FIP1L1-PDGFRα p.T674I-D842L: A Novel and Ponatinib Resistant Compound Mutation in FIP1L1-PDGFRα Positive Leukemia

Els Lierman[1], Sanne Smits[2], Niamh Appleby[3], Eibhlin Conneally[3], Lucienne Michaux[1,2], Peter Vandenberghe[2,4]

Correspondence: Peter Vandenberghe (e-mail: peter.vandenberghe@uzleuven.be).

Myeloid and lymphoid neoplasms with eosinophilia and with the *FIP1L1-PDGFRA* fusion gene, represent the most common cause of chronic eosinophilia of clonal origin.[1] These neoplasms are in general exquisitely sensitive to imatinib, but the p.T674I point mutation in the PDGFRα kinase domain can rarely lead to secondary imatinib resistance.[2,3] Being the homolog of the p.T315I mutation in the BCR-ABL1 kinase domain found in chronic myeloid leukemia (CML) and B-lymphoblastic leukemia with t(9;22), this mutation is, not unexpectedly, also resistant to second generation BCR-ABL1 tyrosine kinase inhibitors (TKI). While in vitro studies had suggested sensitivity of the FIP1L1-PDGFRα p.T674I mutation to the multikinase inhibitor sorafenib, at most a transient response was obtained in 2 cases treated.[3–5] The transient response was followed by the emergence of a panresistant FIP1L1-PDGFRα p.D842 V mutation. The same mutation has also been described after first line imatinib treatment of a *FIP1L1-PDGFRA* positive patient.[6] More recent in vitro studies have suggested that the third generation TKI ponatinib is active against both FIP1L1-PDGFRα p.T674I and p.D842 V.[7] Here, we report the evolution of a *FIP1L1-PDGFRA* p.T674I positive patient under treatment with ponatinib.

A 30-year old male presented with bone pain, neutrophilic and eosinophilic leukocytosis and mildly elevated serum tryptase. Bone marrow examination revealed marked eosinophilia and hypercellularity, without increased blastosis. Cytogenetic examination was normal but FISH showed the pattern of the *FIP1L1-PDGFRA* fusion gene. Initiation of imatinib 100 mg qd led to a complete clinical and hematological remission. Follow-up FISH or molecular testing were not performed as the patient moved away without taking follow-up appointments. Eight months after initial diagnosis he presented with fever and bone pain. His leukocyte count was 65.5×10^9/L with 7.2×10^9/L eosinophils. Bone marrow examination revealed a hypercellular marrow with now 28% myeloblasts, and acquisition of an additional trisomy 8. FISH showed the typical pattern of the *FIP1L1-PDGFRA* fusion gene, in 9/10 metaphases and 80% of interphase nuclei, supporting clonal cytogenetic evolution of his underlying *FIP1L1-PDGFRA* positive neoplasm to acute leukemia. Two courses of intensive chemotherapy with daunorubicin and cytarabine failed to induce hematological remission, with persisting FIP1L1-PDGFRα fusion transcripts in blood and marrow. A morphological and cytogenetic remission in a hypocellular bone marrow was first reached after a third induction course consisting of fludarabine, cytarabine and idarubicin (FLAG-IDA). PCR at this point was not interpretable due to poor RNA quality. As in the meantime a c.2021C>T substitution in the PDGFRα kinase domain had been identified by sequencing, resulting in the p.T674I mutation, ponatinib was started at 45 mg during the neutropenic phase following FLAG-IDA. After recovery, the patient was referred for unrelated allogenic transplant, given anecdotal evidence of allogeneic transplantation in a case of *FIP1L1-PDGFRA* positive leukemia with the p.T674I PDGFRα kinase domain mutation.[3] During his transplant work-up, the patient was found to have a reduced left ventricular ejection fraction of 30% and, therefore, received a reduced intensity conditioning regime. Ponatinib was discontinued at the start of the allogeneic conditioning regimen. After neutrophil engraftment on d23, FIP1L1-PDGFRα fusion transcripts were undetectable in the peripheral blood at d35. Complete donor chimerism was reached on d52 post allograft. Acute graft-versus-host disease did not occur.

