of response was 13 weeks; median survival was 14 weeks.[37] Earlier inhibitors such as lestaurtinib inhibited multiple kinases and produced mostly partial responses.[38] Despite this promising clinical activity, responses are usually transient and insufficient to induce a durable response.[39,40]

The current cohort of patients, treated with a variety of inhibitors, followed the same pattern as reported in these studies; several patients achieved responses, many of them able to be bridged to transplant, but in all instances (except occasionally when transplanted) the responses were eventually lost. Many of these patients were treated during a time when the allelic burden was not routinely measured, but it has been suggested that patients with relapsed disease carry a greater allelic burden than untreated patients and their disease depends more on the aberrant FLT3 signaling, a phenomena that could explain the greater cytotoxicity observed with more specific FLT3 inhibitors. [41] Interestingly, in a subset of patients the mutated clone was no longer detectable upon progression. This subset of patients carried a similar median survival when compared with the group developing the second mutation, but disease burden and proliferation seem to be increased in the later. Undoubtedly this is a small cohort and a retrospective analysis, making these observations only preliminary, but if these observations can be confirmed in larger, prospective series, this might identify a subset of patients whose biology is changed by the introduction of FLT3 inhibitors, perhaps favoring the emergence of clones that are more proliferative and no longer inhibited by FLT3 inhibitors that have lesser (e.g., quizartinib) or no (e.g., sorafenib) activity against unmutated *FLT3*. It is also entirely possible that the *FLT3* mutated clone is present, only below the level of detection of our assay. Still, this would suggest that another clone has become more prominent. Ongoing studies are testing these hypotheses.

Resistance to FLT3 tyrosine kinase inhibition occurs eventually in virtually all patients and has been attributed to compensatory activation of downstream effectors such as STAT5 or

MAPK despite complete inhibition of FLT3 tyrosine phosphorylation.[42-44] Other mechanisms of resistance include up-regulation of FLT3 ligand and FLT3 receptor or up-regulation of anti-apoptotic genes and down-regulation of pro-apoptotic signals, or compensatory activation of secondary signaling pathways such as Ras or PI3K.[45-49] Recently a novel *FLT3*-ITD mutation located in the non-juxtamembrane domains of FLT3 (FLT3_ITD627E) mediated constitutive phosphorylation of FLT3 and of STAT5, and induced transformation of hematopoietic 32D cells leading to a lethal myeloproliferative disease in a syngeneic mouse model.[12] Upon further characterization authors also suggested that (FLT3_ITD627E) induces dramatic up regulation of the anti-apoptotic myeloid cell leukemia 1 protein (MCL-1), an effect that appeared to be due to an enhanced and sustained binding of the adaptor protein GRB-2 to the FLT3_ITD627E receptor, which was independent from the inhibitor midostaurin-induced inhibition of the receptor kinase.[44] Sensitivity to FLT3 inhibitors seems to be variable in different mutants, [35,50,51] and in vitro exposure to different FLT3 tyrosine kinase inhibitors has been associated with development of secondary *FLT3* resistant mutations, which interestingly were not overlapping like in the case of Bcr-Abl kinase inhibitors (imatinib, dasatinib, nilotinib).[52] More recently, Smith et al.[24] reported the emergence of resistance mutations in cells from 8 patients treated with AC220 (quizartinib). D835Y (an activation loop mutation) and F691L (a gatekeeper mutation) were each detected in 3 instances, with 2 others showing D835V.

Our results confirm the emergence of secondary TKD mutations, in our series specifically D835/I836, as a common mechanism of resistance after treatment with FLT3 inhibitors in patients with *FLT3*-ITD AML occurring in approximately 20% of patients. To our knowledge, this is the first report to analyze the development of these mutations while on therapy with FLT3 inhibitors. De novo *FLT3*-TKD mutations are also associated with normal karyotype AML and higher blast percentage, but while several groups have noted a strong association with worse disease free and overall survival,[14,15,53] others noted no significant prognostic impact.[11,54,55] Recently Bacher et al.[56] characterized the mutational status of 3082 patients with newly diagnosed AML; unlike *FLT3*-ITD, *FLT3*-TKD did not influence prognosis, however authors found an unfavorable prognosis in patients harboring double mutations, including those with both *FLT3* ITD and TKD. In our series, none of the patients expressing TKD mutations, whether alone or in combination with ITD responded to FLT3 inhibitors. The emergence of resistant clones with this or other mutations that are resistant to agents being used to treat the patient would likely alter the outcome of patients. New agents, such as crenolanib[57] and PLX3397[58] are being developed that may overcome these resistant mutations.

