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To the editor:

Complex subclone structure that responds differentially to therapy in a patient with essential thrombocythemia and chronic myeloid leukemia

BCR-ABL translocation and JAK2-V617F mutation can sometimes be found concomitantly in the same patient (reviewed in Hummel et al¹). To date, 4 studies have examined the chronology and architecture of BCR-ABL and JAK2-V617F clones at the molecular level. In 3 studies, the 2 mutations were sequentially acquired by the same stem cell, with JAK2-V617F preceding the acquisition of BCR-ABL.²⁻⁴ Another report concluded that BCR-ABL and JAK2-V617F represented 2 distinct clones.⁵

Here, we studied a 56-year-old female patient diagnosed in May 2005 with JAK2-V617F-positive essential thrombocythemia (ET), normal cytogenetics, and absence of BCR-ABL (Figure 1A). With anagrelide (2 mg/day), platelet levels normalized. However, in October 2010, thrombocytosis, leukocytosis (white blood cell count = $30 \times 10^9/L$) and the presence of myelocytes and meta-myelocytes in the peripheral blood was noted. Bone marrow showed typical features of chronic myeloid leukemia (CML) and cytogenetic analysis revealed the Philadelphia chromosome translocation t(9;22)(q34;q11). Molecular analysis confirmed expression of a BCR-ABL fusion (b3a2). Treatment with dasatinib induced remission of CML, but thrombocytosis persisted. Therefore, low-dose hydroxyurea (500 mg every second day) was added. A complete molecular remission with a 10^{-4} to 10^{-5} reduction of BCR-ABL transcripts (CMR^{4,5}) was reached within 12 months.

To allow detecting the translocation in DNA samples, we sequenced the BCR-ABL breakpoint and designed polymerase chain reaction (PCR) primers across the junction. The identity of the amplified fragment was confirmed by sequencing (Figure 1B). DNA derived from single colonies grown from the patient's peripheral blood in methylcellulose was analyzed for the presence of JAK2-V617F by allele-specific PCR,^{6,7} and for BCR-ABL by PCR primers bridging the breakpoint. In October 2010, before dasatinib/hydroxyurea treatment, the majority of the colonies were either wild-type or carried both JAK2-V617F and BCR-ABL. However, 2 colonies harbored only BCR-ABL and 1 colony showed only JAK2-V617F (Figure 1C, left panel). Because the BCR-ABL breakpoint in this patient is unique, this pattern suggested that BCR-ABL was acquired before JAK2-V617F and implied that JAK2-V617F occurred twice. Genotyping of individual colonies with a microsatellite marker for chromosome 9p allowed excluding uniparental disomy as an alternative explanation

for the single-mutant BCR-ABL colonies (Figure 1D). This biclonal pattern was confirmed in colonies derived from a second blood sample taken under dasatinib/hydroxyurea treatment 26 months later. Interestingly, the double-positive colonies completely disappeared, while several colonies positive for either JAK2-V617F or BCR-ABL persisted, suggesting that the double mutant may be more sensitive to treatment (Figure 1C, right panel). Despite the presence of BCR-ABL single-mutant colonies, BCR-ABL was undetectable in messenger RNA from bone marrow and peripheral blood and absent in DNA from granulocytes. We can only speculate that the BCR-ABL single-mutant progenitors can divide in vitro and form colonies but are unable to efficiently expand in vivo in the presence of dasatinib.

This patient displayed a novel pattern with 2 separate clones: 1 that first acquired BCR-ABL followed by a JAK2-V617F mutation within the same clone, and a second clone positive for JAK2-V617F only. Our data show that subclones can respond differentially to therapy. Knowledge of the clonal architecture in patients with MPN/CML could be useful to monitor remission and select therapy.

