## Changes in allele frequencies of *CSF3R* and *SETBP1* mutations and evidence of clonal evolution in a chronic neutrophilic leukemia patient treated with ruxolitinib

In 2008, the World Health Organization (WHO) recognized the BCR-ABL1 negative myeloproliferative neoplasm (MPN) chronic neutrophilic leukemia (CNL) as a distinct entity based on the features of an increased white blood cell count (WBC) with a preponderance of neutrophils, no increase in blasts, no dysplasia, and the exclusion of other clonal or non-clonal etiologies.[1] More recently, it was observed that *CSF3R* is recurrently mutated in patients with CNL,[2] which has led to the addition of this finding to the diagnostic criteria for CNL in the most recent 2016 WHO classification of myeloid neoplasms.[3] CSF3R mutations have therapeutic implications; membrane proximal mutations result in activated signaling through the *JAK-STAT* pathway, and anecdotal reports suggest the *JAK1/2* inhibitor ruxolitinib may have efficacy in this setting.[2,4,5]

Here, we report the case of a 69-year-old CNL patient treated with ruxolitinib. He had no significant past medical history and, four months prior to presentation, developed increasing fatigue, abdominal distention, and easy bruising. He was noted to have a WBC of 100.1x10⁹/L with an absolute neutrophil count (ANC) of 93.1x10⁹/L, a hemoglobin of 10.0 g/dL, and a platelet count of 102x10⁹/L (Figure 1). His peripheral blood smear showed marked leukocytosis, predominately neutrophils (Figure 2a). On physical examination, small areas of ecchymosis on the trunk and back, and significant splenomegaly, palpated 10 cm below the costal margin, were noted.

A bone marrow examination revealed a markedly hypercellular marrow (>95%) with prominent left shifted granulopoiesis. Dysplasia was not noted, and blasts accounted for 1.5% of the bone marrow aspirate; mild reticulin fibrosis was observed (Figure 2b). Cytogenetic studies revealed a 46,Y,t(X;7)(p22.1;q36) in all 20 metaphases examined, believed to be a constitutional finding in this patient. Fluorescence *in situ* hybridization for the *BCR/ABL1* rearrangement was negative. Next generation sequencing of DNA from the bone marrow aspirate using the TruSight myeloid sequencing panel
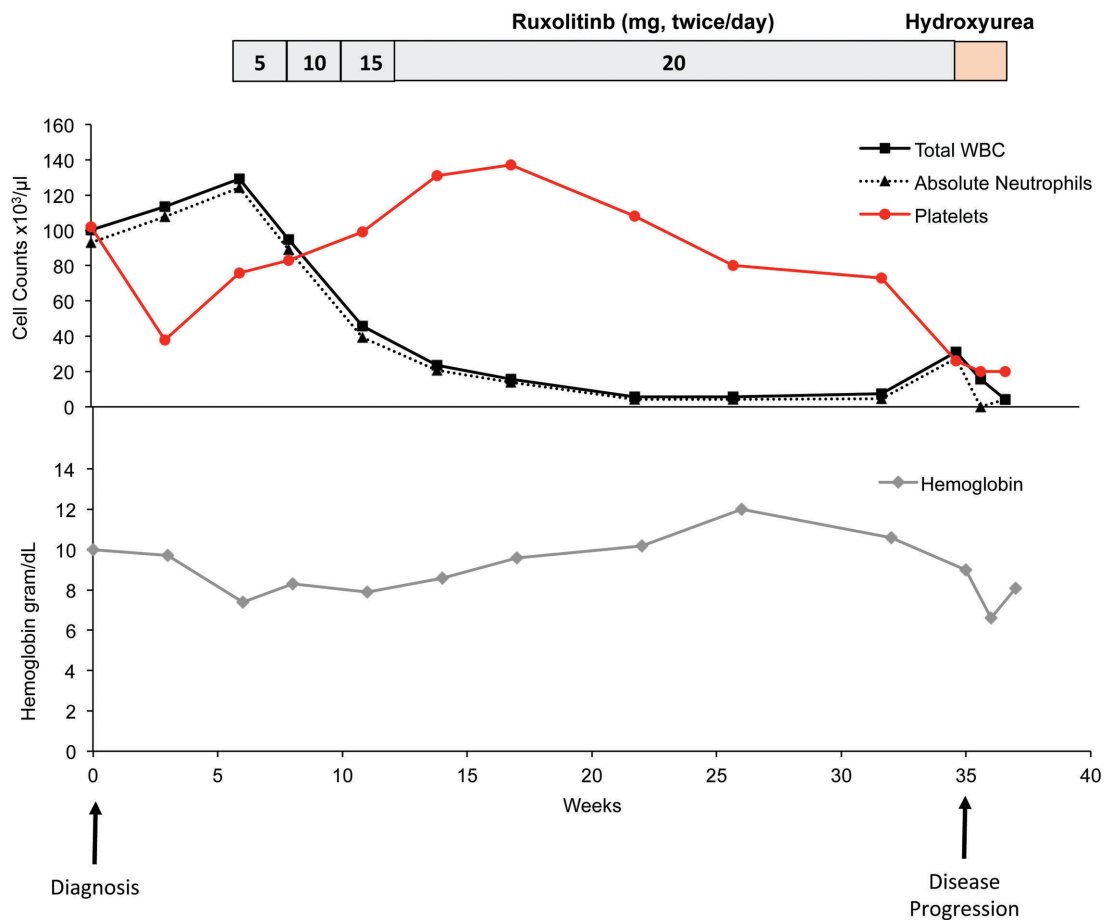


