#2468 Exome Sequencing Informs Mechanisms of Clinical Resistance to the FLT3 Inhibitor Crenolanib

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Abstract

Introduction: Variation of the D835 residue of FLT3 occurs in ~5% of *de novo* AML cases and is a prominent feature of drug resistance in the setting of FLT3-ITD. Crenolanib treatment of AML patients exhibiting FLT3 D835 and/or FLT3-ITD positive tumors has yielded significant clinical responses. However, these clinical responses to single-agent crenolanib were transient, so we performed whole exome sequencing to determine the mechanisms of crenolanib resistance. Mutation of the FLT3 F691 "gatekeeper" residue is a known resistance mechanism to crenolanib and other FLT3 inhibitors *in vitro*. Hence, secondary mutation of FLT3 at the gatekeeper or other sites was hypothesized to be a predominant mechanism of resistance. We report that a minority of patients acquired FLT3 gatekeeper or other secondary FLT3 mutations, however, the majority of cases relapsed in the absence of additional FLT3 mutations indicating alternative mechanisms of drug resistance.

Methods: Genomic DNA was isolated from bone marrow aspirates from 42 AML patients treated with crenolanib. We performed whole exome sequencing using Illumina Nextera exome capture and paired-end sequencing on an Illumina 2500 HiSeq. Paired specimens (pre/post-treatment) were available for 19 of these patients, allowing for analysis of acquired mutations in the context of crenolanib therapy.

Results: Although secondary mutation of FLT3 at the gatekeeper residue, FLT3 F691, was predicted to be a prominent mechanism of resistance, we did not observe FLT3 gatekeeper mutations in most patients. In total, only 3/42 (7%) of cases exhibited a FLT3 gatekeeper mutation despite good coverage of the FLT3 locus (average of 134 fold read depth; range of 77-177 fold average read depth across all 42 cases). In addition, we observed secondary FLT3 mutations at alternative residues, D200N, K429E, and L601F, in 3 other patients. Hence, only 6/42 (14%) of patients exhibited evidence of relapse due to secondary mutation of FLT3, and the majority of patients (38/42; 86%) presented with crenolanib resistance in the absence of secondary FLT3 mutations. Exome analysis revealed that many of these cases acquired mutations involving transcriptional regulators, suggesting alternate pathways of escape as the predominant mechanism of resistance to crenolanib therapy.

Conclusions: Crenolanib is broadly effective against FLT3-ITD and FLT3-D835 mutant AML. Despite predictions of FLT3 gatekeeper mutations (or other secondary FLT3 mutations) as a primary mechanism of resistance, we only observed these events in a minority of patients. Instead, we observed a prominent signal of acquired mutations in transcriptional regulators, suggesting a more elaborate genetic/epi-genetic mechanism of resistance to crenolanib.

Introduction

- Crenolanib is a potent, type I inhibitor of FLT3 kinase (1) and binds to both active and inactive conformation of FLT3 (X-ray crystallography data on file)
- Previous studies showed that crenolanib was effective against drug resistance-conferring kinase domain mutations (2).
- Saturation mutagenesis experiments in the presence of crenolanib had been unable to select kinase domain mutants as a mechanism of resistance to crenolanib (1)
- Gate keeper mutation F691L and another mutation, D698N, were the only two recurring mutant clones out of $300x10^6$ clones screened in the presence of low dose crenolanib (100 nM) (1)
- Type II FLT3 inhibitors, including quizartinib, sorafenib, ponatinib and pexidartinib (PLX3397), have been studied in FLT3 positive AML patients
- These type II inhibitors, binding only to the inactive conformation of FLT3, are intrinsically resistant to activating mutations in the kinase domain (TKD) (Figure 1)
- Acquisition of activating mutations in the kinase domain (D835) are commonly seen after treatment with these type II FLT3 inhibitors (3-4)
- In this study, whole exome sequencing as well as ultra-deep sequencing of FLT3 exon 17 and 20 were conducted to identify potential mechanism of resistance to crenolanib

FLT 3 Mutation	Crenolanib IC_{50} (nM)
ITD	9.1 ± 3.8
D835V	2.9 ± 0.5
D835Y	5.3 ± 2.7
ITD + F691L	55.4 ± 14.4
ITD + D835V	13.1 ± 4.8
ITD + D835Y	13.9 ± 4.3
ITD + D835H	12.3 ± 2.5
ITD + D835F	16.5 ± 0.4

Table 1. IC_{50} of crenolanib on different FLT3 mutations (1).

