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PATHOLOGY ◆ LABORATORY MEDICINE ◆ LABORATORY MANAGEMENT

Frameshift and in-frame *CALR* exon 9 genetic alterations detected in a post-ET myelofibrosis patient before and after stem cell transplantation

CAP TODAY and the Association for Molecular Pathology have teamed up to bring molecular case reports to CAP TODAY readers. AMP members write the reports using clinical cases from their own practices that show molecular testing's important role in diagnosis, prognosis, and treatment. The following report comes from Mayo Clinic, Rochester, Minn. If you would like to submit a case report, please send an email to the AMP at amp@amp.org. For more information about the AMP and all previously published case reports, visit www.amp.org.

Guang Yang, MD, PhD
David S. Viswanatha, MD
Rong He, MD

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by the proliferation of one or more of the myeloid lineages. Philadelphia chromosome-negative classical MPNs include polycythemia vera (PV), primary myelofibrosis (PMF), and essential thrombocythemia (ET). The main recurrent driver mutations include Janus kinase 2 (*JAK2*) V617F, calreticulin (*CALR*), and myeloproliferative leukemia virus oncogene (*MPL*) mutations in PMF and ET, and *JAK2* V617F and *JAK2* exon 12 mutations in PV. Pathological diagnosis of MPNs follows the WHO classification, which provides specific major and minor criteria for each MPN subtype with incorporation of the molecular markers in the major criteria.[1]

Located on chromosome 19p13.2, the *CALR* gene encodes calreticulin (CALR), a multifunctional calcium-binding protein primarily involved in endoplasmic reticulum (ER) protein folding, calcium homeostasis, cellular proliferation, apoptosis, and immunogenic cell death.[2] More than 50 types of pathogenic *CALR* mutations have been described in MPN. All were exon 9 (last exon) insertions and/or deletions (indels) resulting in a 1-bp frameshift leading to a specific alternative reading frame and a neoepitope in the carboxyl-terminus of the mutant CALR protein. The mutant CALR binds and constitutively activates MPL and the downstream JAK-STAT signaling pathway and leads to oncogenic transformation.[2,3]

Rarely, small in-frame *CALR* indels can be seen in healthy individuals or MPN patients. They are often germline polymorphisms, but they have also been reported as subclonal somatic events coexisting with classical MPN driver mutations in MPN patients. These findings indicate that in-frame *CALR* indels are of doubtful clinical significance, in contrast to frameshift indel alterations.[2-6]

As the 2016 WHO classification of myeloid neoplasms added *CALR* mutation into the major diagnostic criteria for PMF and ET, it is important for pathologists and clinicians to understand the genetic, biological, and clinical differences between the two types of *CALR* indels—frameshift versus in-frame—in clinical practice. Here we report an interesting case of an end-stage MPN patient harboring a pathogenic frameshift *CALR* mutation who successfully underwent stem cell transplantation with elimination of the neoplastic clone and acquisition of an in-frame *CALR* deletion from the healthy donor. This case illustrates the essential differences between the frameshift and in-frame indel variants in *CALR*.

Case. A 42-year-old African American female was referred to our institution for further evaluation of progressive anemia, thrombocytopenia, and circulating blasts. She was diagnosed with ET 20 years ago at an outside institution with initial presenting platelet counts in the "one million range." Past history was significant for intolerance to anagrelide, unprovoked deep vein thrombosis, and spontaneous miscarriages. Her CBC showed a hemoglobin of 8.9 g/dL, white blood cell count of 8.8×10^9/L, absolute neutrophil count of 1.3×10^9/L, and platelet

