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Genetic lesions disrupting calreticulin 3′-untranslated region in JAK2 mutation-negative polycythemia vera

To the Editor:

Philadelphia-negative myeloproliferative neoplasms (MPNs) originate from transformed hematopoietic stem cells (HSC), retaining the capacity for multilineage differentiation and effective myelopoiesis.[1] Myeloproliferative neoplasms include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), characterized by an excessive production of terminally differentiated red blood cells, platelets and replacement of the bone marrow by fibrotic tissue. Despite the diverse phenotypes, MPNs share major genetic, molecular and pathophysiological features, including clonal hematopoiesis, triggered by mutually exclusive somatic driver mutations in the Janus kinase 2 (*JAK2*), thrombopoietin receptor (*MPL*) and calreticulin (*CALR*) genes, all deregulating the JAK2/STAT signaling pathway.[1] The *CALR* mutations are found in more than 60% of ET and 80% of PMF not carrying *JAK2* or *MPL* mutations, but are rare or absent in PV.[1-3] They mainly consist of exon-9 deletions and insertions, causing a frameshift that removes the KDEL endoplasmic reticulum-retention motif and generates a common novel positively-charged C-terminal sequence. This constitutively activates the MPL-JAK/STAT signaling pathway, and induces ET-like and PMF-like phenotypes in vivo[1] (and references therein). The frameshift caused by various *CALR* mutations also converts into coding sequence the first 31 nucleotides of the 3′-untranslated region (3′-UTR) mRNA, whose functional role remains unknown. Here, we report the identification and functional characterization of non-canonical *CALR* mutations disrupting such *CALR* 3′-UTR region in JAK2-mutation-negative patients presenting an MPN disease resembling PV.

The 3′-UTRs, the noncoding parts of mRNAs, are emerging as major regulators of phenotypic diversity of higher organisms, mainly by controlling mRNA stability, transport, intracellular localization and translational efficiency.[4] Their secondary structure regulates the binding of RNA-binding proteins or microRNAs to mRNAs.[4,5] By binding phylogenetically conserved regions on 3′-UTR mRNAs, these molecules regulate fundamental biological processes, including hematopoietic lineages development and differentiation; their disruption has been implicated in oncogenesis.[4,5]

We genotyped driver mutations in blood cells from 286 consecutive MPN patients, diagnosed at our institution following 2016 WHO recommendations as described in Appendix S1. Patients' cohort

