A t(1;9) translocation involving *CSF3R* as a novel mechanism in unclassifiable chronic myeloproliferative neoplasm

Atypical chronic myeloid leukemia (aCML), chronic neutrophilic leukemia (CNL), and unclassifiable chronic myeloproliferative neoplasm (MPN-U) constitute rare hematological neoplasms characterized by the abnormal expansion of granulocytes or neutrophils in bone marrow and peripheral blood in absence of the t(9;22)/*BCR-ABL1* translocation. Up to 60% of CNL neoplasms are caused by mutations in *CSF3R*,[1,2] including activating mutations in the membrane proximal region (such as p.T618I or p.T615A),[3] or truncating mutations that eliminate the carboxy-terminal region of the protein,[1,3] including the cytoplasmic domain involved in downstream signaling and receptor recycling.[4] These findings have improved the molecular diagnosis of such neoplasms,[5] although the molecular alterations in the remaining cases are still unknown.

Herein, we report a 57-year-old female patient diagnosed with MPN-U based on peripheral blood leukocytosis of 39.8 x 10^9/L, 236 x 10^9/L platelets, 123 g/L Hb, 2% blasts, granulocyte left shift and hypercellular bone marrow aspirate with granulocytic proliferation without dysplasia (Fig. 1A). She had no hepatosplenomegaly or adenopathies. The patient did not meet all the criteria to be classified as CNL as she had >10% immature granulocytes and <80% neutrophils in blood, and therefore was diagnosed with MPN-U. No *JAK2* p.V671F, *CALR* exon 9, *MPL* exon 10 and 11, or *CSF3R* p.T618I mutations were identified. Leukemic cells were negative for the t(9;22)/*BCR-ABL1* translocation, but we detected a balanced translocation t(1;9)(p34;q34) in 100% of 33 analyzed metaphases (Figure 1B) raising the possibility of a translocation between *ABL1* and a different partner. She was treated with dasatinib (100 mg/day) for one month, but no hematological response was achieved, and treatment was subsequently discontinued. She was then subjected to related allogeneic hematopoietic stem cell transplantation from her HLA-matched brother, with improvement of her clinical condition, no need for transfusions from day +14 post-transplant, reaching morphological remission at day +100 and complete chimerism at day +160. Her condition was stable for 11 months, but then cytopenia and loss of chimerism was detected, and subsequently her clinical condition deteriorated very rapidly, with high transfusion dependency up to 2 units of red blood cells every 48 hours for three months.

To identify the genes involved in this translocation, we performed low coverage (10X) whole genome sequencing of tumor cells. Reads were aligned using BWA,[6] and translocations detected using Lumpy.[7] We identified a single chromosomal translocation between chromosomes 1 and 9 (chr1:36931663 and chr9:131389147) involving the 3'-unstranslated region (3'UTR) of *CSF3R* and intron 49-50 of *SPTAN1* (Figure 2A). Derivative chromosome der(9) comprises the first 49 exons of *SPTAN1*, fused to the last part of the 3'UTR of *CSF3R*, while der(1) contains the complete coding sequence of *CSF3R* plus most of the 3'UTR. However, 296 bases downstream of the stop codon, it becomes fused to intron 49-50 of *SPTAN1* (Figure 2B). Therefore, unlike other mutations of *CSF3R* described in aCML or CNL, this reciprocal translocation does not affect the coding sequence. Due to the role of *CSF3R* in this pathology,[1] we explored whether additional mechanisms might alter the protein sequence of CSF3R.[1,3,8]