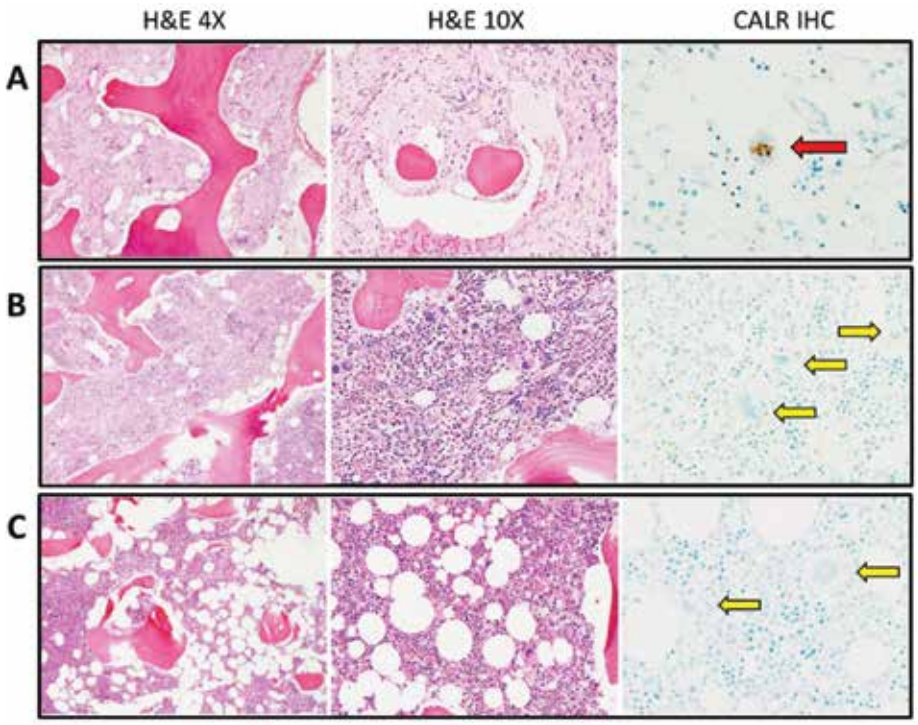

Fig. 1. Bone marrow morphologic findings and CALR immunostains at different time points of disease course. **A.** Pre-transplant bone marrow biopsy showing panhypoplasia, marked fibrosis, osteosclerosis, and dilated sinuses; scattered residual megakaryocytes (red arrow) show positive cytoplasmic immunostaining by CAL2, a monoclonal antibody that recognizes mutant CALR with the C-terminal neoepitope resulting from 1-bp frameshift *CALR* mutations. **B.** Bone marrow biopsy three months after stem cell transplantation showing a representative cellular area with trilineage hyperplasia, cellular streaming, and no tight megakaryocyte clustering. The megakaryocytes (yellow arrows) were negative by CAL2 immunostain. Osteosclerosis is still present. **C.** Bone marrow biopsy 12 months after stem cell transplantation showing a normocellular bone marrow with no morphologic features of involvement by a myeloproliferative neoplasm; the megakaryocytes (yellow arrows) were negative by CAL2 immunostain.

count of 79×10^9/L. Peripheral blood smear showed a leukoerythroblastic picture with two percent metamyelocytes, 21 percent myelocytes, two percent promyelocytes, three percent circulating blasts, and six percent nucleated red blood cells. Dacrocytes were also present. The bone marrow aspirate was hemodilute, and the core biopsy demonstrated a markedly hypocellular marrow with marked panhypoplasia, grade 3/3 myelofibrosis with collagen fibrosis, osteosclerosis, and dilated sinusoids. Rare residual megakaryocytes were present and stained positive by the monoclonal CAL2 antibody, which specifically recognizes the C-terminal neoepitope of the mutant CALR proteins resulting from the 1-bp frameshift pathogenic *CALR* mutations (**Fig. 1A**).[7] Molecular testing was negative for *JAK2* V617F and *MPL* exon 10 mutations, whereas a frameshift 52-bp deletion was detected in *CALR* exon 9 by capillary electrophoresis fragment sizing following polymerase chain reaction using primers flanking exon 9 of *CALR* (**Fig. 2A**).

Chromosome analysis showed del(12p) and del(13q) in 20/20 metaphases. Lactate dehydrogenase was elevated at 531 U/L.