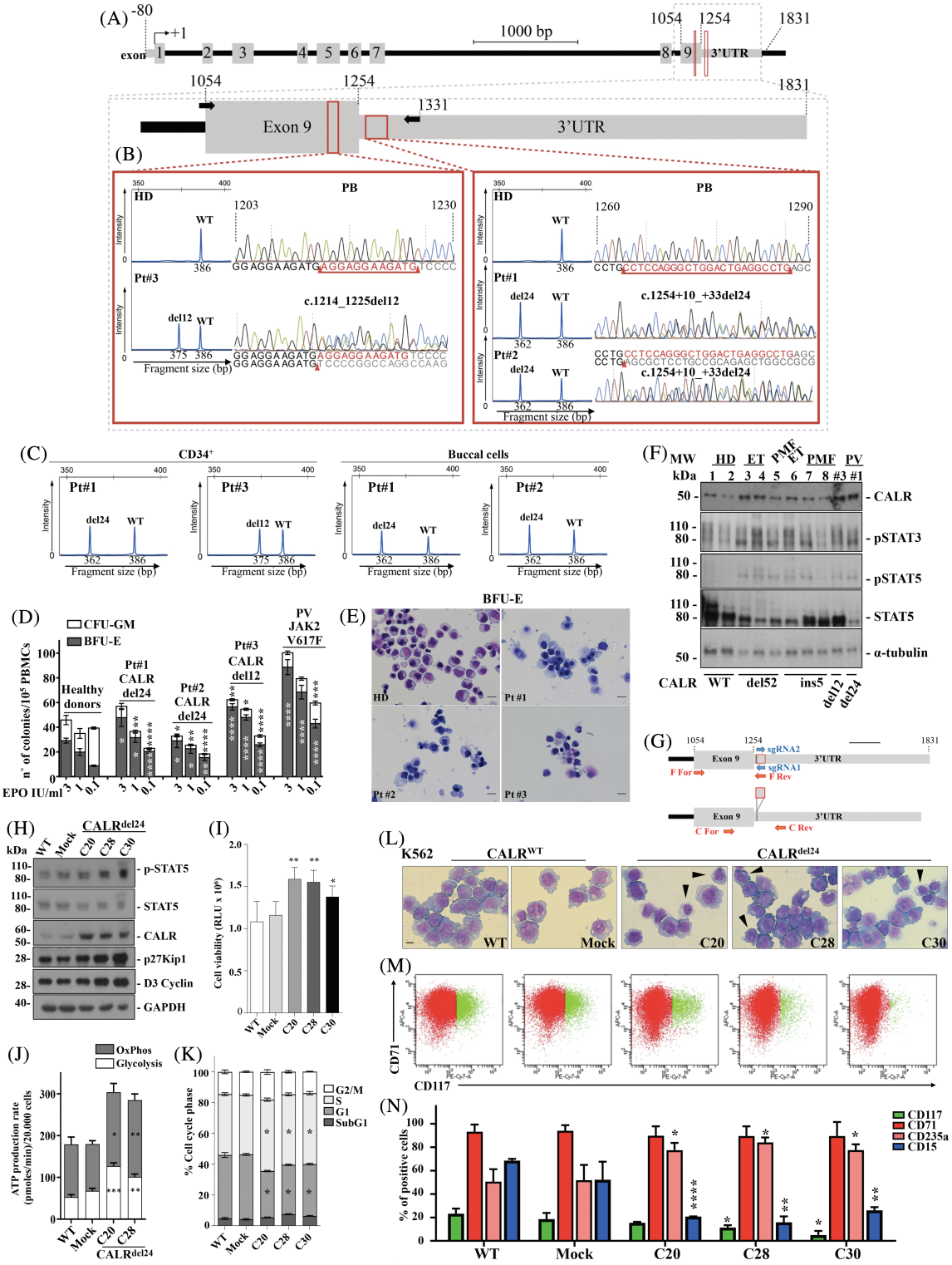

FIGURE 1 Legend on next page.

included 64 PV, 155 ET, 64 PMF and three unclassifiable MPNs (MPN-U) (for clinical features refer to Table S1, and for frequency distribution of MPN driver mutations to Figure S1A). By including in this analysis the first 115 nucleotides of *CALR* 3′-UTR,[6] not always covered by conventional diagnostic methods, we identified *CALR* mutations in 48 (17%) out of 286 MPNs, comprising a case of double positive *JAK2V617F/CALR* and five previously unreported *CALR* exon 9 mutations (Table S2).

Two noncanonical *CALR* mutations, c.1254 + 10_ + 33del24 (*CALR* del24) and E405_D408-del c.1214_1225del12 (*CALR* del12), were identified in blood cells from three patients (patients #1, #2, and #3) with a 2016 WHO diagnosis of MPN-U. These cases lacked *JAK2* mutations but presented clinical, laboratory and histological features recalling PV (Figure 1A-C, Table S3, Figure S1B, and case reports in Appendix S1). Peripheral blood mononuclear cells (PBMCs) from patients#1, #2, and #3 were hypersensitive to erythropoietin (EPO)-induced production of erythroid colonies in vitro (Figure 1D). Figure 1D shows that at very low EPO concentrations (0.1 IU/mL), BFU-E growth was intermediate between that of four healthy donor samples and a control group of 8 *JAK2V617F*-mutated PVs. Moreover, an impairment in the number of CFU-GM colonies was also measured. Morphologic assays performed after 14 days of EPO 3 IU/mL culture, showed a predominance of mature erythroblasts (orthochromatic) in BFU-E colonies from patients #1, #2, #3. By contrast, erythroblasts at earlier maturation stages (proerythroblasts and basophilic erythroblasts) were present in BFU-E colonies from healthy donors (Figure 1E).

