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),[1] represents the most common fusion gene in the Ph-like ABL-class subtype,[2] and is notoriously associated with high rates of induction failure.[1-3] *EBF1-PDGFRB* fusions exhibited 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 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.[5] 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.[6]

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.[7] 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 concentra-

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	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)

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.

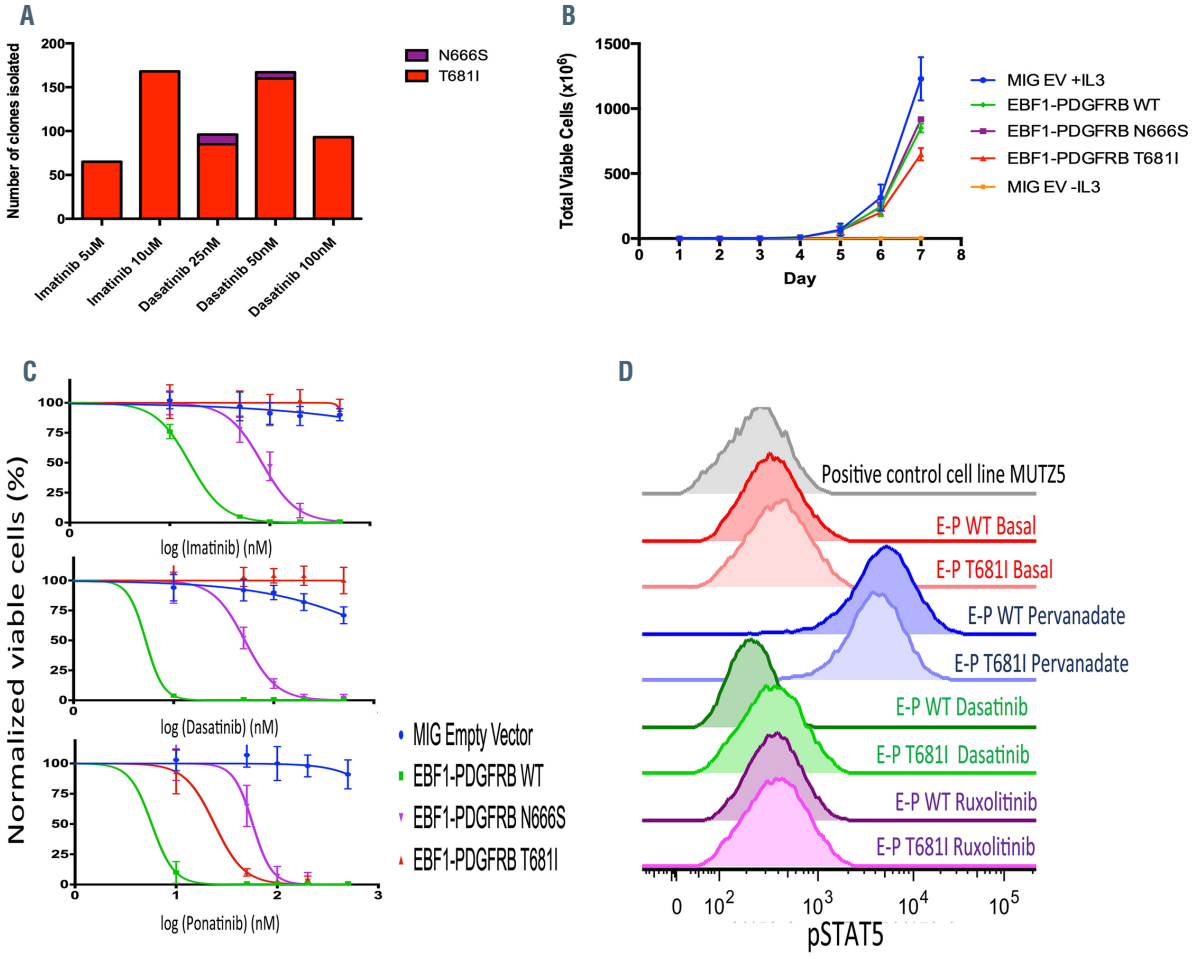

Figure 1. *In vitro* mutational screen of *EBF1-PDGFRB* reveals kinase domain mutations causing varying degrees of resistance to imatinib and dasatinib. (A) The proportion of T681I and N666S kinase domain mutations identified in *EBF1-PDGFRB in vitro* screens to different concentrations of imatinib and 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 and mutant *EBF1-PDGFRB* in response to imatinib, dasatinib and ponatinib. (D) Phosphorylation of STAT5 is elevated at basal in Ba/F3 cells harboring *EBF1-PDGFRB* and can be inhibited in wild-type but not mutant *EBF1-PDGFRB* in response to dasatinib. E-P: *EBF1-PDGFRB*; EV: empty vector; IL3: interleukin-3: WT: wild-type; pSTAT5: phosphorylated STAT5.