Our series represents a retrospective analysis of patients treated with a variety of inhibitors. It is possible that the rate and type of mutations will vary clinically depending on the inhibitor being used as has been described in the laboratory.[52] Importantly, we did not look for other mutations that have been described as mechanisms of resistance of FLT3 inhibitors, e.g. N676K as reported by Heidel et al.[25] Thus, it is likely that the incidence of resistant mutations is higher than reported here.

We conclude that emergence of resistant mutations is a common mechanism of resistance to FLT3 inhibitors used clinically, with such mutations emerging in at least 20% of patients.

This shows that in these cases, survival of AML blasts depends to a great extent on FLT3 signaling. Patients treated with these agents need to be assess for emergence of these mutations, and new agents that may overcome or, ideally, prevent the emergence of such mutations need to be developed.

Acknowledgments

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Key Points

1. Secondary FLT3-TKD mutations can arise after treatment with FLT3 inhibitors in patients with FLT3-ITD mutated AML.

2. D835/I836 mutations were observed in at least 20% of patients after treatment, and are associated with resistance and poor prognosis.
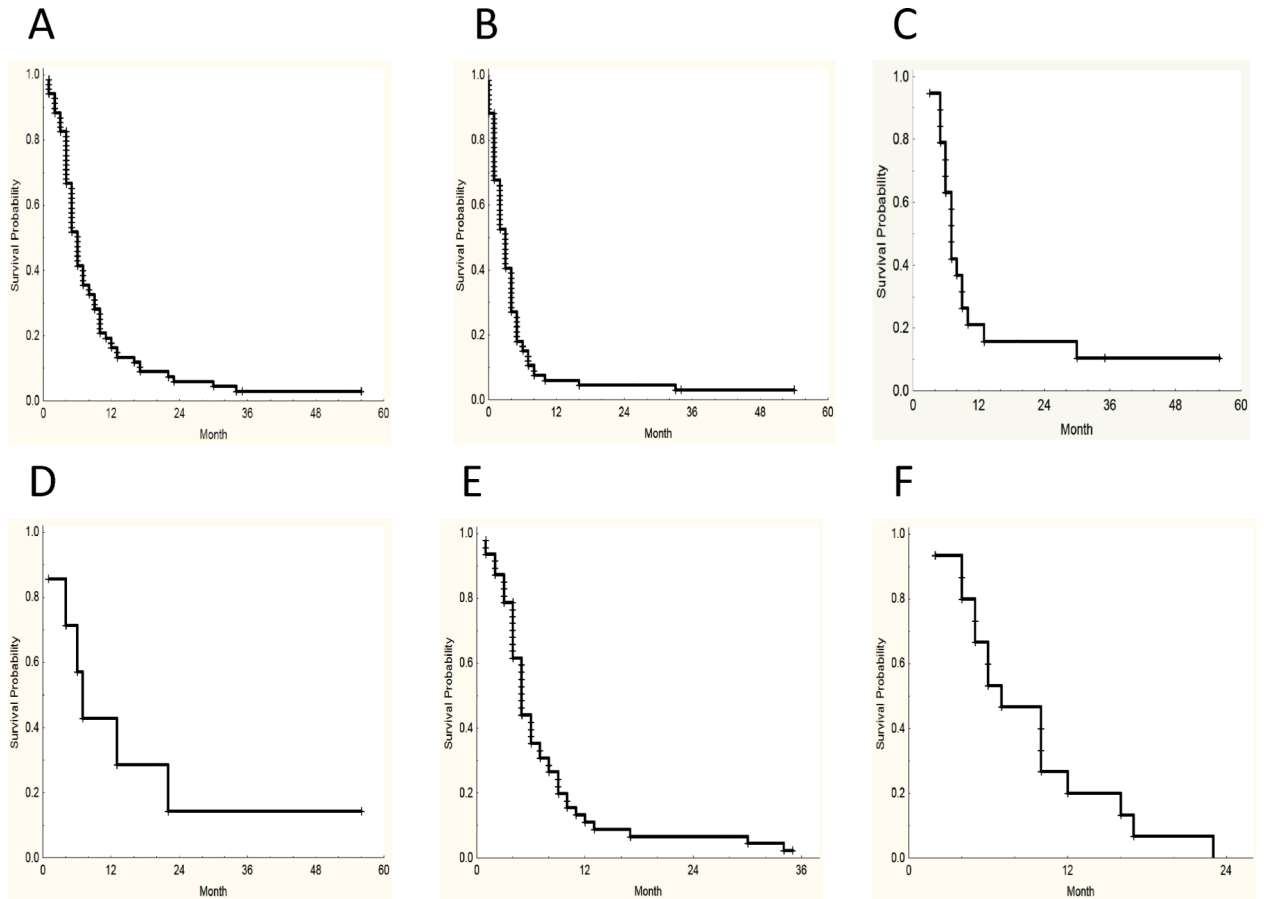
Figure 1. Survival Curves