Patients #1, #2 are siblings and carried the previously unreported *CALR* del24, which, interestingly, does not affect protein sequence but disrupts the proximal region of 3′-UTR mRNA commonly converted into coding sequence by *CALR* mutations in MPNs (Figure 1A, Table S2). Patient #3 displayed the in-frame del12, causing the loss of four negatively charged amino acids (405-408 aa, stretch IV in Table S2) and encoding a novel CALR protein maintaining KDEL unaltered (Figure 1A, Table S2). Note, del12 was previously described in PMFs and found in a *JAK2V617F*$^{+}$ PMF case of our cohort (Table S2). Notably, *CALR* del24 and del12 occur in evolutionarily conserved *CALR* gene regions. Neither of these mutations affect the integrity of the KDEL motif, but are predicted to cause structural variations at the proximal *CALR* 3′-UTR mRNA, commonly converted into coding sequence by *CALR* frameshift mutations (Figure 1A, Figure S2, Table S2).

Polymerase chain reaction followed by fragment length analysis[6] estimated a *CALR* mutant allele burden ≥50% in PB granulocytes of patients #1, #2, and #3 (Figure 1B), whereas the median mutant allele burden of *CALR*-mutated ET or PMF patients was 33% and 44%, respectively (Figure S1C). Both del24 and del12 on *CALR* exon 9 were also detected in CD34 + -hematopoietic cells from patients #1, #2, and #3, supporting their occurrence in early progenitors and clonal hematopoiesis, with mutant allele burdens ≥50% (Figure 1B,C and data not shown). Note, *CALR* del24 with a ≥ 50% mutant allele frequency was also detected in both siblings' buccal cell DNA, suggesting that such mutation is inherited (Figure 1C). Unfortunately, additional samples from patients and their relatives were unavailable for further investigations.

A targeted NGS analysis[7] on granulocyte DNA from these patients confirmed the presence of the del24 or the del12 on *CALR* exon 9 and excluded alterations of genes commonly involved in rare inherited erythrocytosis or thrombocytosis (Table S4). Moreover, the siblings exhibited del24 in *CALR* 3′-UTR with a > 45% variant allele frequency (VAF), and displayed mutations in *ASXL1* (patient #1) or *DNMT3A* (patient #2), two complementary molecular clonal markers of myeloid neoplasms known to lead to clonal hematopoiesis (Table S5).

Recurrent *CALR* deletions/insertions of MPN, cause a frameshift, modifying the protein sequence.[2,3] Instead, *CALR* del24 affects only 3′-UTR mRNA, whereas *CALR* del12 partially alters the coding sequence, but does not affect KDEL. Therefore, *CALR* del24 and

FIGURE 1 Clinical, molecular and functional characterization of noncanonical CALR exon 9 mutations in MPN-U cases resembling policythemia vera (PV). A, Human *CALR* gene (from http://genome.ucsc.edu/). Exons (gray boxes) and del12 and del24 (red boxes) locations in exon 9, comprising 3′UTR, are shown. Nucleotides are numbered relative to the coding region start site (+1). Black arrows indicate the location of primers used for Sanger sequencing and fragment length analysis. B, Sanger sequencing and fragment length analysis in peripheral blood (PB) granulocyte DNAs from a healthy donor (HD) and patients (Pt) #1, #2, and #3. Nucleotides of del12 or del24 are marked in red. Red arrowheads indicate deletion/junction sites. C, Fragment length analysis of *CALR* exon 9 in patients #1, #2, and #3 CD34^{+}- hematopoietic cells and buccal cells. D, Clonogenic assays in four healthy donors, patients #1, #2, and #3 and 8 *JAK2V617F*$^{+}$ PVs cases. The peripheral blood mononuclear cells (PBMCs) were cultured with 3, 1 and 0.1 IU/mL EPO for 14 days. Results show the mean ± SEM. E, Wright-Giemsa-staining of healthy donors (HD) and Pt #1, #2, and #3 BFU-E after 14 days of cultures at EPO 3 IU/mL (−10 μm). F, Immunoblot analysis of granulocyte protein extracts from MPN cases displaying specific *CALR* mutations as indicated. G, Schematic representation of *CALR* del24 genome-editing showing the small guide RNAs 1 and 2 (sgRNA1-2, blue arrows), the target site (red square) and the forward (For) and reverse (Rev) primers used for validation (red arrows). The CALR del24 region (red box, top) and CRISPR-Cas9 generated deletion (bottom) are shown (−100 bp). K562- wild-type (WT), -vector-transduced (Mock) and -genome-edited CALR^{del24}clones 20, 28 and 30 (C20, C28, C30) were tested for: H, Immunoblot analysis; I, Cell viability, determined by ATP assay, indicated as Relative Luminescence Unit (RLU); J, Total rate of cellular ATP production and the fractional contribution from glycolysis and oxidative phosphorylation (OxPhos); K, FACS analysis of the cell cycle by propidium iodide staining method, the percentage of cells in G1, S, G2/M and subG1 phases is represented; L, Wright-Giemsa morphology, arrowheads indicate cells with erythroid terminal maturation, original magnification 60x; M, FACS analysis, red and green indicate CD71 positive and CD71/CD117 double positive cells, respectively; N, FACS analysis of indicated surface markers. All data are shown as mean ± SD of three independent evaluations. Student's *t* - test vs HD or WT K562 was used for comparisons, *, P <.05, **, P <.01, ***, P<.001, ****, P < .0001