tion (IC_{50}) values for wild-type *EBF1-PDGFRB* were 15.74 nM, 5.26 nM and 5.73 nM for imatinib, dasatinib and ponatinib, respectively. The IC_{50} values for the *EBF1-PDGFRB* T681I mutant isoform were 602.5 nM and 23.93 nM for imatinib and ponatinib, respectively, while the IC_{50} 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 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.[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 age of the entire cohort was 12 years (range, 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 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

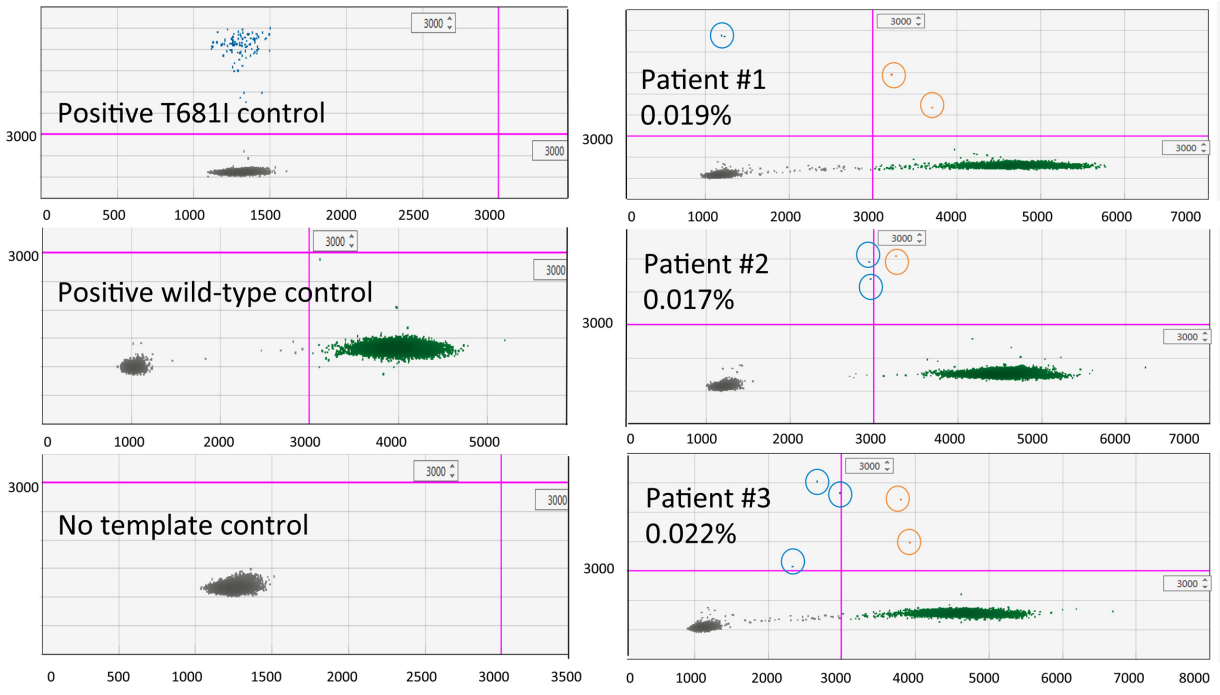

Figure 2. Droplet digital polymerase chain reaction can detect subclonal T681I mutation in clinical samples at diagnosis. Droplet digital polymerase chain reaction (ddPCR) experiments including positive T681I, wild-type and no template controls in the left panel. In the right panel, three *EBF1-PDGFRB* patients were found to have subclonal T681I mutationd at diagnosis by ddPCR. Patient #1 had four droplets containing the mutant T681I out of 20,879 total generated droplets (0.019%); Patient #2 had three positive droplets out of 17,987 generated (0.017%) and patient #3 had five positive droplets out of 22,799 generated (0.022%).