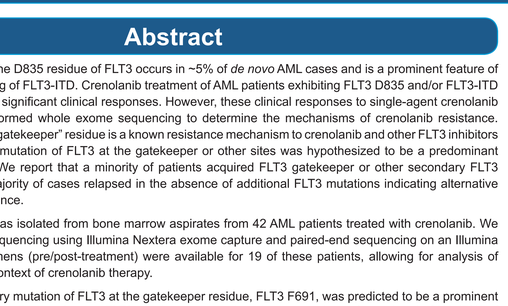

Figure 1. Relative resistance of different FLT3 inhibitors. The values are fold-difference in IC_{50} relative to ITD alone (Figure adapted from *Smith et. al. (2015) Leukemia. doi:10.1038 (2).*

Objectives

- To determine if resistance to crenolanib is due to acquisition of secondary FLT3 mutations
- To determine additional somatic mutations in the exome that may be acquired at the time of crenolanib resistance

Methods

Patient population: Samples in this study were collected from relapsed/refractory AML patients who received crenolanib as monotherapy. The sampled population included patients who have had durable responses to crenolanib, as well as those who have had transient responses to crenolanib.

Whole Exome Sequencing: To determine the potential mechanism of crenolanib resistance, the bone marrow and/or peripheral blood samples from 42 patients who had progressed after treatment with crenolanib were assessed by whole exome sequencing. We performed whole exome sequencing using Illumina Next era exome capture and paired-end sequencing on an Illumina 2500 HiSeq. Paired specimens (before/after resistance) were available for 19 of these patients, allowing for analysis of acquired mutations in the context of crenolanib therapy. For the other 17 patients, samples at the time of relapse were available.

Ultra-deep Sequencing: Exon 17 and exon 20 of FLT3 were also sequenced in 20 patient samples to an average of 224,693 reads by MiSeq Next-Generation Sequencing (Illumina) (3). Samples from 17 patients were paired samples before and after crenolanib treatment.

Ultra-deep Sequencing of FLT3 TKD

No New D835 Mutations Arise After Crenolanib Treatment

- Exon 20 of FLT3 was sequenced in paired samples from six (6) patients who had FLT3 ITD with no TKD mutations at the time of starting crenolanib
- With an average of 203,964 reads, there is no evidence of acquisition or increase in allelic burden in any of the D835Y/H/R/V mutations at the time of relapse

Patient	Cycle/Day	Baseline FLT3	D835Y GAT > T	D835H GAT >C	D835R GAT > CG	D835V GAT > T	Coverage D835Y/H/R	Coverage D835V
1879	C1D1	ITD	11	2	0	19	268886	270143
	C1D14		34	20	0	28	177948	177610
	C2D1		23	15	0	37	191771	191465
1901	C1D1	ITD	32	10	0	28	197143	196055
	C3D1		41	12	0	19	185162	183918
1914	baseline	ITD	16	6	0	17	144746	143752
	C2D1		37	11	0	34	162105	160951
	C3D1		26	9	0	26	193904	192782
	C5D1		29	7	0	29	165747	164710
	C6D1		33	23	0	35	178953	178452
	C8D1		13	16	0	27	160944	160309
	C9D1		45	10	1	33	136920	136600
1497	C1D1	ITD	8	2	0	11	201548	202325
	C4D1		120	14	0	39	185818	185818
1499	C1D1	ITD	9	2	0	14	288254	289771
	C2D1		156	442 (0.14%)	1	76	326270	326270
	C3D1		199	45	1	72	272778	272778
1549	C1D1	ITD	5	2	0	7	203971	204649
	C5D1		156	37	0	68	234699	234699

Table 2. D835 mutation status of patients with FLT3-ITD at baseline (data are read counts at each loci).