del12 mutants lack the key molecular determinants constitutively activating the MPL-JAK/STAT signaling pathway, which underlies ET- and PMF-like phenotypes in cellular/animal models[1] (and references therein).

Nevertheless, immunoblot analysis showed that, with respect to healthy donor samples and similarly to *CALR*-mutated ET and PMF, increased phosphorylation status of STAT3 and STAT5 is detectable in granulocytes isolated from *CALR* del24 and *CALR* del12 cases, suggesting an activation of JAK/STAT signaling (Figure 1F). Moreover, granulocytes from these cases expressed *CALR* at the highest levels, a finding consistent with the disruption of 3′-UTR mRNA sequence.[4]

To address the biological consequences of structural variations in *CALR* 3′-UTR, using the CRISPR-Cas9 technology, we introduced the CALR del24 mutation within *CALR* 3′-UTR in K562 cells, which are able to differentiate along erythroid or megakaryocytic lineages, despite the expression of BCR/ABL (Figure 1G, Appendix S1). The *CALR* genome matched sites and potential off-targets are shown in Table S6. The K562 C20, C28 and C30 clones, carrying *CALR* del24 in 3′-UTR, were selected by PCR (Figure S3A). Efficiency of gene editing was evaluated by Sanger sequencing and fragment length analysis (Figure S3B), as reported in Appendix S1. The three clones displayed the mutation c.1254 + 10_ + 33del24, besides partial deletions of the targeted region on the other *CALR* allele. Mutant allele burdens in K562 C20, C28 and C30 mutated clones were respectively of 57%, 54% and 45%, thus very similar to those measured in patients #1, and #2 primary blood cells (Figure S3B, Figure 1B,C). The *CALR* mRNA and protein levels were increased in *CALR* mutated clones, whereas BCR/ABL1 (e14a2) expression was unaffected (Figure 1H, Figure S3C,D).

When compared to K562 control cells, pSTAT5 was induced in *CALR* mutated clones, supporting an active JAK/STAT signaling in these cells (Figure 1H). Moreover, these clones showed increased cell viability (Figure 1I), glycolytic activity and mitochondrial oxidative phosphorylation (Figure 1J, Figure S3E-H), promotion of G1/S phase transition of the cell cycle and increased levels of cyclin D3 and $p27^{Kip1}$ (Figure 1 H,K). These are all well recognized biological features of *JAK2*-mutated PVs. Importantly, *CALR* del24 also induced morphological changes in K562 cells, including cell size reduction, chromatin condensation, decreased cytosolic basophilia, appearance of mature erythroblasts, which are consistent with their maturation toward erythropoiesis (Figure 1L). Moreover, flow cytometric analysis showed a significant decrease of the percentage of CD117 + -erythroid precursors, while the percentage of CD235a/glycophorin A+ – differentiated erythroid cells increased and CD71 + -pro-erythroblasts remained at the levels measurable in K562 wild-type and mock cells (Figure 1M,N). A significant decrease in the percentage of CD15 + -granulocytic cells in *CALR* mutated K562 clones was also observed.

