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The *EBF1-PDGFRB* T681I mutation is highly resistant to imatinib and dasatinib *in vitro* and detectable in clinical samples prior to treatment

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Running title: TKI-resistant mutations in *EBF1-PDGFRB* Ph-like ALL

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Contributions

THT and MLL designed the study. JN and THT performed the experiments and analyzed the data. AS performed comparative protein structure modeling of PDGFRB. JA, JN and THT performed the drug screens. AVM, YD and MD provided the clinical data. THT and MLL wrote the manuscript. All authors reviewed the manuscripts.

EBF1-PDGFRB accounts for 3% of children with Philadelphia chromosome-like acute lymphoblastic leukemia (Ph-like ALL),[1] represents the most common fusion gene in Ph-like ABL-class subtype,[2] and is notoriously associated with high rates of induction failure.[1-3] *EBF1-PDGFRB* fusions exhibit exquisite sensitivity to ABL tyrosine kinase inhibitors (TKI) in preclinical models,[3] and durable remissions have been reported in patients harboring *EBF1-PDGFRB* when treated with either imatinib or dasatinib.[4] Collectively, these observations provide compelling rationale to investigate the incorporation of ABL TKI in combination with conventional chemotherapy for Ph-like ABL-class ALL patients in clinical trials. However, the emergence of kinase domain (KD) mutations as the primary mechanism of acquired resistance to TKI has been well described and occurs in many adults with relapsed/refractory Philadelphia chromosome-driven leukemias.[5] Mechanisms of TKI resistance in Ph-like ABL-class ALL have not been extensively studied, although we hypothesize that similar resistance mechanisms may occur between the two subsets. Hence, we sought to characterize the spectrum of TKI-resistant KD mutations in *EBF1-PDGFRB* Ph-like ALL as a mechanism of acquired resistance by using a validated *in vitro* saturation mutagenesis screen, as previously described.[6]

Among 245 imatinib-resistant and 416 dasatinib-resistant colonies isolated from our *in vitro* screens, 233 (95%) and 363 (87%) colonies harbored a single KD mutation respectively. The predominant recurrent single KD mutation was the gatekeeper T681I point mutation for both imatinib (n=233/245, 95%) and dasatinib (n=338/416, 81%). The next most common recurrent KD mutation was the N666S (n=18/416, 4%), which conferred resistance to dasatinib only. The mutation T681I in *EBF1-PDGFRB* is analogous to the gatekeeper mutation T315I in *BCR-ABL1*, while the mutation N666S is analogous to the N676S mutation in *FLT3-ITD*.[7] The full spectrum of KD mutations in *EBF1-PDGFRB* to imatinib and dasatinib identified from the *in vitro* saturation mutagenesis screens is reported in **Table S1**.

We then focused on the two most common KD mutations to assess their proliferative properties and characterize their biochemical resistance to the relevant TKIs. Introduction of *EBF1-PDGFRB* T681I and N666S mutant isoforms into Ba/F3 cells rendered them IL-3 independent, illustrating that the transforming capacity of the *EBF1-PDGFRB* fusion gene is preserved in the presence of these mutations. In viability assays, the T681I was highly resistant to imatinib and dasatinib, while the N666S showed intermediate resistance to dasatinib. The IC50s for wild-type *EBF1-PDGFRB* were 15.74 nM, 5.26 nM and 5.73 nM to imatinib, dasatinib and ponatinib respectively. The IC50s for the *EBF1-PDGFRB* T681I mutant isoform were 602.5 nM and 23.93 nM to imatinib and ponatinib respectively, while the IC50 was not reached with the highest concentration of dasatinib used. Moreover, phosphorylation of STAT5 was not abrogated by dasatinib in Ba/F3 constructs harboring the T681I *EBF1-PDGFRB* compared to wild-type *EBF1-PDGFRB* (**Figure 1**).

To understand the molecular mechanism of TKI resistance from KD mutations, we modeled the wild-type and mutant structures of PDGFRB in relationship with the relevant TKI. Co-crystal structure analysis of the T681I mutation demonstrated that substitution from a threonine to the bulkier hydrophobic isoleucine at the gatekeeper position leads to a steric incompatibility between the ligand and the pocket, thus preventing dasatinib from binding both the active and inactive kinase conformations. As for the N666S substitution, the model of PDGFRB N666S demonstrated that the mutation likely disrupts a network of stabilizing hydrogen bonds, which might have long-range effects on the conformation of the ATP binding pocket (**Figure S1**).