The overall findings, including the documented previous diagnosis of ET and the long duration between the initial ET diagnosis and myelofibrosis development, presence of a pathogenic *CALR* mutation, bone marrow morphology and myelofibrosis, peripheral blood leukoerythroblastic picture, anemia, and elevated LDH, were compatible with a diagnosis of post-ET myelofibrosis per the 2016 WHO diagnostic criteria.[1] A repeat bone marrow examination two months later showed similar morphologic findings; however, the CBC showed significantly decreased Hb, ANC, and Plt, with 13 percent circulating blasts and 17 percent blasts in a hemodilute bone marrow aspirate. The patient received cytoreductive chemotherapy with idarubicin and cytarabine, followed by myeloablative conditioning with busulfan and cyclophosphamide. She subsequently underwent peripheral blood stem cell transplant from a matched healthy sibling donor two months later.

Three months after the transplant, the patient's CBC improved to a Hb of 9.3 g/dL, WBC 4.9×10^9/L, ANC 2.9×10^9/L, and Plt 87×10^9/L. Bone marrow examination exhibited a spectrum of changes, with patchy areas showing marked hypocellularity in a fibrotic background similar to the pre-transplant bone marrow, and other areas exhibiting cellular reconstitution with trilineage hyperplasia and cellular streaming. The megakaryocytes did not show distinct tight clustering, although focal loose aggregates were noted; they were negative for mutant CALR by the CAL2 immunostain (**Fig. 1B**). Background osteosclerosis was still present. Chromosome analysis showed a normal karyotype. Repeat *CALR* mutational analysis revealed disappearance of the frameshift 52-bp deletion and, interestingly, acquisition of an in-frame 9-bp deletion in *CALR* (**Fig. 2B**). The sample was forwarded for Sanger sequencing, which confirmed the presence of a 9-bp deletion (NM_004343.3:c.1191_1199del; p.Glu398_Asp400del, **Fig. 2C**). Concurrent chimerism testing showed 100 percent donor and zero percent recipient DNA (**Fig. 3**, page 4). Retrospective *CALR* testing on the donor DNA was performed and further confirmed the donor-origin of the 9-bp deletion (**Fig. 2D**).

A follow-up examination nine

months later showed further improvement of CBC counts: Hb 11.0 g/dL, WBC 4.6×10^9/L, ANC 3.9×10^9/L, and Plt 89×10^9/L. Bone marrow examination was normocellular with unremarkable trilineage hematopoiesis, and no morphologic features of involvement by an MPN. Megakaryocytes showed no clustering and were negative by CAL2 immunostain (**Fig. 1C**). Molecular testing again revealed 100 percent donor DNA, zero percent recipient DNA, and persistence of the in-frame 9-bp deletion. Karyotype was also normal. At the last follow-up, 31 months after the peripheral blood stem cell transplant, the patient showed no sign of recurrence of an MPN.

Discussion. The Philadelphia chromosome-negative classical MPNs ET, PV, and PMF are chronic myeloid neoplasms with significant risks for leukemic transformation and terminal (end stage) myelofibrosis. Decades of research have greatly facilitated our understanding of the genetic basis of MPN pathogenesis and largely defined their mutational landscape by recurrent driver mutations in *JAK2*, *CALR*, and *MPL*, in an essentially mutually exclusive pattern. In ET and PMF, the most common mutation is the missense mutation *JAK2* V617F, seen in 50 percent to 60 percent of patients, followed by *CALR* exon 9 mutation (20 to 30 percent), and *MPL* exon 10 mutation (five to 10 percent in PMF and three to five percent in ET). In PV, at least 95 percent of patients harbor a *JAK2* V617F, and the vast majority of the remainder has *JAK2* exon 12 mutations.[8] These mutations converge on the activation of the JAK-STAT pathway, which also explains their mutually exclusive nature and the observed clinical response to JAK2 inhibitors in MPN patients regardless of the specific driver mutation.[9]