In conclusion, we report the identification of noncanonical mutations affecting *CALR* 3′-UTR in *JAK2* mutation-negative patients with MPN diseases resembling PV. These mutations occur in evolutionarily conserved *CALR* regions and are predicted to disrupt mRNA secondary structure of the proximal *CALR* 3′-UTR region, which commonly generates a novel peptide through the frameshifts of canonical *CALR* deletions/insertions. Notably, the disruption of this *CALR*-3′-UTR region in myeloid cells using CRISPR-Cas9 genome-editing induces well recognized biological features of PVs. If a deletion in proximal *CALR* 3′-UTR can determine such phenotypic changes, we hypothesize that this non-coding mRNA region has hematopoietic functions, thus its disruption might affect PV onset. Functional roles of 3′-UTRs in hematopoietic lineage development and differentiation are emerging[5] and 3′-UTR mRNA structural variations have been related to cancer.[4] Whereas further studies addressing the mechanisms underlying these findings are needed, the systematic screening of *CALR* 3′-UTR region might reveal novel aspects of MPN pathogenesis and clarify if our cases are a rare finding or a recurrent genotype ignored so far.

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Haploidentical transplants for patients with graft failure after the first allograft

To the Editor:

Successful engraftment with donor hematopoietic cells is an essential goal of allogeneic hematopoietic stem cell transplantation (AHSCT) to ensure adequate long-term hematopoiesis and provide effective graft-vs-tumor effect. Graft failure (GF), defined as either absence of initial donor cell engraftment (primary GF, PGF) or loss of donor cells after initial engraftment (secondary GF, SGF), is an infrequent, but serious complication after AHSCT with incidence of 5%-10% in patients with hematologic malignancies.[1-3] Patients with GF are at high risk of early non-relapse mortality (NRM) due to prolonged cytopenia, increase risk of life threatening infections and hemorrhage. Second AHSCT is most of the time the only salvage option which needs to be performed urgently as higher NRM and relapse may occur without a functioning graft. Haploidentical (HAPLO) donor may be the alternative donor choice for a second AHSCT due to its wide availability for almost all patients in need of an urgent transplant.

Here we analyzed outcomes of consecutive 31 adult patients with a diagnosis of hematologic malignancy, who received an unmanipulated HAPLO AHSCT as a salvage treatment for either PGF (N = 18) or SGF (N = 13). This was after the first allograft between October1995 and September2017 at the University of Texas MD Anderson Cancer Center. Details on outcome definitions and statistical analyses are available in supplemental material.

The main diagnosis was acute myeloid leukemia (AML; N = 13, 42%). Donor type for the first AHSCT was HAPLO (N = 19, 61.3%), umbilical cord blood (N = 8, 25.8%), matched-related (N = 2, 6.5%) and matched unrelated donor (N = 2, 6.5%). Before the first transplant, four of 22 (18.2%) tested patients had donor-specific anti-HLA antibodies (DSA) positivity (>2000 mean fluorescence intensity) and all of these four patients had PGF despite receiving desensitization treatment with plasma exchange, rituximab, IVIg +/− donor buffy coat infusion,[3] while T cell rejection was noted in 15 patients.

For the rescue transplant, patients received hematopoietic stem cells from a HAPLO donor, 7/19 (36.8%) from the same HAPLO donor. Fifteen patients (48.4%) received non-myeloablative conditioning with fludarabine, cyclophosphamide and 2 Gy of total body irradiation (Flu-Cy-TBI) with post-transplant cyclophosphamide, mycophenolate mofetil and tacrolimus. The median time from the first to second stem cell infusion was 48 days (range 27-147 days), or 20 days from GF diagnosis. (supplemental material 2). None of the patients had DSAs at the time of second transplant against the HAPLO donor.

Of 31 patients, 27 patients had neutrophil engraftment with a median time of 16 days (range 8-27 days), and the corresponding cumulative incidence at 28 days of 87.5% (Figure 1A). Three patients died before day+28 without neutrophil engraftment and one patient died of PGF at 43 days post-transplant. Among seven patients who received stem cells from the same HAPLO donor, only four patients (57%) engrafted successfully but eventually died of NRM within 100 days, and three patients (42.8%) died of NRM before engraftment. The cumulative incidence of platelet engraftment at 60 days was 48.4% (Figure 1A) and the median time was 20 days (range 13-87 days). Among 27 engrafted patients, 25 and two patients had full donor and mixed chimerism, respectively.

Cumulative incidence of acute graft-versus-host disease (GVHD) grade 2-4 and 3-4 at 100 days was 35.5% and 13.8%, respectively. Chronic GVHD incidence was only 14.9% at 3 years. Cumulative incidence of NRM at 1 year was 59% and of relapse was 14.9% at 3 years