We then hypothesized that KD mutations might be present at very low levels at diagnosis in patients with *EBF1-PDGFRB* when assessed by more sensitive technologies and emerge as the dominant clone at relapse under the selective pressure of therapy as suggested by a few adult studies.[8,9] We designed a droplet digital polymerase chain reaction (ddPCR) assay to identify the T681I mutation in diagnostic patient samples prior to any TKI exposure. Among the 23 diagnostic *EBF1-PDGFRB* patient samples we analyzed, the gatekeeper T681I mutation was identified in 13% (n=3/23) by our ddPCR assay (**Figure 2**). This cohort comprised 13 patients enrolled on the Children's Oncology

Group ALL trials (AALL0232: n=1, AALL1131: n=12) and 10 patients on United Kingdom ALL trials (UK ALL 97/99: n=3, UK ALL 2003: n=7) (**Table 1**). The median age of the entire cohort was 12 years [8-16], and the median white blood cell count at diagnosis was 39.0 [17-80.7] x 10^9 cells/L. The median duration of follow-up was 60 [14-81] months. None were treated with TKI. Baseline characteristics, leukemia response and clinical outcomes among the 3 *EBF1-PDGFRB* patients with subclonal T681I mutation detected by ddPCR at diagnosis were not significantly different compared to the 20 patients without subclonal T681I mutation, although there was a trend towards a higher likelihood of relapse in the T681I-positive group versus T681I-negative group (100% vs. 35%; p=0.0678) (**Table S2**).

To the best of our knowledge, our study is the first to report that KD mutations represent a potential mechanism of acquired resistance in children with *EBF1-PDGFRB* Ph-like ALL. The gatekeeper T681 was the predominant KD mutation in our *in vitro* screens that was resistant to both imatinib and dasatinib, but could be rescued by ponatinib as predicted. The paucity of KD mutations in *EBF1-PGDFRB* recovered in the dasatinib mutational screen was similar to other *BCR-ABL1* mutational screens, since dasatinib is active against most of imatinib-resistant KD mutations.[10] However, to our surprise, the gatekeeper mutation was the only KD mutation in *EBF1-PDGFRB* retrieved in the imatinib mutational screen, while over 90 imatinib-resistant KD mutations have been reported with *BCR-ABL1*.[11] This finding could be explained by a higher dose that was used in our screen compared to previous reports, but it is also known that imatinib is much more potent in *PDGFR* family fusions compared to the *BCR-ABL1* fusion. The IC50 of imatinib to *EBF1-PDGFRB* in our hands was 15.74nM, while Cools *et al*. reported that IC50 of imatinib to cells expressing *FIP1L1-PDGFRA* was 3.2nM, whereas the IC50 for *BCR-ABL1* was 582 nM.[12] Thus, mutations that impart a modest degree of imatinib resistance may not have been detected by our screens.

The analogous N666S mutation has not been previously reported in *BCR-ABL1 in vitro* screens with either imatinib or dasatinib. However, the residue N666 in *EBF1-PDGFRB* is adjacent to its analogous residue V299 in *BCR-ABL1*, which represents the

third most common contact residue where KD mutations to dasatinib arise, after both T315 and F317 amino acid residues respectively.[10] Smith *et al.* identified the N676S mutation in *FLT3-ITD* in their *in vitro* mutagenesis screen with the FLT3 inhibitor PLX3997, but only N676K/T mutations rather than N676S were isolated from adult acute myeloid leukemia patients with acquired clinical resistance to PLX3997.[7] Moreover, *FLT3* N676K mutations have been identified in core-binding factor leukemia at diagnosis and may represent a cooperating mutation in leukemogenesis. The *FLT3* N676K mutant alone can induce cytokine-independent growth in Ba/F3 cells and confer resistance to FLT3 inhibitors.[13]

In contrast to the report by *Zhang et al.*,[14] our *EBF1-PDGFRB in vitro* saturation mutagenesis screen did not identify the C843G KD mutation that was seen in *AGGF1-PDGFRB* Ph-like ALL. In their experiments, the reported IC50 of *AGGF1-PDGFRB* C843G and *EBF1-PDGFRB* C843G to dasatinib was 0.78 nM and 0.121 nM, respectively. Thus, we may not recover this mutant in our screens even at 25nM of dasatinib, the lowest dasatinib concentration used in our screen, which is more than 200-fold above the measured IC50 of *EBF1-PDGFRB* C843G.