RT-PCR using RNA from neoplastic cells resulted in the

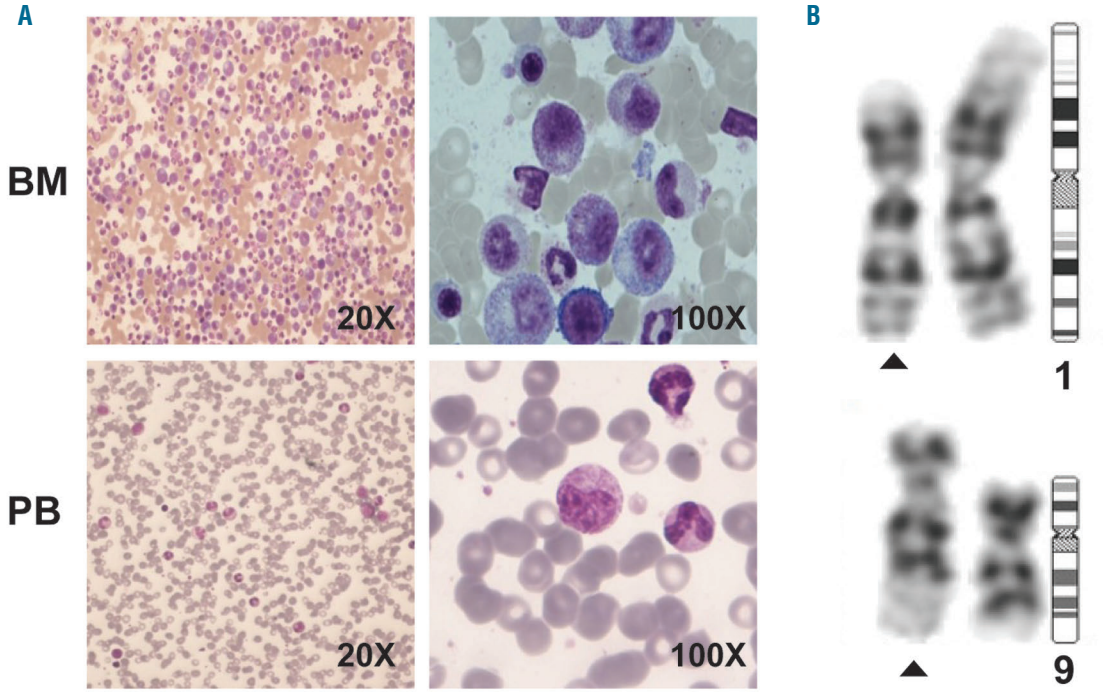

Figure 1. Morphologic and cytogenetic analysis of MPN-U cells. (A) Bone marrow (BM) and peripheral blood (PB) morphology showing hypercellularity and increased granulopoiesis, with no dysplasia or blastosis (magnification 20X and 100X, respectively). (B) Cytogenetic analysis of MPN-U cells showing the presence of a t(1;9)(p34;q34) balanced translocation. Derivative chromosomes der(1) and der(9) are indicated by arrowheads.