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-PDGFRB* Ph-like ALL. The gatekeeper T6811 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-PDGFRB* 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.[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 the 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 than in the *BCR-ABL1* fusion. The IC_{50} of imatinib for *EBF1-PDGFRB* in our hands was 15.74 nM, while Cools *et al.* reported that the IC_{50} of imatinib for cells expressing *FIP1L1-PDGFRA* was 3.2 nM, whereas the IC_{50} 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 T315 and F317 amino acid residues.[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 IC_{50} 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 25 nM of dasatinib, the lowest dasatinib concentration used in our screen, which is more than 200-fold above the measured IC_{50} of *EBF1-PDGFRB* 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.[8,15] The frequency of T315I mutation at diagnosis ranges from 12.5% to 17%,[15] which is in keeping with the frequency of the analogous gatekeeper T681I mutation in our cohort of *EBF1-PDGFRB* 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.[15] Patients with subclonal T681I mutations 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 numbers of patients 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 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-PDGFRB* Ph-like ALL. The T681I gatekeeper KD mutation was the most common KD mutation in *EBF1-PDGFRB* 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 T681I KD mutation.

Thai Hoa Tran,[1,2] Jonathan V. Nguyen,[2] Adrian Stecula,[3] Jon Akutagawa,[2] Anthony V. Moorman,[4] Benjamin S. Braun,[2] Andrej Sali,[3] Charles G. Mullighan,[5] Neil P. Shah,[6] Yunfeng Dai,[7] Meenakshi Devidas,[8] Kathryn G. Roberts,[5] Catherine C. Smith[6] and Mignon L. Loh[2]

[1]Division of Pediatric Hematology-Oncology, Charles-Bruneau Cancer Center, CHU Sainte-Justine, University of Montreal, Montreal, Quebec, Canada; [2]Department of Pediatrics, Benioff Children's Hospital and the Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA; [3]Department of Bioengineering and Therapeutic Sciences, Department of Pharmaceutical Chemistry, California Institute for Quantitative Biosciences, University of California San Francisco, San Francisco, CA, USA; [4]Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK; [5]Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN, USA; [6]Division of Hematology-Oncology and the Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA; [7]Department of Biostatistics, College of Medicine and Public Health & Health Professions, University of Florida, Gainesville, FL, USA and [8]Department of Global Pediatric Medicine, St. Jude Children's Research Hospital, Memphis, TN, USA

Correspondence: THAI HOA TRAN - thai.hoa.tran@umontreal.ca

doi:10.3324/haematol.2020.261354

Received: June 11, 2020.

Accepted: February 2, 2021.

Pre-published: February 25, 2021.

Disclosures: no conflicts of interest to disclose.

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 manuscript.

Acknowledgments: we thank the Children's Oncology Group ALL Biology Committee and the UK Childhood Leukemia Cell Bank for providing the patients' precious samples.

Funding: this work was supported by National Institutes of Health grants U10 CA98543 and U10 CA180886 (COG Chair's grants), U10 CA98413 and U10 CA180899 (COG Statistics and Data Center grants), U24 CA114766 and U24-CA196173 (COG Specimen Banking), and by the American Lebanese Syrian Associated Charities. MLL is the Benioff Chair of Children's Health and the Deborah and Arthur Ablin Endowed Chair for Pediatric Molecular Oncology at Benioff Children's Hospital. THT and MLL were supported by the Innovation Grant of Alex's Lemonade Stand Foundation as well as the Frank A. Campini Foundation.

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):3352-3361.
2. den Boer ML, Cario G, Moorman 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(16):1351-1355.
5. Soverini S, Branford S, Nicolini FE, 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 PLX3397. Cancer Discov. 2015;5(6):668-679.
8. Pfeifer H, Wassmann 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, Wassmann B, et al. Presence of the BCR-ABL mutation Glu255Lys prior to STI571 (imatinib) treatment in patients with Ph+ acute lymphoblastic leukemia. Blood. 2003;102(2):659-661.
10. Burgess MR, Skaggs BJ, Shah NP, Lee FY, Sawyers 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. 2003;112(6):831-843.
12. Cools J, DeAngelo DJ, Gotlib J, et al. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. N Engl J Med. 2003;348(13):1201-1214.
13. Opatz S, Polzer H, Herold T, et al. Exome sequencing identifies recurring FLT3 N676K mutations in core-binding factor leukemia. Blood. 2013;122(10):1761-179.
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):2128-2137.