However, on d60, bone pains recurred along with mild eosinophilia (0.6×10^9/L). Bone marrow and trephine biopsy revealed a hypercellular marrow with increased myeloblasts (>5%), eosinophilia, and focal fibrosis. Conventional karyotyping showed further subclonal cytogenetic evolution of the original clone to 47,XY,+8[7]/47,XY,del(5)(q22q31),+8[3]. By Sanger sequencing only p.T674I positive FIP1L1-PDGFRα transcripts were identified in the bone marrow. In addition, sequencing

of the complete PDGFRα kinase domain revealed a novel c.2524_2525delinsCT change resulting in a p.D842L mutation in about 50% of the FIP1L1-PDGFRα transcript, indicating a subclone with a compound mutation (Fig. 1). No other mutation was found in the kinase domain of PDGFRα. To our knowledge this is the first time a p.D842L mutation is identified in a FIP1L1-PDGFRα background and the first report on drug resistance via compound mutations in the FIP1L1-PDGFRα fusion transcript. In addition, the PDGFRα p.D842L mutation was not previously described in other malignancies. On day 60, the patient was restarted on ponatinib 30 mg/daily, along with low dose prednisone, without response. Two donor lymphocyte infusions were infused equally without response. Ponatinib was continued throughout this period. About 6 months following his allograft, the patient went to palliative care and died in the hospice.

The FIP1L1-PDGFRα fusion with the double p.T674I-D842L mutation was cloned and compared with the single mutant p.T674I, p.D842L, the previously published p.D842V and the wildtype fusion.[5] Expression of these fusions induced growth factor independent growth of Ba/F3 cells (data not shown). The effect of sorafenib, ponatinib, quizartinib and crenolanib on the growth of the transduced Ba/F3 cells were compared (Fig. 1). Midostaurin was not tested based on previously published data indicating resistance of the p.D842V mutant.[5] Growth of Ba/F3 cells expressing the FIP1L1-PDGFRα wildtype and the p.T674I mutant was strongly inhibited by all four tested inhibitors. Crenolanib inhibited growth of Ba/F3 cells expressing the FIP1L1-PDGFRα p.T674I-D842L double mutant with an IC50 of 196 nM, well below the average steady-state concentration of crenolanib that is obtained in vivo.[8] Ba/F3 cells expressing the FIP1L1-PDGFRα p.T674I-D842L double mutant were also sensitive to ponatinib in vitro but only at an IC50 of 234 nM, which exceeds the average steady-state plasma concentration of ponatinib (101 nM).[9] The double mutant cells were resistant to sorafenib and quizartinib. Ba/F3 cells expressing the FIP1L1-PDGFRα p. D842V mutant responded to crenolanib and ponatinib (IC50 of 9 and 24 nM resp.), moderately to quizartinib but were resistant to sorafenib, as previously reported.[5] In contrast, the FIP1L1-PDGFRα p.D842L single mutant was sensitive to crenolanib, ponatinib, sorafenib and quizartinib (IC50 of 11, 22, 237 and 162 nM resp.) (Fig. 1).

The effect of ponatinib and crenolanib on the phosphorylation of FIP1L1-PDGFRα and its downstream targets STAT5 and ERK1/2 was explored. Ponatinib inhibited FIP1L1-PDGFRα tyrosine phosphorylation for wildtype FIP1L1-PDGFRα and the p.T674I mutant in the low nanomolar range. The p.D842V/L mutants and p.T674I-D842L compound mutant were clearly less responsive in line with the results of the growth experiments. Inhibition of STAT5 and ERK1/2 phosphorylation followed that of FIP1L1-PDGFRα (Fig. 2). Also with crenolanib, the phosphorylation of FIP1L1-PDGFRα, STAT5 and ERK1/2 was specifically inhibited with the FIP1L1-PDGFRα p.T674I and p.T674I-D842L mutants being clearly less responsive (Fig. 2).