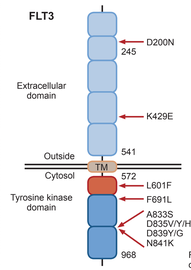

Figure 2. Domain organization of FLT3 showing position of all mutations described in this study.

FLT3 Gatekeeper Mutation F691L Was Infrequent in Crenolanib-treated AML Patients

- Gatekeeper mutation F691L of FLT3 is known to be insensitive to crenolanib, based on pre-clinical assay (Table 1)
- To determine if F691L is a resistance mechanism of crenolanib treatment, the exon 17 of FLT3 was sequenced in samples from 20 AML patients who received one (1) to four (4) months of crenolanib
- Only one (1) out of 20 (5%) of crenolanib-treated AML patients developed gatekeeper mutation F691L at the time of relapse
- None of the other patient had any apparent increase in the F691L clone

Patient	Cycle/Day	Baseline FLT3	F691L TTT > C	F691L TTT > G	Coverage TTT >C	Coverage TTT >G
1879	C1D1	ITD	9	2	180345	180129
	C1D14		23	19	171803	171653
	C2D1		29	22	202623	202551
1550	C1D1	ITD+D835	11	4	314202	314192
	C2D1		37	21	197222	197183
	C3D1		25	17	177890	177832
	C5D1		35	23	228619	228493
1885	C1D1	D835	21	13	201611	201574
	C2D1		18	17	198619	198574
1887	C1D1	D835	17	15	180745	180699
	C2D1		20	18	172999	172937
	C3D1		23	20	184047	184024
	C5D1		17	13	176002	175969
	follow-up		19	11	184083	184022
1901	C1D1	ITD	31	18	163187	163148
	C3D1		19	16	159558	159532
1908	C1D1	ITD+D835	8	2	206330	206228
	C2D1		11	6	81091	81077
	C3D1		14	8	167835	167774
	C5D1		19	15	189878	189794
1911	C1D1	ITD+D835	23	22	159186	159134
	C2D1		26	16	160907	160861
1914	baseline	ITD	23	18	153825	153829
	C2D1		13	14	153144	153094
	C3D1		15	16	149792	149732
	C5D1		19	11	142185	142162
	C6D1		19	16	171383	171335
	C8D1		24	17	190831	190821
	C9D1		22	18	178269	178221
1551	C2D1	ITD+D835	32	15	185383	185361
	C3D1		19	14	170146	170109
	C4D1		27	18	194165	194104
1496	C1D1	D835	12	3	264062	264013
	C2D1		43	35	286414	286483
	C3D1		119	3156 (1.1%)	291153	290912
	C5D1		37	22	310249	309862
1497	C1D1	ITD	10	2	168555	168498
	C2D1		39	16	271716	271349
	C4D1		29	27	320876	320189
1498	C1D1	D835	11	3	227084	227049
	C2D1		60	30	389541	389128
1499	C1D1	ITD	14	3	285602	285576
	C2D1		49	14	332312	332030
	C3D1		50	36	313583	312484
1500	C1D1	ITD+D835	9	5	277343	277294
	C2D1		2398 (1.6%)	86946 (57%)	149756	151969
1501	C1D1	ITD+D835	13	7	202386	202332
	C2D1		31	17	248251	248451
	C3D1		32	19	212494	212520
1502	C2D1	ITD+D835	37	105	311675	311630
	C3D1		30	90	268368	267768
1503	baseline	ITD+D835	34	14	273017	273002
	C2D1		49	40	279834	279662
1504	baseline	ITD+D835	56	69	213212	213045
	C2D1		43	45	259562	259518
1548	C3D1	ITD	42	30	297581	297620
1549	C1D1	ITD	8	2	248287	248247
	C5D1		49	26	253419	253455

Table 3. F691 mutation status of all patients (data are read counts at each loci).