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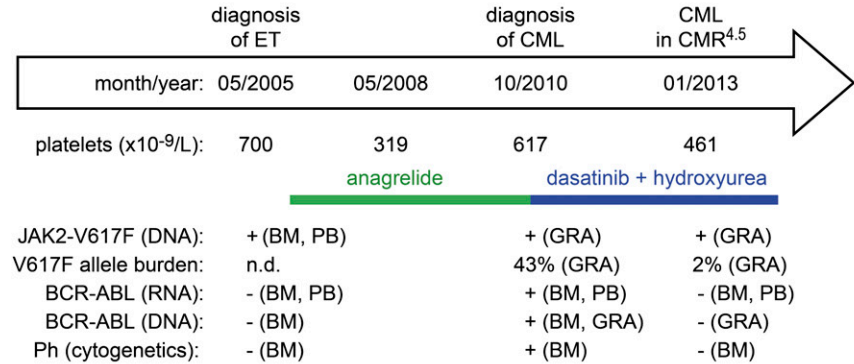
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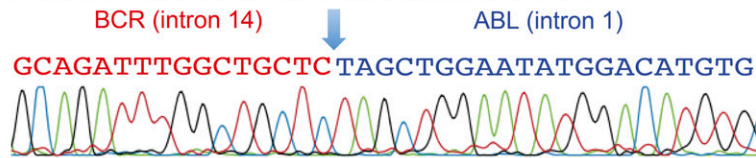
Figure 1. Patient characteristics, BCR-ABL breakpoint sequence, and genotyping of single colonies.

(A) Clinical diagnoses, treatment history, and diagnostic workup of a 56-year-old female patient with coexisting JAK2-V617F mutation and BCR-ABL rearrangement. BM, bone marrow; CMR^{4,5}, complete molecular remission with a log₄ to log₅ reduction of BCR-ABL transcripts; GRA, granulocytes; n.d., not determined; Ph, Philadelphia chromosome. (B) Sequencing chromatogram of a 157-bp amplicon derived from granulocyte DNA revealed the breakpoint sequence in the intron 14 of BCR and intron 1 of ABL. The same sequences were obtained in colonies positive for BCR-ABL. (C) Analysis of single colonies for mutation in JAK2-V617F and BCR-ABL rearrangement in the genomic DNA before and after tyrosine kinase (dasatinib) therapy. Mononuclear cells from peripheral blood were grown in methylcellulose in the presence of erythropoietin. Single burst-forming units erythroid (BFU-E) and non-BFU-Es (ie, CFU-G and CFU-GM) were picked and analyzed individually for the presence of JAK2-V617F mutation by allele-specific PCR. Each colony is represented by a dot that is placed into 1 of 6 quadrangles representing the 6 possible genotypes: wild-type (WT), heterozygous (het) and homozygous (hom) for JAK2-V617F on the vertical axis, and for BCR-ABL on the horizontal axis. Percentages are shown in red for each possible genotype. (D) Genotyping of DNA from single colonies. Ethidium-bromide-stained PCR fragments for the BCR-ABL breakpoint and for BCR (loading control) were separated by agarose gel electrophoresis (top panel). The chromatograms of the allele-specific PCR assay (AS-PCR) show the presence or absence of the wild-type “G” or mutant sequence “T” in codon 617 of JAK2. The results of individual colonies marked with small letters in panel C are shown. Results of microsatellite analysis for a marker on chromosome 9p are shown below. Note that all colonies tested retained both alleles (1 and 2), excluding uniparental disomy as the reason for the loss of BCR-ABL/JAK2-V617F double-mutant colonies. n.a., not available.

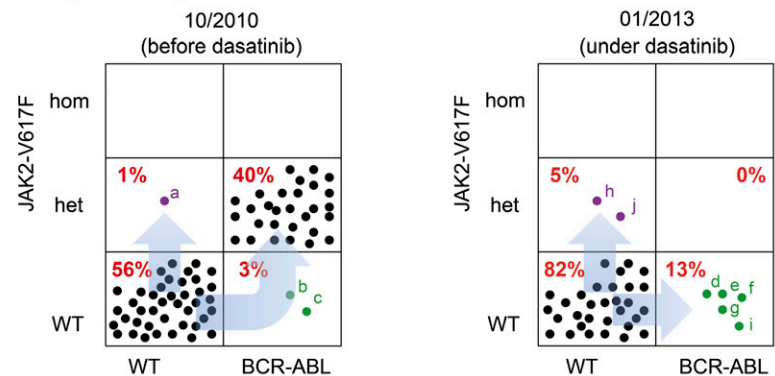
A Clinical course and laboratory data



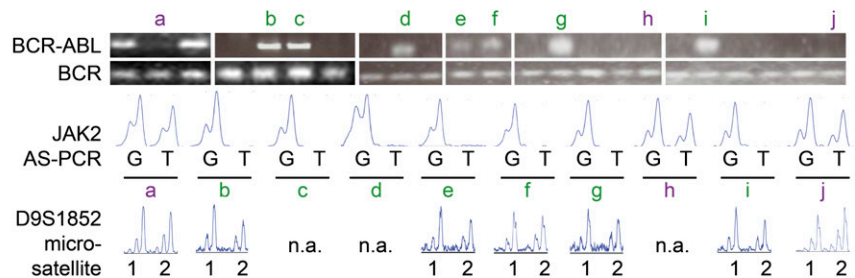
B Sequence of the BCR-ABL breakpoint (genomic DNA)