The detection of drug-resistant KD mutations at diagnosis has been reported in 21% to 40 % of TKI-naïve chronic myelogenous leukemia with advanced disease and Ph^+ ALL samples.[8,15] The frequency of T315I mutation at diagnosis ranges from 12.5% to 17%,[15] which is in keeping with the analogous gatekeeper T681I mutation frequency in our *EBF1-PDGFRB* patients' cohort. Nevertheless, the clinical and prognostic significance of pre-existing KD mutation detected by sensitive technologies prior to TKI remains unclear. Willis *et al.* showed that mutation detection at low levels by allele-specific oligonucleotide polymerase chain reaction does not invariably predict relapse, or negatively impact cytogenetic response or event-free survival.[15] Patients with subclonal T681I mutation detected by ddPCR at diagnosis had a trend towards increased risk of relapse compared to the T681I-negative subgroup; however, these analyses were hindered by small patient numbers and should be validated in larger cohorts of uniformly-treated patients. Furthermore, confirmation of the T681I mutation in relapsed

samples would be essential in future studies to validate that relapse was driven by the clonal expansion of drug-resistant mutations under the selective pressure of TKI therapy. However, none of our 23 patients were treated with TKI and relapsed samples post-TKI treatment were not available for testing.

In conclusion, KD point mutations represent a potential mechanism of acquired resistance in *EBF1-PDGFRB* Ph-like ALL. The T681I gatekeeper KD mutation was the most common resistant KD mutation in *EBF1-PDGFRB* Ph-like ALL to both imatinib and dasatinib, and could be identified in clinical samples at diagnosis by ddPCR. Validation of our *in vitro* saturation mutagenesis screens would be important in future clinical trials of Ph-like ALL and concerted efforts should focus on exploring novel therapies targeting the T681I KD mutation.

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Table 1. Clinical characteristics and outcomes of the 23 *EBF1-PDGFRB* patients with or without subclonal T681I mutation at diagnosis by ddPCR