Although *JAK2* V617F and *MPL* mutations were documented in MPN more than a decade ago, the discovery of *CALR* in MPN was more recent. In 2013, two European groups independently reported highly prevalent somatic mutations in the last exon of *CALR* in the majority of *JAK2* and *MPL*-negative ET and PMF patients but not PV patients.[2,3] *CALR* mutations identified were exclusively insertions and/or deletions resulting in a 1-bp frameshift following the stereotypical pattern of (3n+1) bp for deletions and (3n+2) bp for insertions. The two most frequent mutations were the 52-bp deletion p.L367fs*46 (type 1) and 5-bp insertion p.K385fs*47 (type 2), and they account for approximately 53 percent and 32 percent of the *CALR*-mutated MPN cases, respectively. These pathogenic frameshift mutations result in a specific alternative reading frame and generate a C-terminal neomorphic mutant CALR protein. *CALR* frameshift mutations have been found in the neoplastic hematopoietic stem and progenitor cells in MPN. Studies have shown that these mutations lead to constitutive signaling through the JAK-STAT pathway by direct activation of thrombopoietin receptor MPL, independent of thrombopoietin ligand activation. The C-terminal neoepitope of mutant CALR is indispensable for the activation of MPL and presumptively mediates the pivotal interaction through induction of CALR conformational change. In a retroviral mouse model, *CALR*

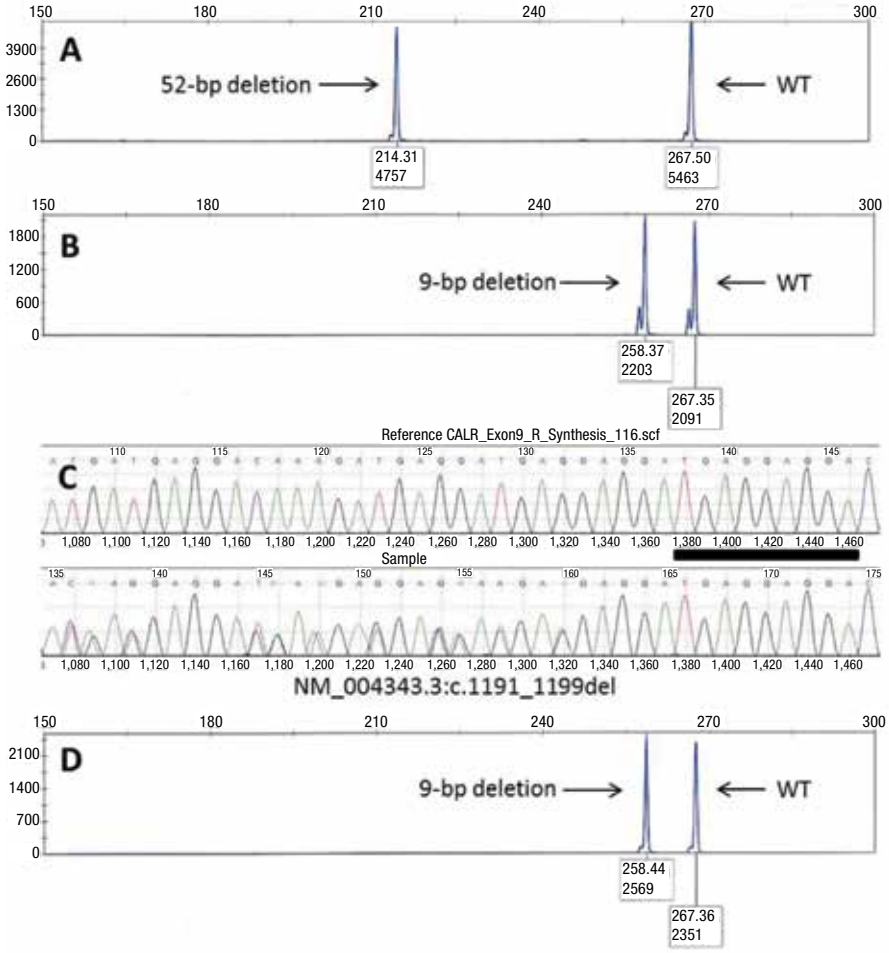

Fig. 2. *CALR* mutational analysis by capillary electrophoresis fragment sizing and Sanger sequencing. **A.** Pretransplant bone marrow showing the presence of a frameshift 52-bp deletion. **B.** Bone marrow specimen three months after stem cell transplantation showing disappearance of the 52-bp deletion and acquisition of an in-frame 9-bp deletion. **C.** The in-frame 9-bp deletion was further confirmed by Sanger sequencing. Top, reference sequence; bottom, patient sample sequence. The deleted sequence is marked by the black bar. **D.** Donor DNA sample showing an identical in-frame 9-bp deletion. (WT, wild type)

