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

EBF1-PDGFRB accounts for 3% of cases of childhood Philadelphia chromosome-like acute lymphoblastic leukemia (Ph-like ALL), represents the most common fusion gene in the Ph-like ABL-class subtype, and is notoriously associated with high rates of induction failure. EBF1-PDGFRB fusions exhibited exquisite sensitivity to ABL tyrosine kinase inhibitors (TKI) in preclinical models, and durable remissions have been reported in patients harboring EBF1-PDGFRB when treated with either imatinib or dasatinib. Collectively, these observations provide a compelling rationale for investigating 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. The 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.

Among 245 imatinib-resistant and 416 dasatinib-resistant colonies isolated from our in vitro screens, 233 (95%) and 363 (87%) colonies, respectively, harbored a single KD mutation. 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 N666S (n=18/416, 4%), which conferred resistance to dasatinib only. The T681I mutation in EBF1-PDGFRB is analogous to the gatekeeper mutation T315I in BCR-ABL1, while the N666S mutation is analogous to the N676S mutation in FLT3-ITD. The full spectrum of KD mutations in EBF1-PDGFRB identified from the in vitro saturation mutagenesis screens with imatinib and dasatinib is reported in Online Supplementary 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 TKI. Introduction of EBF1-PDGFRB T681I and N666S mutant isoforms into Ba/F3 cells rendered them independent of interleukin-3, illustrating that the transforming capacity of the EBF1-PDGFRB fusion gene is preserved in the presence of these mutations. In viability assays, the T681I mutation was highly resistant to imatinib and dasatinib, while the N666S mutation showed intermediate resistance to dasatinib. The half maximal inhibitory concentration

Table 1. Clinical characteristics and outcomes of the 23 EBF1-PDGFRB patients with or without a subclonal T681I mutation at diagnosis, as determined by droplet digital polymerase chain reaction.

| ID# | Age at diagnosis (years) | WBC at diagnosis x10^9/L | 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                       | 396.0                    | 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            | IF | >1          | BM      | 32               | Yes             | Alive (6.8 years) |
| 8   | 19                       | 54.9                     | 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                        | 212.1                    | 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                        | 49.9                     | 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              | Died of disease (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) |

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.
The proportion of T681I and N666S kinase domain mutations identified in EBF1-PDGFRB in vitro screens to different concentrations of imatinib and dasatinib. The IC\textsubscript{50} values for the EBF1-PDGFRB T681I mutant isosform were 602.5 nM and 23.93 nM for imatinib and ponatinib, respectively, while the IC\textsubscript{50} was not reached with the highest concentration of dasatinib used. Moreover, phosphorylation of STAT5 was not abrogated by dasatinib in Ba/F3 cells harboring the T681I EBF1-PDGFRB mutant 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 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 PDGFRB N666S model 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 (Online Supplementary 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.\cite{8,9} We designed a droplet digital polymerase chain reaction (ddPCR) assay to identify the T681I mutation in patients’ diagnostic samples prior to any exposure to a TKI. Among the 23 diagnostic EBF1-PDGFRB patients’ 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 ten patients on United Kingdom ALL trials (UK ALL 97/99: n=3, UK ALL 2003: n=7) (Table 1). The median duration of follow-up was 60 (14-81) months. None of the patients was treated with TKI. Baseline characteristics, leukemia response and clinical outcomes among the three EBF1-PDGFRB patients with subclonal T681I mutation detected by ddPCR at diagnosis were not significantly different from those of the 20
patients without a subclonal T681I mutation, although there was a trend towards a higher likelihood of relapse in the T681I-positive group versus the T681I-negative group (100% vs. 35%; P=0.0678) (Online Supplementary 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-PDGFBR Ph-like ALL. The gatekeeper T681I mutation was the predominant KD mutation in our *in vitro* screens which was resistant to both imatinib and dasatinib, but could be rescued by ponatinib as predicted. The paucity of KD mutations in EBF1-PDGFBR recovered in the dasatinib mutational screen was similar to that in other BCR-ABL1 mutational screens, since dasatinib is active against most imatinib-resistant KD mutations. However, to our surprise, the gatekeeper mutation was the only KD mutation in *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-PDGFBR recovered in the dasatinib mutational screen was similar to that in other BCR-ABL1 mutational screens, since dasatinib is active against most imatinib-resistant KD mutations. However, to our surprise, the gatekeeper mutation was the only 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-PDGFBR recovered in the dasatinib mutational screen was similar to that in other BCR-ABL1 mutational screens, since dasatinib is active against most imatinib-resistant KD mutations. However, to our surprise, the gatekeeper mutation was the only KD mutation in *in vitro* screens that was resistant to both imatinib and dasatinib, but could be rescued by ponatinib as predicted. In contrast to the report by Zhang et al., our EBF1-PDGFBR in *vitro* saturation mutagenesis screen did not identify the C843G KD mutation that was seen in AGGF1-PDGFBR Ph-like ALL. In their experiments, the reported IC₅₀ of AGGF1-PDGFBR C843G and EBF1-PDGFBR C843G to dasatinib was 0.78 nM and 0.121 nM, respectively. Thus, we may not recover this mutant in our screens even at 25 nM of dasatinib, the lowest dasatinib concentration used in our screen, which is more than 200-fold above the measured IC₅₀ of EBF1-PDGFBR C843G.