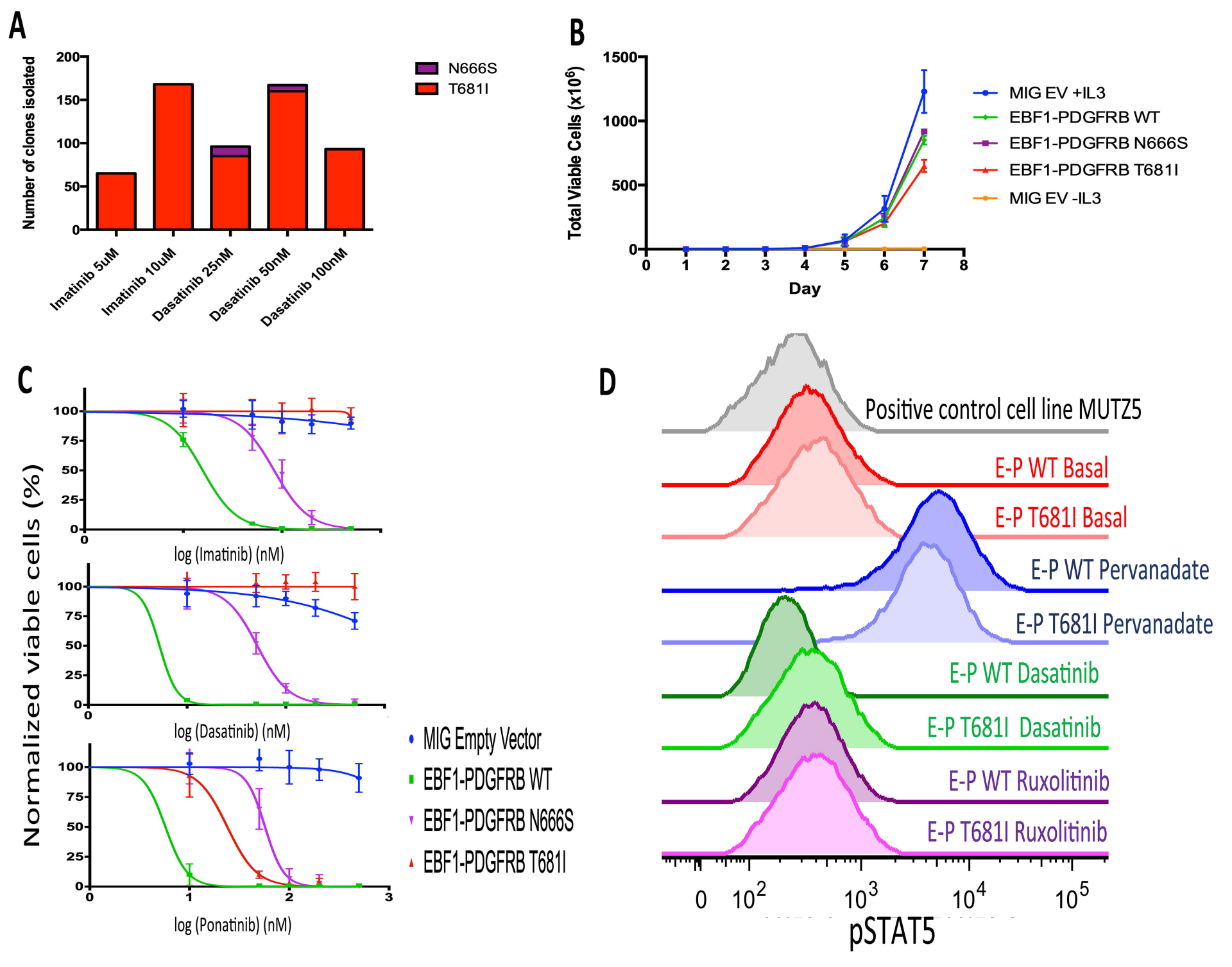
ID#	Age at diagnosis	WBC at diagnosis	BM blasts (%)	CR	EOI MRD (%)	Relapse	Months to relapse	HSCT	Status
1	3	18.4	95	Yes	>1	No		Yes	Alive (11.1 years)
2	12	114.3	98	IF	>1	BM	12	Yes	Died of disease (1.2 years)
3	14	419.8	92	IF	>1	No		Yes	Died in remission (1.2 years
4	7	79.9	85	IF	>1	No		Yes	Died in remission (1.5 years
5	17	396	69	Yes	0.1-0.99	CNS	27	No	Alive (7.4 years)
6*	17	13.4	96	Yes	>1	BM	28	Yes	Alive (6.8 years)
7	12	32.5	89	Yes	>1	BM	32	Yes	Alive (6.8 years)
8	19	54.8	97	IF	>1	No		Yes	Alive (6.2 years)
9	14	41.7	90	Unknown	>1	No		Yes	Alive (6.0 years)
10	11	28.2	85	IF	>1	No		Yes	Alive (5.2 years)
11	6	80.7	91	IF	>1	No		Yes	Alive (5.6 years)
12	14	3.3	90	Unknown	Unknown	No		No	Induction death (19 days)
13	9	39	74	IF	>1	No		Yes	Alive (5.0 years)
14	6	212	98	Yes	Unknown	BM/CNS	31	No	Died of disease (5.0 years)
15	12	17	68	Yes	Unknown	No		No	Alive (7.6 years)
16*	12	5	Unknown	Yes	Unknown	BM	39	No	Alive (7.4 years)
17	4	49	95	Yes	>0.1	No		No	Alive (7.5 years)
18	19	8	99	Yes	>10	CNS	40	No	Alive (6.8 years)
19	18	3	Unknown	Yes	>10	No		Yes	Alive (5.2 years)
20*	14	26	94	Yes	>10	BM	18	No	Died of disease (1.8 years)
21	8	34	99	Yes	>10	BM	50	No	Alive (3.8 years)
22	16	68	95	Unknown	>0.01	No		No	Died in remission (4 months
23	16	102	72	IF	>10	No		No	Died of disease (3 months)

ddPCR: droplet digital polymerase chain reaction; ID#: Identification number; WBC: white blood cell, BM: bone marrow; CR: complete remission defined as M1 marrow or <5% blasts on microscopic assessment; EOI MRD: end of induction minimal residual disease; HSCT: hematopoietic stem cell transplantation; IF: induction failure; CNS: central nervous system, *patients with subclonal T681I mutation