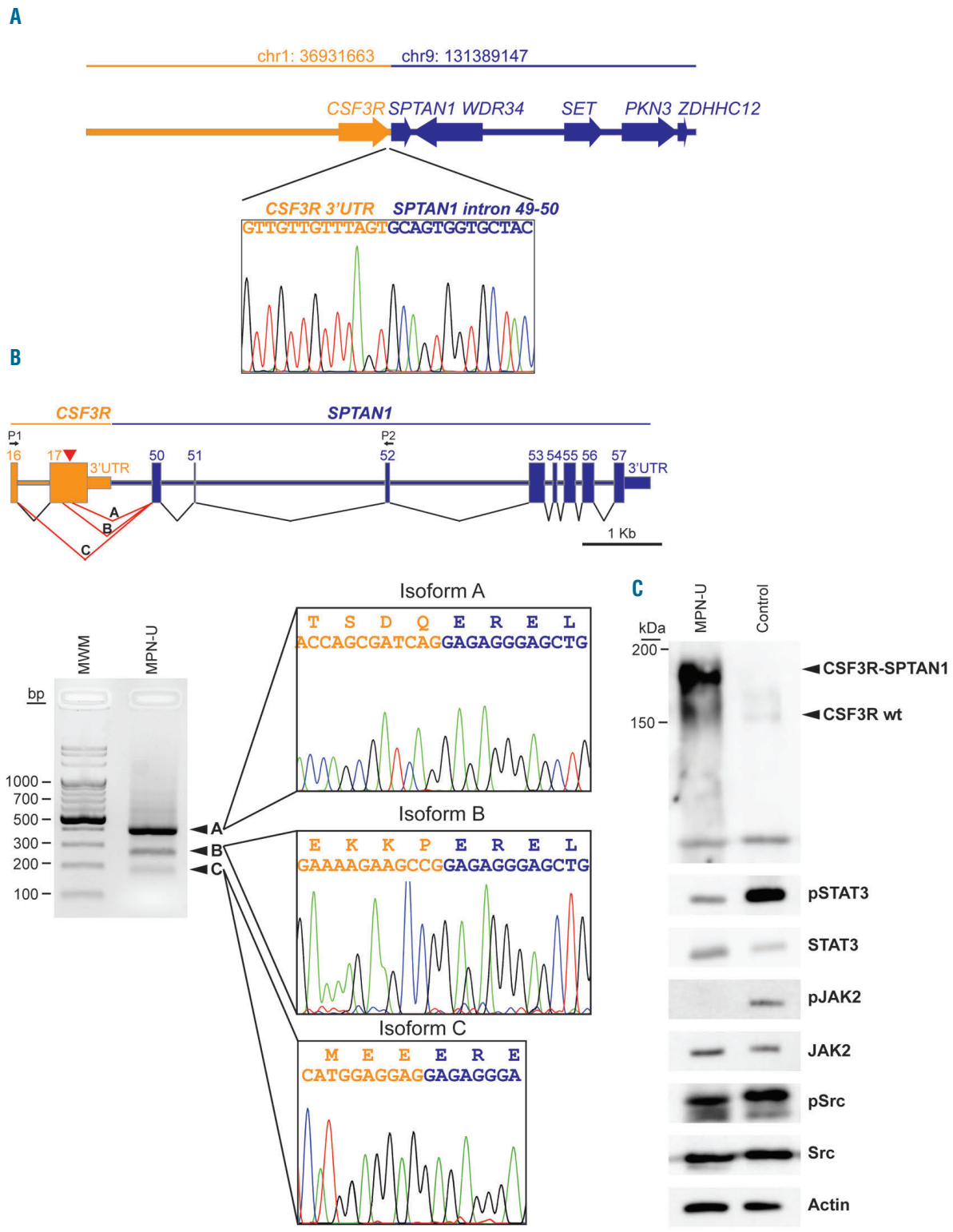

Figure 2. Characterization of the t(1;9)(p34;q34) *CSF3R-SPTAN1* translocation. (A) Characterization of the t(1;9)(p34;q34) *CSF3R-SPTAN1* translocation. (A) Schematic representation of the genomic loci involved in the translocation showing 100 Kb at each side of the breakpoint detected by whole genome sequencing for the der(1) chromosome. Results from whole genome sequencing were validated by PCR with two specific primers (5'-GTCACCAGCATCTCCCTCTC, and 5'-TCACACCTGTAATCCCAGCA), and the electropherogram shows the sequence corresponding to the breakpoint. (B) Representation of the *CSF3R-SPTAN1* fusion gene and splicing events showing three abnormal splicings (named A, B and C) between both genes. The red arrow on *CSF3R* exon 17 indicates the region in which truncating mutations in this gene have been described in MPN-U. Below, RT-PCR analysis of RNA from the MPN-U tumor cells using the oligonucleotides shown in (A) (P1: 5'-CTGGGTGCCCACAATCAT; P2: 5'-CCCGACTCTTCCACCATACA) resulting in the amplification of three bands (A, B and C) (bp: base pairs; MWM: Molecular Weight Marker). On the right, electropherograms corresponding to each band and representing the different splicing events. Orange letters correspond to the *CSF3R* transcript, blue letters to *SPTAN1*. (C) Western blot analysis with an anti-CSF3R antibody (AF-381-PB, R&D Systems), showing the presence of a more intense band corresponding to the expected *CSF3R-SPTAN1* chimeric isoform with a higher molecular weight in cell extracts from the MPN-U patient but not in control cells without this alteration (26-year-old female patient with venous thrombosis and no mutations in *JAK2, BCR-ABL1* or *CSF3R*). Below, Western blot analysis of different downstream effectors in protein extracts from the same cells used above. Actin was used as loading control.

amplification of several chimeric isoforms. The most abundant (isoform A, Figure 2B) is generated from an abnormal splicing between a cryptic splicing donor site located in the last exon of *CSF3R* coding region and the canonical splicing acceptor site of exon 50 of *SPTAN1*. This *CSF3R* splicing donor site is the same as that previously described in isoform IV receptor.[8,9] The other two minor chimeric isoforms detected (B and C) were generated by another cryptic donor site in exon 18 or the canonical donor site of exon 17 from *CSF3R* with the canonical acceptor site of exon 50 of *SPTAN1*.

Therefore, in this *CSF3R-SPTAN1* fusion gene, a strong splicing acceptor site of *SPTAN1* exon 50 is brought in close proximity (<1.2 Kbp) to the isoform IV donor site within the last exon of *CSF3R*, resulting in a major chimeric transcript. Total RNA from neoplastic cells was used to prepare a library using the TruSeq™ RNA Sample Prep Kit v2 (Illumina), and sequenced in paired-end mode (2x100 bp) in a HiSeq 2000 instrument (Illumina). RNA-seq analysis revealed that the expression of mutant *CSF3R-SPTAN1* isoform A was equivalent to wild type *CSF3R* isoform I (190 *vs.* 200 reads), suggesting that virtually all transcripts derived from the translocated allele experienced splicing with *SPTAN1*. Wild type isoform IV CSF3R transcripts accounted for 1.5% of all transcripts at this splicing site, similar to previous reports (<4%).[8]