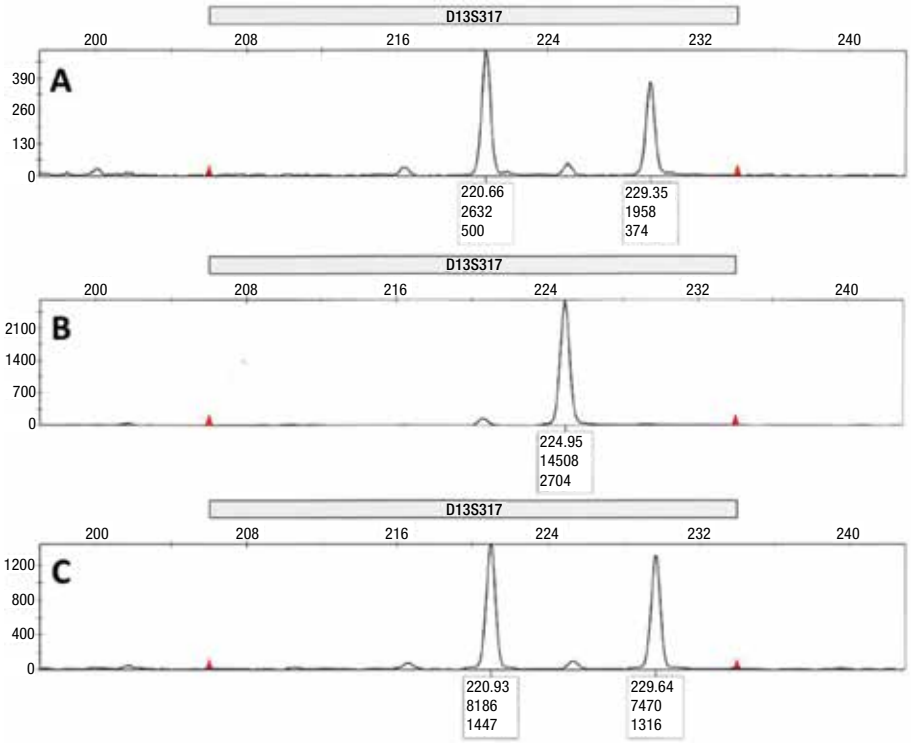

Fig. 3. Chimerism analysis in the post-transplant sample showing 100 percent donor DNA and zero percent recipient DNA. **A.** Donor DNA. **B.** Pre-transplant recipient DNA. **C.** Post-transplant recipient DNA.

mutants were sufficient to induce an MPN phenotype mimicking ET with later progression to myelofibrosis.[10-12]

Clinically, in cases morphologically equivocal for MPN and/or with unexplained cytosis or myelofibrosis, identification of a driver mutation aids in establishing a diagnosis of MPN in the appropriate clinical setting. The mutational status of *JAK2*, *MPL*, and *CALR* is also prognostically informative in PMF because a triple-negative (*JAK2/CALR/MPL*-wild type) mutational status is an adverse molecular signature in PMF, and type 1/like *CALR* mutations have been shown to be associated with more favorable clinical outcome. In ET, *CALR* mutations are associated with a lower risk of thrombosis.[2,3] In light of this apparent clinical and pathologic validity, the 2016 WHO classification of myeloid neoplasms listed *CALR* along with *JAK2* and *MPL* as one of the major diagnostic criterion for ET and PMF.[1]