Legend to figures

Figure 1. *In vitro* mutational screen of *EBF1-PDGFRB* (E-P) reveals kinase domain mutations causing varying degrees of resistance to imatinib and dasatinib. A. The proportion of T681I and N666S KD mutations identified in *EBF1-PDGFRB in vitro* screens to different concentrations of imatinib and dasatinib. **B**. Proliferation assays demonstrating the cytokine-independent proliferation of wild-type and mutant *EBF1-PDGFRB* Ba/F3 cells. **C.** Drug-sensitivity profiles of Ba/F3 cells harboring wild-type (WT) and mutant *EBF1-PDGFRB* to imatinib, dasatinib and ponatinib. **D**. Phosphorylation of STAT5 is elevated at basal in Ba/F3 cells harboring *EBF1-PDGRB* and can be inhibited in wild-type but not mutant *EBF1-PDGRB* to dasatinib.

Figure 2. Droplet digital PCR can detect subclonal T681I mutation in clinical samples at diagnosis. Droplet digital PCR (ddPCR) experiments including positive T681I, wild-type and no template controls on the left panel. On the right panel, three *EBF1-PDGFRB* patients were found to have subclonal T681I mutation at diagnosis by ddPCR. Patient #1: 4 droplets containing the mutant T681I over 20 879 total generated droplets (0.019%); Patient #2: 3/17 987 droplets (0.017%); Patient #3: 5/22 799 droplets (0.022%).