C Analysis of single colonies



D Genotyping of DNA from single colonies



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To the editor:

Platelet secretion defect in a patient with stromal interaction molecule 1 deficiency

During the last few years, the Ca²⁺ sensor stromal interaction molecule 1 (STIM1) and the channel protein Orai1 have emerged as critical components of store operated Ca²⁺ entry in platelets.¹ In human platelets, both proteins were detected in dense granules and lysosome-related organelles.^{2,3} However, it remains unclear whether Orai1 and STIM1 are required for human platelet function in vivo. Platelet activation requires agonist-induced elevation of intracellular Ca²⁺, which is released from intracellular stores and enters from the extracellular space. As essential second messenger, Ca²⁺ regulates fundamental cellular processes in platelets such as coagulant activity and granule release.⁴ Platelets from Stim1-deficient mice showed a marked defect in agonist-induced Ca²⁺ responses and impaired thrombus formation.⁵ However, *Stim1*^{-/-} mice showed only mild bleeding time prolongation. Mice lacking *Stim1* in platelets formed unstable platelet-rich thrombi and had delayed and reduced fibrin generation in injured arterioles.⁶ Integrin-αIIbβ3-mediated aggregation was not impaired in Stim1-deficient platelets.^{4,6}

Patients bearing mutations in STIM1 present with immunodeficiency, autoimmune disorders, congenital myopathy, and ectodermal dysplasia.⁷ In 5 published patients, enhanced bleeding diathesis was not reported,⁸⁻¹⁰ however, it seems that these patients were not challenged by surgeries in mucocutaneous areas (ie, tonsillectomy). The sixth patient presented with mucocutaneous bleeding symptoms.⁹ The prognosis of patients lacking functional Orai1 or STIM1 is poor unless treated by hematopoietic stem cell transplantation.

Here, we investigated platelet function of a patient with a homozygous R429C mutation in STIM1. The girl presented with recurrent autoimmune hemolytic anemia and thrombocytopenia, recurrent bacterial and viral infections, an enamel defect of her teeth, and mild muscular hypotonia.¹⁰ Until the age of 5 years, she did not show increased bleeding symptoms. However, she had never been challenged by a surgery in mucocutaneous areas.

Platelet aggregation after stimulation with epinephrine was slightly impaired (Figure 1A). Platelet aggregation/agglutination after stimulation with adenosine 5'-diphosphate, collagen, and ristocetin was within normal limits. Bleeding time was within the upper normal range (patient: 5.5 minutes; normal: 6.0 minutes). Flow cytometry analyses

of patient's platelets revealed that platelet α (CD62P)-granule and δ (CD63)-granule secretion in response to thrombin stimulation was impaired (Figure 1B-C). Surface expression of GPIb/IX and GPIIb/IIIa, ristocetin-induced binding of Von Willebrand factor, and binding of soluble fibrinogen were normal, as well as hemoglobin, platelet count, platelet size, and lactate dehydrogenase.

Platelet analyses of the father (heterozygous STIM1 R429C mutation) revealed a reduced Ca²⁺-store content, a partially impaired store-regulated Ca²⁺ entry, as well as a secretion defect, in agreement with his heterozygous status.

These data extend findings in mice and show that STIM1 also plays an important role in human platelet physiology. We identified a granule secretion defect affecting α- and δ-granules in platelets from the patient with a STIM1-mutation. This storage pool defect seems similar to that observed in patients with familial hemophagocytic lymphohistiocytosis type 5 who present with mucocutaneous bleedings.¹¹ Although no major bleeding symptoms in patients with STIM1-mutations have been reported so far, our findings show that a mild bleeding diathesis seems to be an additional feature of the complex clinical syndrome associated with STIM1-deficiency.

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