The FIP1L1-PDGFRA p.T674I mutation was originally reported as an imatinib-resistant mutation in myeloid and lymphoid neoplasms with eosinophilia and with the FIP1L1-PDGFRA fusion gene. We previously identified sorafenib and ponatinib with in vitro activity against this mutation and now report the activity of two novel compounds quizartinib and crenolanib.[4,7] Clinical effect of sorafenib was curtailed by

emergence of a p.D842V clone.[5] Based on the in vitro activity against p.T674I and p.D842V single mutants we previously proposed ponatinib as a potential candidate for the treatment of p.T674I positive FIP1L1-PDGFRα disease. The present case illustrates that the acquisition of resistant compound mutations can lead to disease progression under ponatinib. As such, we demonstrate yet another molecular mechanism of resistance development in FIP1L1-PDGFRA p.T674I positive leukemia.

The BCR-ABL1 p.T315I-L387M compound mutation has been described in imatinib resistant CML patients, with p. L387 being the homolog position to p.D842 in PDGFRα. It was demonstrated that, in CML, p.T315I mutant-inclusive compound mutants are less sensitive to ponatinib than p.T315I mutated BCR-ABL1.[9] As shown in this report, the same appears to apply for the novel FIP1L1-PDGFRα p.T674I-D842L compound mutant in comparison with FIP1L1-PDGFRα p.T674I, the former being less sensitive to ponatinib as well as to the other tested inhibitors. Based on in vitro data in this report, crenolanib might be a good candidate for treatment of novel FIP1L1-PDGFRα p.T674I positive patients, but the evidence until now is that activity in vitro against FIP1L1-PDGFRα p.T674I, does not predict clinical activity and that the grim prospects of patients with the FIP1L1-PDGFRα p.T674I mutation have so far not substantially improved.

Bone marrow and peripheral blood samples were obtained for diagnostic cytogenetic and molecular work-up. Cytogenetic analysis followed standard protocols. RNA was isolated using the RNeasy minikit (Qiagen, Venlo, the Netherlands) and cDNA was synthesized (Vilokit, InVitrogen, Merelbeke, Belgium). After nested RT-PCR (limit of detection: 0.05%) of the PDGFRα kinase domain, the final PCR product was sequenced with the ABI3730 sequencer (Applied Biosystems, Foster City, CA). Primer sequences can be obtained upon request.

Ponatinib, sorafenib, crenolanib[10] and quizartinib[11] were purchased from Selleckchem (Munich, Germany) and stored in dimethyl sulfoxide (DMSO) as a 10 mM stock at −20°C. Dilutions were made in DMSO immediately before use.

Plasmids containing PDGFRα p.D842L and PDGFRα p. T674I-D842L were obtained from GenScript (Piscataway, NJ) and cloned into an in-house developed retroviral pMSCVpuro-FIP1L1 vector. Next, mutant PDGFRα was replaced within this construct to yield a wildtype PDGFRα kinase domain or a p.T674I mutant kinase domain. Finally, the previously described pMSCVpuro-FIP1L1-PDGFRα p. D842V construct was used.[5]

Viral vector production and transduction of Ba/F3 cells was performed with different pMSCVpuro vectors containing the mutated FIP1L1-PDGFRα fusions described above.[12] Cell culture and dose-response curves were done as reported earlier.[4] Dose-response curves were fitted using GraphPad Prism5 software (La Jolla, CA).