A
Number of clones isolated
200
150
100
50
0
N666S
T681I
Imatinib 5uM
Imatinib 10uM
Dasatinib 25nM
Dasatinib 50nM
Dasatinib 100nM
B
Total Viable Cells (x10^6)
1500
1000
500
0
0 1 2 3 4 5 6 7 8
Day
MIG EV +IL3
EBF1-PDGFRB WT
EBF1-PDGFRB N666S
EBF1-PDGFRB T681I
MIG EV -IL3
C
Normalized viable cells (%)
100
75
50
25
0
log (Imatinib) (nM)
log (Dasatinib) (nM)
0 1 2 3
log (Ponatinib) (nM)
MIG Empty Vector
EBF1-PDGFRB WT
EBF1-PDGFRB N666S
EBF1-PDGFRB T681I
D
Positive control cell line MUTZ5
E-P WT Basal
E-P T681I Basal
E-P WT Pervanadate
E-P T681I Pervanadate
E-P WT Dasatinib
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E-P WT Ruxolitinib
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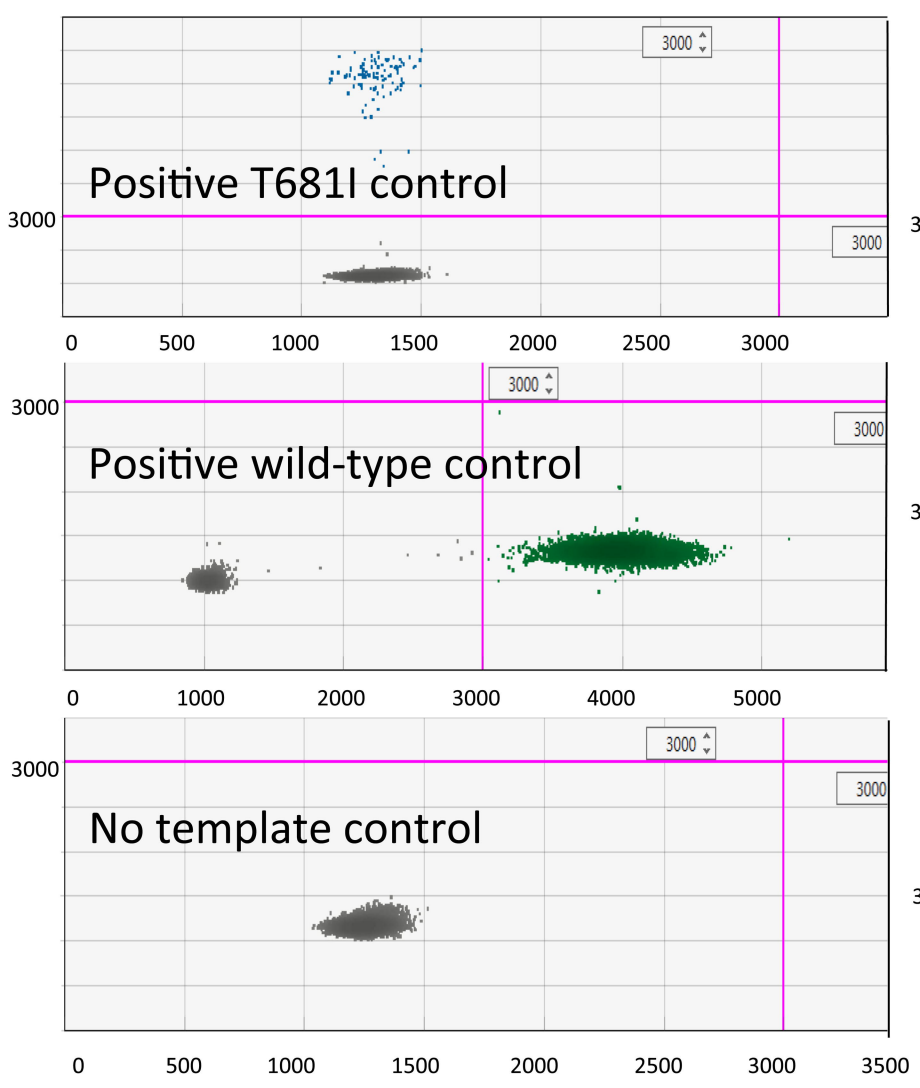
pSTAT5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Positive wild-type control
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No template control
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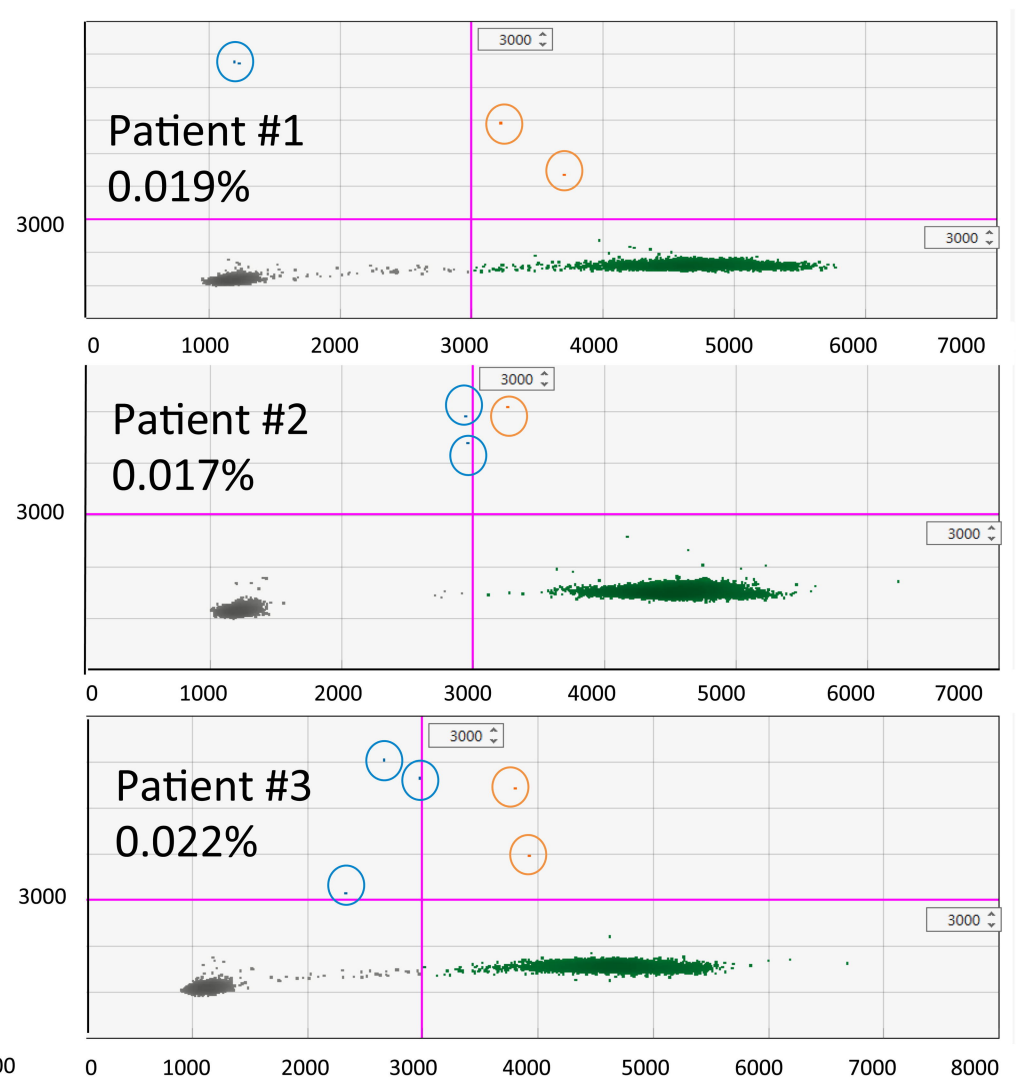
Patient #1
0.019%
3000
0
1000
2000
3000
4000
5000
6000
7000
Patient #2
0.017%
Patient #3
0.022%
8000