Crenolanib Has Activity Against FLT3 A833S, D839Y/G and N841K A-loop Mutations

- Low allelic burdens of A833S, D839Y/G and N841K were detected at baseline in four (4) patients, and these clones were eliminated during the course of crenolanib treatment

Patient	Cycle/Day	A833S GCT > T	D839Y GAT > T	D839G GAT > G	N841K AAC > A	Coverage A833S	Coverage D839Y	Coverage D839G	Coverage N841K
1550	C1D1	7	483 (0.29%)	435 (0.27%)	6	161435	165034	161689	160984
	C1D1	9	36	33	8	140308	141173	140007	136938
	C2D1	10	13	12	7	166423	167185	165389	162533
	C3D1	10	8	11	5	141608	143459	140103	138620
	C5D1	8	11	9	15	477283	477803	476402	474245
1908	C1D1	4	9	13	311 (0.44%)	76676	77140	74859	71166
	C2D1	8	11	15	7	158847	158848	155074	153738
	C3D1	9	14	10	9	174660	174130	171036	169603
	C5D1	7	12	7	6	175004	174990	172601	170114
1497	C1D1	8	1945 (0.96%)	7	7	201582	203442	199954	197003
	C4D1	4	31	12	6	185833	187380	182863	180028
1498	C1D1	5949 (3.33%)	19	6	5	178598	178153	174655	171101
	C2D1	11	27	15	12	279026	280699	275706	274450

Table 4. A833, D839, N841 mutation detected in 4 patients (data are read counts at each loci).

Full Exome Sequencing

Detected Three Novel Mutations of FLT3

- Three (3) novel mutations were identified, and two (2) of them were present at the time of treatment initiation
- The importance of L601F, K429E and D200N (see position in figure 2) is currently under investigation

Patient	Cycle/Day	Additional FLT3 Mutation	VAF	Coverage	BM blast count
1888	C1D1	L601F	5 (8.5%)	89	83%
	C2D1	L601F	6 (9.5%)	63	68%
1897	C1D27	K429E	95 (74%)	129	91%
1907	C1D1	D200N	26 (23%)	113	10%
	C1D28	D200N	8 (14%)	58	7%

BM: bone marrow, VAF: Variant allele frequency
Table 5: Additional FLT3 mutations were detected by exome sequencing in AML patients treated with crenolanib.

F691L Was Found in 7% of Patients

- Consistent with the ultra-deep sequencing results, three (3) out 42 (7%) patients developed a F691L mutation during crenolanib treatment
- Among the three (3) patients with F691L mutation, only two (2) had a significant allelic burden

Patient	Cycle/Day	FLT3 mutation	VAF	Coverage	BM blast count
1904	C1D28	F691L	17 (34%)	50	73%
1551	C2D1	F691L	0	48	10%
	C3D1		2 (2.2%)	92	4%
1500	C2D1	F691L	9 (45%)	20	78%

BM: bone marrow, VAF: Variant allele frequency;
Table 6: FLT3 F691L was detected by exome sequencing in AML patients treated with crenolanib.

Additional Gene Mutations May Contribute to Crenolanib Resistance

- Overall mutational spectrum identified with whole exome sequencing data from 29 resistance samples showed higher frequency in WT1, NRAS, SF3B1, STAG2, ASXL1, IDH2, TET1 and CCND3 compared to mutation frequency seen in AML patient samples with FLT3 ITD or D835 mutations from TCGA database (n=54) (Table 7).
- VAF changes (subtraction) before and after crenolanib treatment from 19 paired cases were calculated (Table 8), and NRAS, ASXL1, and STAG2 showed higher VAF after crenolanib resistance (Figure 3).

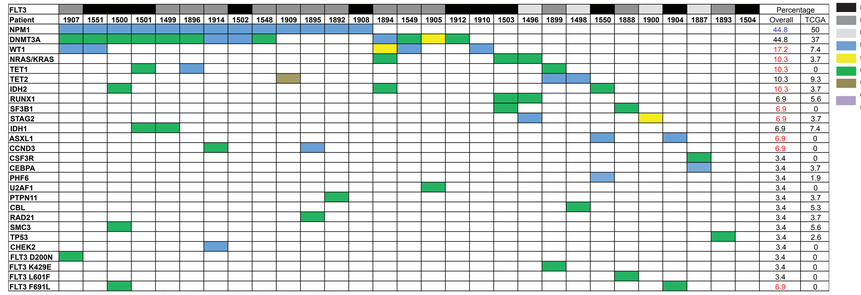

Table 7. Overall mutational spectrum of all patients at their latest sampling time point.