Interestingly but not surprisingly, besides the pathogenic frameshift mutations seen in MPN, rare small in-frame (3n) indels (≤18-bp) of *CALR* have been described in both healthy individuals and in MPN patients. These are commonly germline polymorphisms but can also occur as somatic events. The Klampfl study reported three cases with in-frame *CALR* indels: 1/524 healthy individuals with a 3-bp deletion, and 2/292 *JAK2* V617F-positive PMF and ET patients with germline *CALR* deletions (an 18-bp deletion in 1/108 PMF and a 12-bp deletion in 1/184 ET).[3] Nangalia, et al., also reported infrequent germline 9-bp and 12-bp *CALR* deletions confirmed through mining of public databases, exome sequencing of healthy donors, and paired constitutional T-cell population sequencing.[2] The findings were further supported by subsequent reports of approximately one percent (8/809) cases suspicious/diagnosed of MPN showing a small in-frame *CALR* deletion of 3- or 9-bp, all at a variant allele fraction of about 50 percent, consistent with germline origin.[5] Two cases co-harbored classical MPN driver mutations, with *MPL* W515L in one and *JAK2* V617F in the other. The p.Glu398_Asp400del 9-bp deletion seen in our patient has been documented in the public databases as a low-level polymorphism with an overall population frequency of 0.13 percent, and variable frequencies in different ethnicity groups, ranging from zero percent in the Finnish Europeans up to 1.3 percent in the African population.[13]

In addition to germline polymorphic variants, *CALR* in-frame deletions may also present as a somatic event in MPN. This was first documented as a rare case of subclonal acquisition of an 18-bp *CALR* deletion on the same allele of a precedent 1-bp *CALR* frameshift deletion in an MPN patient; in this case, the specific mutant reading frame and the C-terminal neoepitope of the frameshift mutation was retained.[3] Lim, et al., also reported four types of 3-bp *CALR* deletions in 5/59 *JAK2* V617F-positive ET patients using TA cloning following negative Sanger sequencing. The somatic nature of these 3-bp deletions was supported by their low allele burden levels (1.4 percent to 10 percent).[4] In both studies, these subclonal somatic in-frame deletions were of doubtful clinical significance and most likely represented passenger events, in light of the presence of coexisting classical MPN driver mutations in these MPN cases. Although Lim, et al., showed a higher frequency of *CALR* in-frame deletion in *JAK2* V617F-mutated MPN, it is worth noting that the relatively small study cohort and the sensitive technique of TA cloning used in the study may have contributed to the differences seen between studies. Biologically, it is evident that these in-frame indels produce neither a reading frameshift nor the C-terminal neoepitope indispensable for the oncogenic activation of MPL in the pathogenesis of mutant *CALR* MPN. In-frame *CALR* indels are therefore of doubtful clinical significance. Unfortu-

nately, the fundamental differences between the frameshift and in-frame *CALR* indels are sometimes underappreciated clinically, leading to erroneous molecular pathologic interpretation of the latter.[6]

In the current case, the patient presented with post-ET myelofibrosis with blast transformation. She harbored a frameshift 52-bp deletion in *CALR*, conforming to the stereotypic (3n+1) bp pattern of pathogenic *CALR* deletions. She was also negative for *JAK2* V617F and *MPL* mutations, consistent with the observed mutually exclusive nature of the three MPN driver mutations. After a successful peripheral blood stem cell transplant, the patient's frameshift 52-bp deletion was eliminated, but interestingly an in-frame 9-bp deletion in *CALR* appeared. We confirmed the donor origin of this in-frame *CALR* deletion through both concurrent chimerism testing, which demonstrated 100 percent donor and zero percent recipient, and retrospective confirmation of the presence of the 9-bp in-frame deletion in the donor sample. The patient remained disease-free at the last follow-up 31 months status post-transplant. This case showcases the genetic, biologic, and clinical differences between a frameshift *CALR* mutation with established pathogenic role in MPN and an in-frame *CALR* deletion of doubtful clinical significance acquired from a healthy donor. It would be important for practicing pathologists and clinicians to recognize the salient differences between these two types of *CALR* indel variants to avoid errant interpretation and subsequent misdiagnosis.