The detection of drug-resistant KD mutations at diagnosis has been reported in 21% to 40% of cases of TKI-naïve chronic myelogenous leukemia with advanced disease and in Ph' ALL samples. The frequency of T315I mutation at diagnosis ranges from 12.5% to 17%, which is in keeping with the frequency of the analogous gatekeeper T681I mutation in our cohort of EBF1-PDGFBR patients. 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 have a negative impact on cytogenetic response or event-free survival. Patients with subclonal T6811 mutations detected by ddPCR at diagnosis had a trend towards increased risk of relapse compared to the T6811-negative subgroup; however, these analyses were hindered by small numbers of patients and should be validated in larger cohorts of uniformly treated patients. Furthermore, confirmation of the T6811 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 was treated with TKI and relapse samples after TKI treatment were not available for testing.

In conclusion, KD point mutations represent a potential mechanism of acquired resistance in EBF1-PDGFBR Ph-like ALL. The T6811 gatekeeper KD mutation was the most common KD mutation in EBF1-PDGFBR Ph-like ALL that was resistant 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 T6811 KD mutation.

**References**

1. Reshmi SC, Harvey RC, Roberts KG, et al. Targetable kinase gene fusions in high-risk B-ALL: a study from the Children's Oncology Group. Blood. 2017;129(25):3552-3561.

2. den Boer ML, Cario G, Mooiman AV, et al. Outcome of ABL-class acute lymphoblastic leukemia in children in the pre-tyrosine kinase inhibitor era: an international retrospective study of the Ponte di Legno group. Lancet Haematol. 2021;8(1):e55-e66.

3. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med. 2014;371(11):1005-1015.

4. Tanasi I, Ba I, Sirvent N, et al. Efficacy of tyrosine kinase inhibitors in Ph-like acute lymphoblastic leukemia harboring ABL-class rearrangements. Blood. 2019;134(15):1551-1555.

5. Soverini S, Branford S, Nicolaia FL, et al. Implications of BCR-ABL1 kinase domain-mediated resistance in chronic myeloid leukemia. Leuk Res. 2014;38(1):10-20.

6. Smith CC, Wang Q, Chin CS, et al. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature. 2012;485(7397):260-263.

7. Smith CC, Zhang C, Lin KC, et al. Characterizing and overriding the structural mechanism of the quizartinib-resistant FLT3 "gatekeeper" F691L mutation with PLX3997. Cancer Discov. 2015;5(6):668-679.

8. Pfeifer H, Wawasann B, Pavlova A, et al. Kinase domain mutations of BCR-ABL frequently precede imatinib-based therapy and give rise to relapse in patients with de novo Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). Blood. 2007;110(2):727-734.

9. Hofmann WK, Komor M, Wawasann B, et al. Presence of the BCR-ABL mutation t(2;22)(q13;q13) prior to STI571 (imatinib) treatment in patients with Ph+ acute lymphoblastic leukemia. Blood. 2003;102(2):659-661.

10. Burgess MK, Skaggs BJ, Shah NF, Lee FY, Sawyer CL. Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance. Proc Natl Acad Sci U S A. 2005;102(9):3395-3400.

11. Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. Cell. 2005;121(6):851-843.

12. Geola J, DeAngelio DJ, Golub J, et al. A tyrosine kinase created by fusion of the PDGFRα and HIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. N Engl J Med. 2005;353(15):1201-1214.

13. Oparz S, Polzer H, Hesoldt T, et al. Exome sequencing identifies recurring FLT3 N676K mutations in core-binding factor leukemia. Blood. 2013;122(10):1761-1769.

14. Zhang Y, Gao Y, Zhang H, et al. PDGFRB mutation and tyrosine kinase inhibitor resistance in Ph-like acute lymphoblastic leukemia. Blood. 2018;131(20):2256-2261.

15. Willis SG, Lange T, Demehri S, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. Blood. 2005;106(6):2182-2187.