**Figure 1. Graphical analysis of white blood cell count, absolute neutrophil count, platelets and hemoglobin at diagnosis, with escalating doses of ruxolitinib, and at the time of progression.** Ruxolitinib resulted in a rapid and significant reduction in white blood cells (10⁹/L), absolute neutrophil count (10⁹/L) as well as improvement in platelets (10⁹/L) and stabilization of hemoglobin (g/dL). At disease progression there was an increase in the white blood cells and neutrophils, mitigated by hydroxyurea as well as a decline in platelets and hemoglobin.

with 568 amplicons interrogating 54 genes was performed with genomic DNA isolated using the Promega Maxwell® RSC Blood DNA Kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA quantitation was performed using NanoDrop (Thermal Fisher Scientific, Waltham, MA). 100 micrograms of DNA were processed using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA), and the normalized libraries were pooled and loaded on a MiSeq for sequencing using the 600-cycleMiSeq Reagent Kit v3 to generate 2×250 read lengths. Mutations in *SETBP1* (c.G2608A; p.G870S) and *CSF3R* (c.C1853T; p.T618I) were observed, and a diagnosis of CNL was made.

The patient initiated ruxolitinib 5 mg twice daily, and after two weeks experienced decreased fatigue and abdominal fullness; the ruxolitinib was rapidly titrated to 20 mg twice daily over a six-week period. After four months of treatment, the patient had stabilization of the blood counts, with a WBC of 5.5x10$^9$/L, hemoglobin 10.2 g/dL and platelets of 137x10$^9$/L (Figure 1), he no longer had a palpable spleen and, clinically, had returned to his usual baseline with respect to energy and activity tolerance. He declined a follow up bone marrow examination to formally assess his response. The ruxolitinib was continued.

Nine months after initiating ruxolitinib, he developed a rapid increase in fatigue and abdominal distention. His WBC increased to 31.2 x10$^9$/L, and the hemoglobin and platelets dropped to 8.3 g/dL and 26 x10$^9$/L, respectively, with an ANC of 27.4 x10$^9$/L and 2% blasts in the peripheral blood. (Figure 1). The spleen was again palpable, now 8 cm below the costal margin, and a computed tomography scan confirmed splenomegaly (24.5 cm in length). A bone marrow examination revealed a hyper-

**Table 1.** Gene mutations and allele frequencies with read depths at diagnosis and progression.

| Mutation | Region* | Allele frequency at diagnosis | Read depth at diagnosis | Allele frequency at progression | Read depth at progression |
|---|---|---|---|---|---|
| *CSF3R* T618I | chr1: 36933434 | 36.4% | 4,894 | 7.0% | 7,602 |
| *SETBP1* G870S | chr18: 42531913 | 44.8% | 12,721 | 6.0% | 17,659 |
| *GATA2* K392fs | chr3:128,200,087 | Undetected | 5,249 | 37.4% | 6,838 |
| *KIT* D816V | chr4:55,599,321 | Undetected | 5,575 | 33.8% | 4,657 |
| *RUNX1* 337_341del | chr21: 36164772 | 5.0% | 17,239 | 38.5% | 19,460 |