Cells were treated with inhibitors for 90 minutes and lysed in ice-cold lysis buffer (Cell Signaling/Bioke, Leiden, the Netherlands). Gel electrophoresis was performed using NuPage Bis-Tris 4 to 12% gels (Invitrogen, Carlsbad, CA). Western blotting was done with the following antibodies: anti-phospho-PDGFRα, anti-PDGFRα, anti-phospho-STAT5, anti-STAT5a, anti-phospho-ERK1/2 and anti-ERK1/2 (Cell Signaling Technology, Danvers, MA), and anti-mouse/anti-rabbit peroxidase-labeled antibodies (Amersham Biosciences, Munich, Germany).
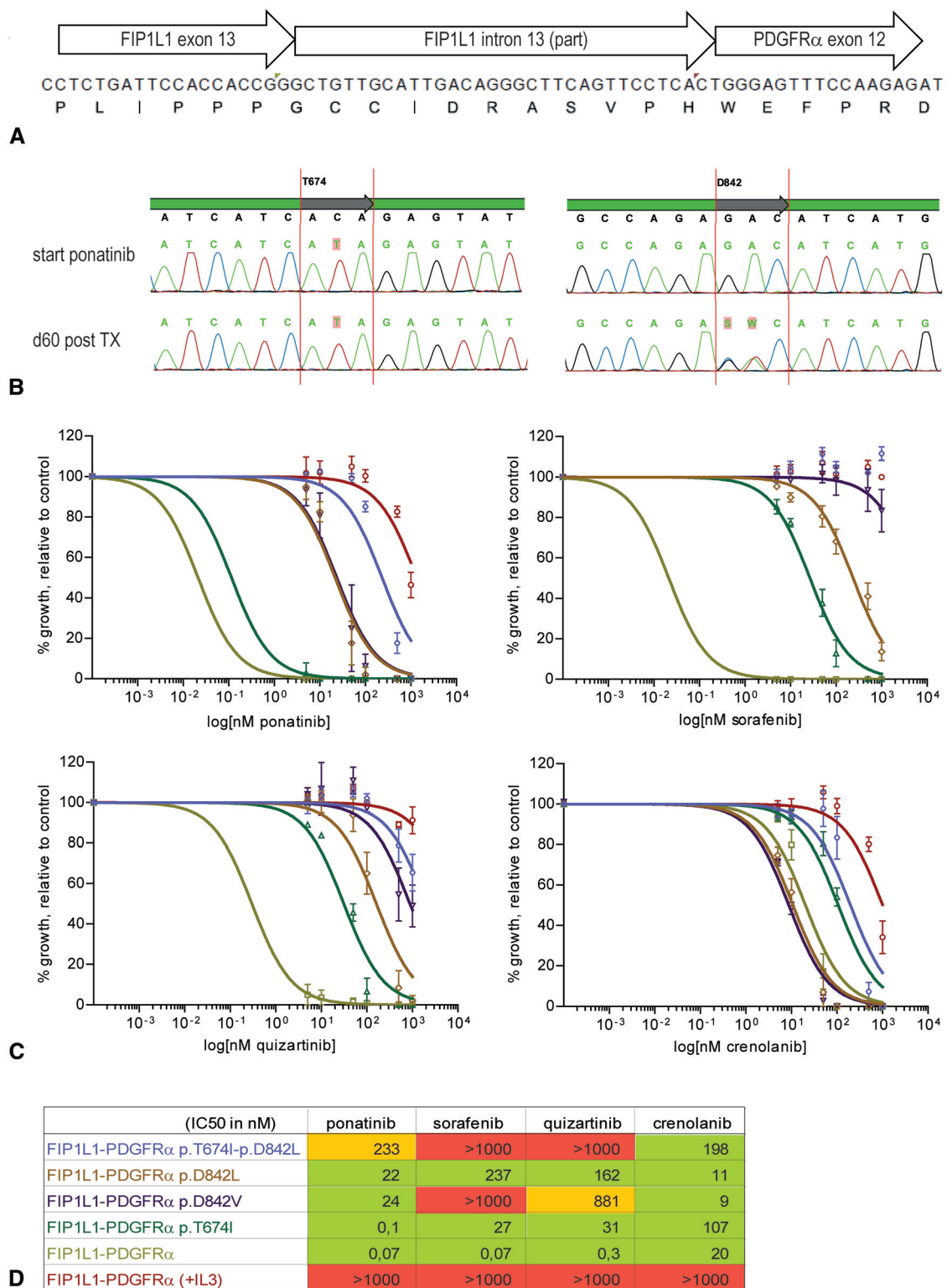
Figure 1. Molecular identification of mutated FIP1L1-PDGFRα and its response to treatment. A. Schematic representation of the FIP1L1-PDGFRα fusion transcript, identified in this patient. B. Electropherogram depicting the mutation status of position p.T674 en p.D842 during disease course. C. Dose-response curves of Ba/F3 cells expressing FIP1L1-PDGFRα wildtype or one of the following FIP1L1-PDGFRα mutants: p.T674I, p.D842V, p.D842L, p.T674I-p.D842L, in the presence of varying concentrations of ponatinib, sorafenib, quizartinib or crenolanib for 24 hours. The growth of FIP1L1-PDGFRα wildtype expressing Ba/F3 cells in the presence of IL-3, and