Supplemental Data

Table S1. The full spectrum of kinase domain mutations identified in *in vitro* saturation mutagenesis screens of *EBF1-PDGFRB* with imatinib and dasatinib.

	Table 1: In-Vitro Saturation Mutagenesis Screens				
KD mutations / TKIs	Incidence (n)				
	Imatinib 5uM	**Imatinib 10uM**	**Dasatinib 25nM**	**Dasatinib 50nM**	**Dasatinib 100nM**
Recurrent single KD mutations	**T681I** (65/67)	**T681I** (168/178)	**T681I** (85/112) **N666S** (11/112)	**T681I** (160/209) **N666S** (7/209)	**T681I** (93/95)
Single KD mutations			**I654T** (1/112) **L667P** (1/112) **D850N** (1/112) **D854G** (1/112) **G861S** (1/112) **F864P** (1/112)		**D850V** (1/95)
Non-T681I compound KD mutations			**N666S/G846S** (1/112) **G687R/M909I** (1/112)	**M741I/D850Y** (1/209)	
Recurrent T681I compound KD mutations		**T681I/S731Y** (4/178) **T681I/E943K** (2/178)		**T681I/G687E** (2/209) **T681I/A764T** (3/209)	

KD: kinase domain; TKIs: tyrosine kinase inhibitors

Table S2. Baseline characteristics and outcomes of *EBF1-PDGFRB* patients stratified by subclonal T681I mutation status.

	All patients (n=23)	T681I-positive (n=3)	T681I-negative (n=20)	p-value
Median age at diagnosis, years	12 [8-16]	14 [12-17]	12 [7.5-16]	0.4703
Median WBC at diagnosis, x10^9/L	39.0 [17.0-80.7]	13.4 [5.0-26.0]	45.4 [23.3-91.4]	0.0889
CR rate, %	60.0	100.0	52.9	0.2421
EOI MRD>1%, %	84.2	100.0	84.2	1.000
HSCT, %	52.5	33.3	55.0	0.5901
Relapse, %	43.5	100.0	35.0	0.0678
Median time from diagnosis to relapse, months	31 [27-39]	28 [18-39]	31.5 [27-40]	0.7086
Mortality, %	34.8	33.3	35.0	1.0000
5-year EFS, %	30.4	0	35.0	0.4467
5-year OS, %	64.9	66.7	64.6	0.8472

WBC: white blood cell; CR: complete remission; EOI MRD: end of induction minimal residual disease; HSCT: hematopoietic stem cell transplantation; EFS: event-free survival; OS: overall survival.

Figure S1. Comparative structure models of PDGFRB. (**A,B)** Cartoon representation of the ATP binding site indicating the predicted pose of dasatinib (shown as sticks with a transparent molecular surface). **A**. The gatekeeper residue T681 (shown as sticks in green) is predicted to form a hydrogen bond (shown as a black line) with the amide nitrogen on dasatinib. **B**. The mutant T681I (shown as sticks in orange) is predicted to be sterically incompatible (shown by red lines) with dasatinib binding. (**C,D**) Cartoon representation of the loop between helix alphaC and strand beta4. **C**. The N666 residue (shown as sticks in green) is predicted to form the basis of a network of hydrogen bonds (shown as black lines) stabilizing the loop region. **D.** The mutant N666S (shown as sticks in orange) is predicted to lack the ability to form the equivalent set of favorable interactions (shown by the absence of hydrogen bonds).