In summary, the chimeric protein formed by isoform A is composed of *CSF3R*, with the exception of the last 87 carboxy-terminal residues, which are substituted by the terminal 285 residues of *SPTAN1*. Interestingly, described exon 17 mutations in *CSF3R* truncate the cytoplasmic tail in the same region (residues 741-791)[1] affected by this abnormal splicing (residue 749). Western blot analysis of neoplastic cells showed elevated levels of a high molecular weight *CSF3R* band not present in control cells without this alteration (Figure 2C). Despite the expression of the wild type and the chimeric transcripts being similar by RNA-seq, the amount of chimeric protein detected by Western blot was considerably higher than the wild type receptor, supporting the idea that this abnormal splicing might interfere with receptor internalization and degradation similar to previously described truncating mutations in *CSF3R*[1,4,10]. An important observation regarding treatment of the patient was that administration of the *CSF3R* ligand (G-CSF) for neutrophil mobilization during cytopenia periods had a strong negative clinical effect, with general deterioration, including sweating and even loss of consciousness, within 24 h post G-CSF administration.

Analysis of downstream effectors such as *STAT3* or *JAK2* showed reduced phosphorylation when compared to a control sample, while Src phosphorylation remained mainly unaffected (Figure 2C). This latter result differs from the observed increase in pSrc for truncating mutations such as p.S783fs.[1] This difference could explain why the patient did not show any clinical response to the Src inhibitor dasatinib, and might shed light onto the complex relationship between apparently similar mutations and clinical response. In fact, the different behavior between truncating mutations in the cytoplasmic tail of *CSF3R* and the *CSF3R-SPTAN1* fusion described in this work might be explained by the fact that in this chimera, the last 87 residues of *CSF3R* are replaced by 285 residues of *SPTAN1*. In isoform IV, the same last 87 residues are replaced by 34 different residues,[11] resulting in similar molecular characteristics as we observed in *CSF3R-SPTAN1*, including higher protein stability, reduced pSTAT3, and no response to dasatinib.[9]

Cells expressing isoform IV are sensitive to *JAK2* inhibitors such as ruxolitinib or NVP-BSK805.[9] Based on these findings, after our patient experienced lack of clinical response to dasatinib and failure to the allogeneic transplant, treatment with the *JAK2* inhibitor was proposed and approved. Ruxolitinib was started at 5 mg twice daily for 20 days and at 15 mg twice daily thereafter. This greatly improved her clinical condition from the first month, reducing the dependency to transfusion by 10-fold (from 2 units of red blood cells every 48 hours, to one unit every 10 days), and there was a great improvement in her general condition, including reduction of asthenia and sweating. Monitoring the disease by qPCR with specific oligonucleotides to detect the translocation at the genomic DNA level showed that the number of leukemic cells remains stable, and at this moment, five months after initiation of ruxolitinib treatment, in spite of her clinical improvement, no significant reduction in the number of cells with the translocation is detected. This is somehow expected, as reduction of the mutation burden in CNL after treatment is usually slow.[12] Interestingly, the lack of sensitivity to dasatinib and the response to ruxolitinib is more similar to that reported for patients with the p.T618I proximal mutation[12,13] than for patients with truncating mutations in the C-terminal tail of *CSF3R*. This data suggests that despite the fact that this novel mutation replaces the C-terminal domain of *CSF3R*, its molecular and clinical behavior is similar to activating mutations such as p.T618I.

We did not observe additional mutations in *CSF3R* or in other genes previously associated with MPN-U, with the exception of a truncating mutation in *ASXL1* (p.K618fs15*) in ~58% of tumor cells. Mutations in this gene are known to co-occur in CNL with those in *CSF3R* in approximately 57% of cases,[14] further supporting the plausible oncogenic nature of the *CSF3R* mutation described in this study.

Our data shows that current analysis aimed at sequencing only the coding region of *CSF3R* might not be sufficient to capture all the oncogenic alterations in this gene, and some MPN-U, CNL or aCML cases without coding mutations in *CSF3R* might harbor them in the non-coding region. However, management of these patients is different to those with simple truncating mutations in exon 17, as our patient did not show any response to dasatinib treatment, whilst being clinically responsive to ruxolitinib. These data underscore the importance of mutations in non-coding regions in tumor development, and show that therapy response differs from the more frequent coding-mutations in *CSF3R*.

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