varying concentrations of these inhibitors is also shown. The proliferation relative to untreated controls is shown. Experiments were performed in triplicate. For explanation of the colors, see Figure 1D. D. The IC50 values (in nM) obtained for the different conditions shown in Figure 1C. Box colours indicate sensitivity to the different inhibitors: green box: sensitive, orange box: decreased sensitivity, red box: resistant.
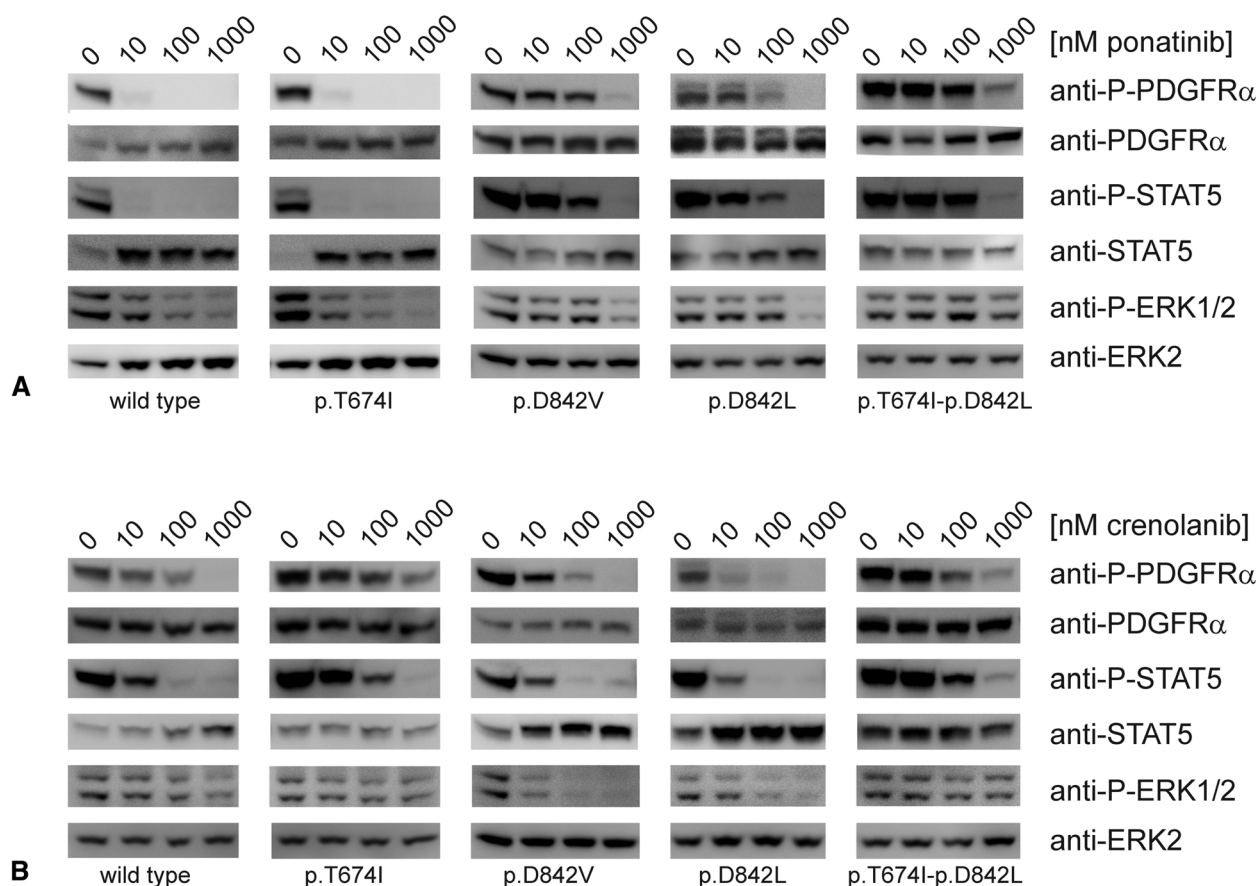
Figure 2. Phosphorylation status of FIP1L1-PDGFRα and its downstream targets STAT5 and ERK1/2. A. Western blot analysis of $2^{*}10^{6}$ Ba/F3 cells expressing FIP1L1-PDGFRα wildtype or one of the following FIP1L1-PDGFRα mutants: p.T674I, p.D842V, p.D842L, p.T674I-p.D842L after treatment with ponatinib for 90 minutes. The phosphorylation status of FIP1L1-PDGFRα and its downstream targets STAT5 and ERK1/2 is shown. B. Western blot analysis of $2^{*}10^{6}$ Ba/F3 cells expressing FIP1L1-PDGFRα wildtype or one of the following FIP1L1-PDGFRα mutants: p.T674I, p.D842V, p.D842L, p.T674I-p.D842L after treatment with crenolanib for 90 minutes. The phosphorylation status of FIP1L1-PDGFRα and its downstream targets STAT5 and ERK1/2 is shown.