FLT3	1907	1551	1499	1501	1914	1909	1892	1908	1549	1905	1912	1910	1496	1503	1887	1888	1900	1550	1504	Overall
NPM1	0.22	0.09	0.02	0.06	-0.15	-0.1	-0.05	-0.12	0	0	0	0	0	0.19	0	0	0	0	0	1
DNMT3A	0.19	0.44	-0.02	-0.06	-0.23	0	0	0	0.01	0.13	0.13	0	0	0	0	0	0	0	0	1
WT1	0.22	0.05	0	0	0	0	0	0	0.12	0	0	0.07	0	0	0	0	0	0	0	4
NRAS	0	0	0	0	0	0	0	0	0	0	0	0	0.31	0.04	0	0	0	0	0	2
RUNX1	0	0	0	0	0	0	0	0	0	0	0	0	0.12	0.06	0	0	0	0	0	2
SF3B1	0	0	0	0	0	0	0	0	0	0	0	0	0	-0.08	0	0.08	0	0	0	0
STAG2	0	0	0	0	0	0	0	0	0	0	0	0	0.65	0	0	0.14	0	0	0	2
IDH1	0	0	0.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
CSF3R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-0.19	0	0	0	0	-1
BCORL1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.19	0	0	0	1
CEBPA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	1
IDH2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.13	0	1
PHF6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.28	0	1
TET2	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	1
U2AF1	0	0	0	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0	1
ASXL1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.64	0	1
PTPN11	0	0	0	0	0	0	0.11	0	0	0	0	0	0	0	0	0	0	0	0	1
FLT3 D200N	-0.09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-1
FLT3 L601F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.04	0	0	0	0	1
FLT3 F691L	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-0.07	0	0	-1

Table 8. VAF changes (subtraction) before and after crenolanib resistance from 19 paired cases were calculated.

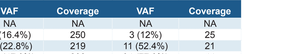

Figure 3. Plot of allele burden of genes before crenolanib treatment against after crenolanib treatment.

Clonal Evolution Patterns Observed in the Relapsed / Refractory AML Patients

FLT3 ITD + D835 clone was reduced, but a drug resistant subclone emerged during treatment

- Patient 1550 responded to crenolanib and achieved CRi after one cycle of crenolanib (28 days).
- At remission, low level of IDH2 and PHF6 mutant were detected, suggesting the presence of a potentially drug resistant subclone.
- At relapse, patient acquired ASXL1, and a clone with ASXL, IDH2 and PHF6 mutations expanded and became the dominant clone.

Patient	Cycle/Day	WBC (k/ul)	Peripheral Blast (%)	BM Blast (%)	ITD and TKD Status	ITD Allelic burden (%)	D835 Allelic Burden (%)	IDH2		ASXL1		PHF6	
								VAF	Coverage	VAF	Coverage	VAF	Coverage
1550	C1D1	2.3	16	92	ITD + D835	5.6	6.39	NA	NA	NA	NA	NA	NA
	C2D1	0.6	0	2	ITD + D835	0	0	13 (3%)	377	0	11	15 (13%)	116
	C3D1	0.9	0	3	ITD + D835	0	0	7 (2%)	345	0	7	11 (9.8%)	112
	C5D27	1.2	0	26	ITD + D835	0	0	47 (16.7%)	281	7 (63.6%)	11	45 (41%)	109

WBC: White blood cells; BM: Bone marrow; VAF: Variant allele frequency; NA: Not available
Table 9. Blood count, blast count and mutation status of a patient with a drug resistant subclone.