A: Overall Survival from beginning of treatment (Median 6 months), B: Overall Survival from FLT3 Inhibitor treatment discontinuation (Median 3 months), C: Survival Patients Transplanted (Median 7 Months), D: Survival Patients with Undetectable Mutation After Treatment (Median 7 months), E: Survival Patients with Unchanged Mutation Status (Median 5 months), F: Survival Patients Developing Both Mutations (Median 7 months).

Table 1

Baseline Patient Characteristics

Patient Characteristics	Median [Range], or No. (%)
Females	35 (51)
Median Age	54 [18-87]
Median No. of Prior Treatments	2 [1-6]
Cytogenetic	Diploid: 24 (35) Complex: 12 (17) Miscellaneous: 11 (16) +8, -7, 11q: 5 (7)
ITD	60 (87)
D835/I836	5 (7)
ITD and D8535/I836	4 (6)
NPM1 Mutation	15 (22)
RAS Mutation	9 (13)
CEBPA Mutation	3 (4)

Table 2

Patient Characteristics of patients that developed D835 Mutation upon clinical resistance to FLT3 inhibitors

Patient	Age	# Prior Treatments	Baseline						At Disease Progression					Treatment after FLT3 Inhibitor	Response
			WBC (x10⁹/L)	PBB (%)	BMB (%)	Ratio Allele Burden ITD	Cytogenetic	Best response to FLT3 inhibitor	WBC (x10⁹/L)	PBB (%)	BMB (%)	Ratio Allele Burden ITD/D835	Cytogenetic		
1	37	2	2.4	33	66	ND	Misc	CRp	20.5	81	56	ND	ND	IA+ Sorafenib	NR
2	87	1	7.1	89	80	ND	Dip	BMBR	23	81	82	ND	Dip	Azacytadine + Sorafenib	NR
3	69	2	5.4	20	86	Size 371 and 39.7%	Dip	CR	14	71	91	0.47/0.5 3	Dip	Phase 1 Protocol	NR
4	25	4	1.7	33	60	ND	Com	NR	10.1	97	76	ND	Com	Phase 1 Protocol	NR
5	79	1	21	51	52	0.007 and 0.167	Dip	NR	5.1	25	63	0.029 and 0.318/0. 210	Dip	Azacytadine + Sorafenib	NR
6	38	5	9.8	85	95	ND	Dip	NR	17.9	63	70	ND	Dip	Sorafenib	NR
7	43	1	60	95	92	0.384	-7	CRp	28	86	47	0.388/0. 265	Dip	Plerixafor + Sorafenib	NR
8	23	4	74	92	86	ND	ND	NR	67	96	88	ND	Com	Phase 1 Protocol	NR
9*	21	2	3	13	55	ND	-7q	NR	1.8	0	23	0.231/0. 384	-7q	SCT	Relapsed within 2 months
10	67	2	60	58	66	0.26	Dip	BMBR	13.9	83	55	0.023/0. 33	Dip	Plerixafor + Sorafenib. FA	NR
11	38	4	7.8	90	90	ND	Com	NR	43	91	80	ND	Com	Decitabine + GO	NR
12*	33	3	0.2	0	25	0.065	Ins	BMBR	23	67	74	0.124/0. 134	Misc	SCT	Relapsed within 3 months
13	52	2	19.2	89	93	ND	Misc	CRp	105	55	80	ND	Misc	Phase 1 Protocol	NR
14	75	2	65.2	1	40	ND	Dip	CR	37.6	6	32	0.856/0.393	Dip	Plerixafor + Sorafenib	NR
15	64	2	1.6	6	20	ND	Dip	NR	2.6	80	78	Ratio 0.01/0.159	ND	ND	NR

WBC: White Blood Cells, PBB: Peripheral Blood Blasts, BMB: Bone Marrow Blasts, CR: Complete Remission, CRp: Complete Remission with incomplete platelet recovery, NR: No Response, BMBR: Bone Marrow Blast Reduction, ND: Not Done, Dip: Diploid, Misc: Miscellaneous, Insu: Insufficient, Com: Complex, I: Idarubicin, F: Fludarabine, A: Ara C, GO: gemtuzumab Ozogamicin, SCT: Stem Cell Transplant.

*
These 2 patients had documented combined ITD/D835 mutation at the time of disease progression after transplantation.