*Coordinates map to Genome Reference Consortium GRCh37/hg18.*
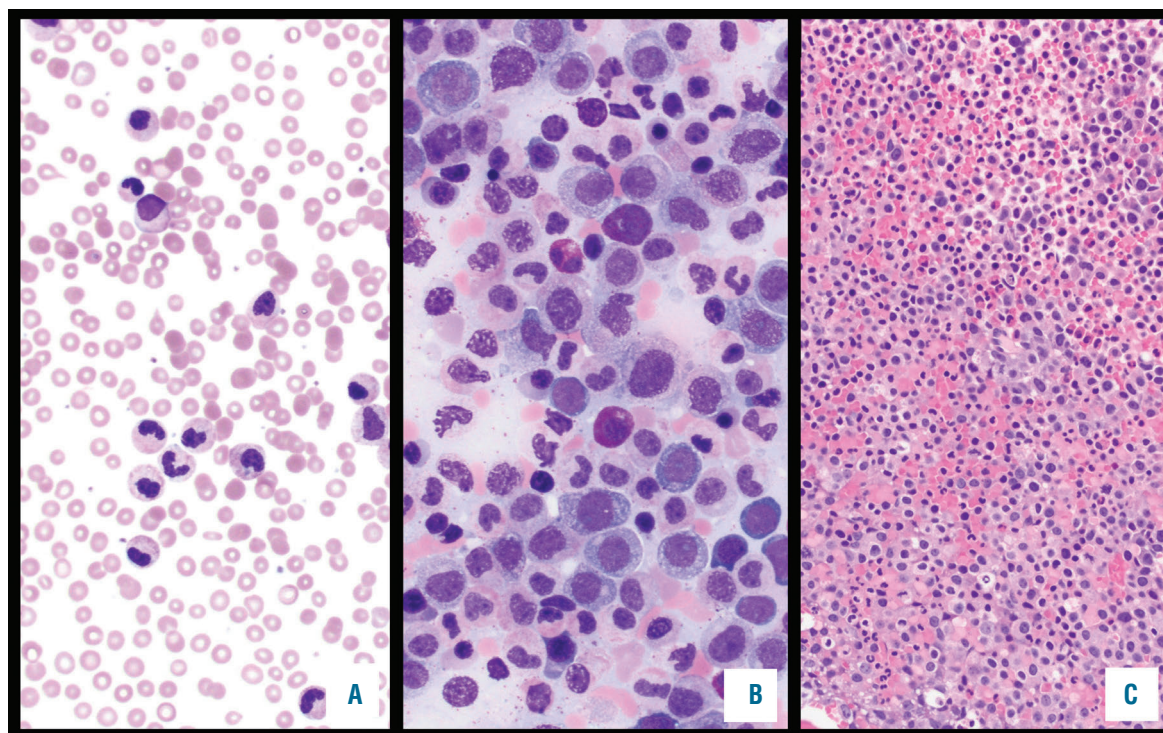


**Figure 2. Peripheral blood smear and bone marrow aspirate and biopsy for the patient at the time of diagnosis.** The peripheral smear (A, 600x) is consistent with leukocytosis with a left shift, characterized by a prominent increase in mature neutrophils. This was in accordance with the aspirate smear (B, 600x), which revelaled no overt dysplasia or increase in blasts, with mild reticulin fibrosis. The core biopsy specimen (C, 200x) is markedly hypercellular (>95%) for age, and is packed with sheets of left shifted myeloid forms.

cellular (>95%) marrow, with prominent neutrophils that still met diagnostic criteria for CNL; the only new finding was a small increase in the blast percentage, from 1.5% to about 5% of marrow cells. Cytogenetic findings were unchanged. The molecular panel was repeated, and three additional mutations were detected: *KIT* (c.2447A>T; p.D816V), *GATA2* (c.1176_1177insA; p.K392fs), and *RUNX1* (c.1009_1022del; p.337_341del); the prior mutations in *CSF3R* and *SETBP1* were no longer observed at the clinically reportable level of detection (~7% allele frequency). Given the newly detected KIT mutation, the biopsy was reviewed carefully for the presence of an increased population of mast cells and no evidence of increased or atypical mast cells was observed.

Re-analysis of the sequencing data to investigate mutations at or below the 7% clinically reportable cutoff did reveal evidence of the *CSF3R* and *SETBP1* mutations, at 7% and 6%, respectively (Table 1). In addition, re-analysis of the sequencing data was also performed on the original bone marrow aspirate, revealing the sub-clinical presence of the *RUNX1* mutation that was not originally detected, but no evidence for the presence of the *GATA2* or *KIT* mutations that were subsequently noted (Table 1).