The founder clone recurred through uncharacterized mechanisms

- Patient 1914 responded to crenolanib and achieved CR after one cycle of crenolanib (28 days).
- During the time of complete remission, substantial allelic burdens of FLT3-ITD, NPM1, DNMT3A, CCND3 and CHEK2 mutation were detected, implying the likely presence of a drug resistant subclone.
- Patient received an allogeneic stem cell transplantation after cycle 3 and received crenolanib maintenance therapy 34 days after the transplant. The aberrant clone was completely eradicated, as indicated by the absence of FLT3-ITD as well as NPM1, DNMT3A and CCND3 mutations during cycle 5 and 6.
- Four months after transplant, patient relapsed due to minimal residual disease, with mutation profile identical to the founder clone, FLT3-ITD and mutations in NPM1, DNMT3A and CCND3 and CHEK2.

Patient	Cycle/Day	WBC (k/ul)	Peripheral Blast (%)	BM Blast (%)	ITD and TKD Status	ITD Allelic Burden (%)	D835 Allelic Burden (%)	NPM1		DNMT3A		CCND3		CHEK2	
								VAF	Coverage	VAF	Coverage	VAF	Coverage	VAF	Coverage
1914	Screening	1.1	30	80	ITD	80	0	NA	NA	NA	NA	NA	NA	NA	NA
	C2D1	22.8	0	1	ITD	80	0	NA	NA	NA	NA	NA	NA	NA	NA
	C3D1	7.2	0	3	ITD	NA	0	75 (56%)	134	44 (37.6%)	117	49 (36%)	136	49 (37.4%)	131
	C5D1	1.2	0	1	ITD	0	0	0	138	0	109	0	128	4 (3.6%)	111
	C6D1	3.7	0	NA	ITD	0	0	0	186	0	179	0	160	0	159
	C8D1	4	0	12	ITD	0	0	10 (5.7%)	176	2 (1.4%)	147	12 (7.3%)	165	7 (5%)	140
	Follow-up	27.4	60	NA	ITD	30	0	27 (32.9%)	82	18 (22.8%)	79	48 (41.7%)	115	33 (31.7%)	104

WBC: White blood cells; BM: Bone marrow; VAF: Variant allele frequency; NA: Not available
Table 10. Blood count, blast count and mutation status of a patient with a recurred founder clone.

FLT3 D835 clone was reduced, but a drug resistant subclone expanded during treatment

- Patient 1496 had hematological improvement after 4.5 months of crenolanib treatment.
- Crenolanib was efficient in eliminating the D835 mutant clone, as evidenced by the substantial reduction of the D835 mutant allelic burden.
- However, there was a significant increase in the allelic burden of RUNX1, NRAS and STAG2 during the course of treatment. This expanding subclone was likely to be a FLT3 independent mutant clone, and therefore resistant to crenolanib.

Patient	Cycle/Day	WBC (k/ul)	Peripheral Blast (%)	BM Blast (%)	ITD and TKD status	D835 Allelic Burden (%)	RUNX1		NRAS		STAG2	
							VAF	Coverage	VAF	Coverage	VAF	Coverage
1496	Screening	2.4	35	47	D835	20	NA	NA	NA	NA	NA	NA
	C2D1	0.9	25	24	D835	9	129 (58.9%)	219	41(16.4%)	250	3 (12%)	25
	C3D1	1.0	21	26	D835	4	113 (62.4%)	184	50 (22.8%)	219	11 (52.4%)	21
	C5D1	2.0	29	74	D835	2	170 (70.8%)	240	135 (47.9%)	282	34 (77.3%)	44

WBC: White blood cells; BM: Bone marrow; VAF: Variant allele frequency; NA: Not available
Table 11. Blood count, blast count and mutation status of a patient with a drug resistant subclone.

Discussions

- Crenolanib is broadly effective against FLT3-ITD and FLT3-D835 mutant AML. No secondary D835 mutations were identified
- Despite predictions of FLT3 gatekeeper mutations (or other secondary FLT3 mutations) as a primary mechanism of resistance, we observed these events only in a minority (5 to 7%) of patients
- Instead, we observed a prominent signal of mutations in spliceosome (SF3B1), chromatin modifiers (ASXL1), cohesion complex (STAG2) and RAS pathway, suggesting a more elaborate genetic/epi-genetic mechanism of resistance to crenolanib

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