References

1. Swerdlow SH, Campo E, Harris NL, et al. WHO classification of tumours of haematopoietic and lymphoid tissues (Revised 4th edition). Lyon: IARC; 2017.
2. 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;348:1201–1214.
3. Metzgeroth G, Erben P, Martin H, et al. Limited clinical activity of nilotinib and sorafenib in FIP1L1-PDGFRA positive chronic eosinophilic leukemia with imatinib-resistant T674I mutation. *Leukemia.* 2012;26:162–164.
4. Lierman E, Folens C, Stover EH, et al. Sorafenib is a potent inhibitor of FIP1L1-PDGFRα and the imatinib-resistant FIP1L1-PDGFRα T674I mutant. *Blood.* 2006;108:1374–1376.
5. Lierman E, Michaux L, Beullens E, et al. FIP1L1-PDGFRα D842V, a novel panresistant mutant, emerging after treatment of FIP1L1-PDGFRα T674I eosinophilic leukemia with single agent sorafenib. *Leukemia.* 2009;23:845–851.
6. Score J, Walz C, Jovanovic JV, et al. Detection and molecular monitoring of FIP1L1-PDGFRA-positive disease by analysis of patient-specific genomic DNA fusion junctions. *Leukemia.* 2009;23:332–339.
7. Lierman E, Smits S, Cools J, et al. Ponatinib is active against imatinib-resistant mutants of FIP1L1-PDGFRA and KIT and against FGFR1-derived fusion kinases. *Leukemia.* 2012;26:1693–1695.
8. Galanis A, Ma H, Rajkhowa T, et al. Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. *Blood.* 2014;123:94–100.
9. Zabriskie MS, Eide CA, Tantravahi SK, et al. BCR-ABL1 compound mutations combining key kinase domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia. *Cancer Cell.* 2014;26:428–442.
10. Heinrich MC, Griffith D, Mckinley A, et al. Crenolanib inhibits the drug-resistant PDGFRA D842V mutation associated with imatinib-resistant gastrointestinal stromal tumors. *Clin Cancer Res.* 2012;18:4375–4384.
11. Kampa-Schittenhelm KM, Heinrich MC, Akmut F, et al. Quizartinib (AC220) is a potent second generation class III tyrosine kinase inhibitor that displays a distinct inhibition profile against mutant-FLT3, -PDGFRA and -KIT isoforms. *Mol Cancer.* 2013;12:1doi:10.1186/1476-4598-12-19.
12. Schwaller J, Frantsve J, Aster J, et al. Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproli- ferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. *EMBO J.* 1998;17:5321–5333.