The patient was admitted for respiratory failure from sepsis, developed multi-organ failure and expired.

The clinical experience with ruxolitinib in *CSF3R*-mutated myeloproliferative neoplasms is limited, but anecdotal reports suggest it can be active,[2,4,5] and this is the focus of an ongoing clinical trial (*clinicaltrials.gov Identifier: 02092324*). Ninety percent of CNL patients, and about 40% of atypical chronic myeloid leukemia (aCML) patients have mutations in *CSF3R*; membrane proximal mutations activate *JAK/STAT* signaling,[2] suggesting the *JAK1/2* inhibitor ruxolitinib may be active, supported by pre-clinical studies.[6]

At diagnosis, our patient had clinically detectable mutations in *CSF3R* and *SETBP1*, among the most commonly mutated genes in BCR-ABL1 negative myeloproliferative neoplasms.[2,7] Retrospectively, the presence of the *RUNX1* mutation, at low levels, was appreciated. His clinical and laboratory response with ruxolitinib ended abruptly; repeated genomic analysis at the time of disease progression revealed the development of new mutations in *GATA2* and *KIT*, neither of which were detected at the time of diagnosis. The development of these mutations, and/or the increased allele burden of *RUNX1*, were possible contributors to ruxolitinib resistance and subsequent disease progression. *KIT* is involved in activated signaling,[8] while *GATA2* and *RUNX1* are myeloid transcription factors; in addition, *RUNX1* has been identified as a driver mutation in the evolution of a myeloproliferative neoplasm to acute myeloid leukemia.[9] The proportional decreased allele frequenices for *CSF3R* and *SETBP1* after treatment with ruxolitnib (Table 1) suggest that a single sub-clone harbored these mutations and was suscepitble to inhibition of the *JAK/STAT* signaling pathway, as was predicted. Although a previous case report did not show a reduction in *CSF3R*-T618I allele frequency in a patient who clinically responded to ruxolitinib,[5] it is possible that depth of responses may vary, and that this patient experienced a deeper response.

In conclusion, we report here for the first time changes in the allele frequencies of *CSF3R*-T618I and *SETBP1*-G870S with response to ruxolitnib as well as insights into the clonal evolution of CNL under selective pressure from ruxolitinib. Ruxolitinib has activity in *CSF3R*-mutated myeloproliferative neoplasms, and can decrease the allele frequency of this mutation and others within a sub-clone. Although from this case we cannot state definitively whether disease progression was independent of treatment or due to selective pressure from ruxolitinib, we show that clonal evolution in CNL can occur and can be explained by the acqusition of driver gene mutations. Similar studies with more CNL patients undergoing treatment with targeted therapies are warranted to further understand this process.

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## References

1. Swerdlow SH, Harris NL, Jaffe ES, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 4th ed. Lyon, 2008.
2. Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. N Engl J Med. 2013;368(19):1781-1790.
3. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.
4. Stahl M, Xu ML, Steensma DP, Rampal R, Much M, Zeidan AM. Clinical response to ruxolitinib in CSF3R T618-mutated chronic neutrophilic leukemia. Ann Hematol. 2016;95(7):1197-1200.
5. Dao KH, Solti MB, Maxson JE, et al. Significant clinical response to JAK1/2 inhibition in a patient with CSF3R-T618I-positive atypical chronic myeloid leukemia. Leuk Res Rep. 2014;3(2):67-69.
6. Fleischman AG, Maxson JE, Luty SB, et al. The CSF3R T618I mutation causes a lethal neutrophilic neoplasia in mice that is responsive to therapeutic JAK inhibition. Blood. 2013;122(22):3628-3631.
7. Meggendorfer M, Bacher U, Alpermann T, et al. SETBP1 mutations occur in 9% of MDS/MPN and in 4% of MPN cases and are strongly associated with atypical CML, monosomy 7, isochromosome i(17)(q10), ASXL1 and CBL mutations. Leukemia. 2013;27(9):1852-1860.
8. Chaix A, Arcangeli ML, Lopez S, et al. KIT-D816V oncogenic activity is controlled by the juxtamembrane docking site Y568-Y570. Oncogene. 2014;33(7):872-881.
9. Engle EK, Fisher DA, Miller CA, et al. Clonal evolution revealed by whole genome sequencing in a case of primary myelofibrosis transformed to secondary acute myeloid leukemia. Leukemia. 2015; 29(4):869-876.