An unusual, activating insertion/deletion MPL mutant in primary myelofibrosis

Triple negative myeloproliferative neoplasms (TN-MPN) are defined as patients with essential thrombocythemia or primary myelofibrosis (PMF) who test negative for the principal MPN phenotype driver mutations: JAK2 V617F, CALR exon 9 frameshift mutations and MPL variants at S505 or W515. Recent studies have demonstrated that some cases of TN-MPN harbour non-canonical mutations in JAK2 or MPL that may be constitutional or acquired somatically.

We studied a female patient who presented at the age of 67 with pruritus, night sweats, bone pain and leucocytosis. Her peripheral blood counts were hemoglobin 12.4g/dl, white blood cells 21.5x10⁹/L, platelets 375x10⁹/L with a leucoerythroblastic film. JAK2 V617F was not detected at diagnosis, and subsequent testing for MPL W515 and CALR mutations was also negative. A diagnosis of PMF with osteomyelosclerosis was made and she was treated with localised radiotherapy for the bone pain and with photochemotherapy for the pruritis. Her disease remained stable until 2013 when she redeveloped constitutional symptoms, along with falling haemoglobin, falling platelets and an increase in circulating blasts to 3%. She was treated with ruxolitinib, followed by pacritinib and subsequently ruxolitinib again with a reduction in spleen size but her latest assessment in 2017, 17 years after her initial diagnosis, indicated further disease progression with 10% blasts in the peripheral blood.

We retested a sample taken in early 2016 for MPN phenotype driver mutations using an amplicon-based next generation sequencing pipeline on an Illumina MiSeq. An unusual insertion/deletion mutation was detected in MPL exon 10 that had been missed by targeted MPL W515 analysis. The mutation, designated HLdelinsVISLVT, is a deletion of 6bp and insertion of 18bp resulting in the loss of two amino acids (His 499 and Leu 500) and gain of six amino acids (Val Ile Ser Leu Val Thr). The net effect is to shunt the transmembrane domain down by +4 residues, with position 515 being a leucine (Figure 1). An estimated 90% of the alleles were mutant, suggesting loss of
heterozygosity, and the abnormality was not detected in cultured T cells indicating that it was acquired.

To analyse the consequences of the mutation, we inserted sequence encoding 4 amino acids at position 501 of human TpoR, leading to construct TpoR 501-SLVT-504 (insSLVT). On this background, we substituted residues His 499 and Leu 500 for Val and Ile, respectively, to create construct VIinsSLVT, identical to that seen in the patient. To test the biologic activities of these constructs, we employed dual luciferase assays in JAK2-deficient gamma2A cells where we reconstituted TpoR signaling by transfecting cDNAs coding for TpoR, JAK2 and STAT5. These assays assess STAT5 transcriptional activity, as described, using a firefly luciferase reporter (spi-Luc) driven by STAT5 transcriptional activity and Renilla luciferase driven by a constitutive promoter for normalization. As shown in Figure 2, both TpoR mutants insSLVT and VIinsSLVT exhibited strong constitutive activation of STAT5, with levels equivalent to stimulation of wild type TpoR stimulated with 10 ng/ml Tpo. Thus, the insertion of 4 amino acid residues is sufficient to activate the receptor. The H499V mutation is unlikely to be active by itself, especially since we previously showed that H499L does not alter the activity of human TpoR and L500I is a conservative mutation.

We then asked whether activation is simply due to the appearance at position 515 of a Leu residue instead of the natural Trp 515, or to more global conformational changes resembling those induced by Tpo ligand, involving rotation and rearrangement of extracellular, transmembrane and intracellular domain. Trp515 normally maintains the tilt of transmembrane helices and prevents their dimerization in an active conformation, explaining why 17 of the 20 natural amino acids can activate if substituted at residue W515 of human TpoR. If the mechanism of activation for the HLdelinsVISLVT mutant was the same as that for W515 mutants, replacing Leu 515 with Trp would inhibit activation. As depicted in Figure 2, there was only a very weak inhibition (not statistically significant) when we restored Trp at 515. These data suggest that HLdelinsVISLVT might activate constitutive signalling via
a different mechanism than that adopted by W515 mutants, as TpoR is known to be able to signal from several dimeric interfaces.\textsuperscript{8,9} In addition, the HLdelinsVISLVT mutant might exhibit different membrane insertion features, or more profound secondary structure changes compared to wild type TpoR and W515 mutants. More biophysical studies would be required to identify the actual structure of the HLdelinsVISLVT mutant.

In summary, we have identified an unusual \textit{MPL} mutant that activates TpoR signalling in a patient diagnosed with triple negative PMF and a strikingly long disease course, despite having apparently high risk PMF. Other notable features were presentation with bone pain and osteomyelosclerosis, with diffuse dense sclerosis throughout the skeleton as well as rather severe pruritus for MF. Our findings reinforce the utility of testing triple negative MPN mutations for non-canonical mutations and point to a novel mechanism of TpoR activation by the complex mutation found in our case.

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Conflicts of interest
None of the authors have any relevant conflicts of interest or disclosures.

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Figure legends

Figure 1. Wild type and mutant MPL sequences indicating the 6bp deletion (underlined) and 18bp insertion (underlined, italics) along with a confirmatory sequence trace derived from total blood leukocytes (bottom trace) compared to the wild type sequence (top trace). The Human Genome Variation Society (www.HGVS.org/varnomen) recommended nomenclature for this mutation is c.1495_1500delinsGTGATCTCCTTGGTGACG p.(His499_Leu500delinsValIleSerLeuValThr) but for brevity we refer to it as HLdelinsVISLVT.

Figure 2. Transforming activity of the HLdelinsVISLVT mutation. Data represent 18 replicates from 6 independent experiments for hTpoR, hTpoR insSLVT and hTpoR VIinsSLVT, and 12 replicates from 4 independent experiments for hTpoR VISLVT/L515W. Data were normalized in each experiment with control conditions. The standard error of the mean is indicated; *** = significant difference (Kruskall-Wallis test with Steel’s post-test at 5% significance level); ns = not significant.