The commonly used methods for *CALR* mutational analysis are Sanger sequencing and next-generation sequencing, the latter generally providing better analytical sensitivity. As the pathogenic *CALR* mutations in MPN are indels, they are also readily detected by fluorescent capillary electrophoresis fragment sizing analysis. One caveat is that the accuracy of capillary electrophoresis fragment sizing is usually ±1–3-bp depending on the size of the PCR products and the platform used, requiring rigorous assay validation to ensure indel sizing accuracy. For rare *CALR* indels with sizes not covered by validated test run controls, supplemental sequencing is recommended to confirm the exact sizes and accurately differentiate between a frameshift versus in-frame *CALR* indel. In the case presented, as our test runs included positive controls of the most common 52-bp deletion and 5-bp insertion, the 9-bp deletion seen by fragment analysis was further Sanger sequenced for size confirmation. Additionally, as the pathogenic frameshift *CALR* mutations generate a C-terminal neoepitope, several antibodies have been successfully developed against this novel peptide region and validated for clinical immunohistochemical use. The immunostain may thus serve as a surrogate marker for the presence of pathogenic frameshift *CALR* mutations and provides a convenient alternative for molecular *CALR* testing in situations where the latter test is not readily available.[7,14]

Another pertinent observation is that in diseases with pathologic alterations in bone marrow microenvironment (for example, MPN exhibiting myelofibrosis and osteosclerosis), molecular marker tracking may provide a more accurate reflection of therapeutic response than morphologic evaluation, particularly in the early stage after stem cell transplantation, given the delay for the engrafted cells to modify and reconstruct the microenvironment. In our case, although the patient lost the pathogenic frameshift 52-bp *CALR* deletion three months after peripheral blood stem cell transplant and showed 100 percent donor DNA by chimerism analysis, the bone marrow findings provided no clear morphologic evidence of disease resolution. The abnormal MPN-like morphological findings eventually subsided in the subsequent bone marrow biopsy nine months later, lagging behind the molecular finding.

Conclusion. Here we present a rare case of a post-ET myelofibrosis patient harboring a pathogenic frameshift *CALR* mutation who underwent a successful peripheral blood stem cell transplantation. Along with clearance of her neoplastic frameshift mutation, she acquired an in-frame *CALR* deletion from the healthy donor. This case illustrates the salient differences between the pathogenic frameshift *CALR* mutations and the in-frame *CALR* indels of doubtful clinical significance. It also emphasizes the importance for pathologists and clinicians to recognize these differences to avoid diagnostic errors and mismanagement in clinical practice. □

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Dr. Yang is a molecular genetic pathology fellow, Division of Laboratory Genetics and Genomics; Dr. Viswanatha is an associate professor, co-director of the molecular hematopathology laboratory and clinical genome sequencing laboratory, Division of Hematopathology and Division of Laboratory Genetics and Genomics; Dr. He is an associate professor, co-director of the molecular hematopathology laboratory, Division of Hematopathology—all in the Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minn.

Test yourself

Here are three questions taken from the case report. Answers are online now at www.amp.org/casereports *and will be published next month in* CAP TODAY.

1. Which of the following statements is false about driver mutations in Philadelphia chromosome-negative myeloproliferative neoplasms?

a. The most common driver mutation is *JAK2* V617F.
b. *CALR* exon 9 mutations are the second most common mutations in polycythemia vera.
c. *MPL* exon 10 mutations can be seen in primary myelofibrosis.
d. *JAK2* V617F, *CALR*, and *MPL* mutations are essentially mutually exclusive of each other in essential thrombocythemia.

2. In the workup of a myeloproliferative neoplasm, which of the following *CALR* genetic alterations does not provide supporting evidence of involvement by an MPN?

a. 52 base pair deletion
b. 5 base pair insertion
c. 12 base pair deletion
d. 34 base pair deletion

3. Which one of the following statements is true about *CALR*?

a. It is located on chromosome 18.
b. The two most common *CALR* mutations in myeloproliferative neoplasms are 52-bp deletion and 5-bp insertion.
c. *CALR* mutations in myeloproliferative neoplasms lead to inactivation of signaling through the JAK-STAT pathway.
d. In-frame insertion/deletions of *CALR* are common pathogenic mutations seen in myeloproliferative neoplasms.