LETTER TO THE EDITOR

Paroxysmal nocturnal hemoglobinuria and concurrent JAK2V617F mutation

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Paroxysmal nocturnal hemoglobinuria (PNH) is a hemolytic and pro-thrombotic disorder associated with the clonal expansion of hematopoietic stem cells harboring somatic mutations in the PIG-A gene.1 Mutations in PIG-A result in a lack of surface expression of all glycosylphosphatidylinositol (GPI)-anchored proteins, including the complement inhibitors CD55 and CD59,2 which is responsible for the hemolytic (and probably also the pro-thrombotic) phenotype.3,4 However, long-term colony-forming assays and in vivo murine models have failed to show that PIG-A mutations are alone sufficient to drive clonal expansion.5,6

There are two leading hypotheses to account for clonal expansion of PIG-A-null stem cells: (i) clonal selection and (ii) second mutations. The first model posits that aplastic anemia—which is epidemiologically associated with PNH—results in an immune-mediated marrow injury that selectively spares PNH stem cells.7 The second hypothesis is borrowed from models of oncopogenesis and may be supported by the observation that patients with PNH can harbor clonal cytogenetic abnormalities.8 However, no specific gene mutations other than PIG-A have been reported in patients with PNH—with one exception. In two patients, a rearrangement of chromosome 12 with a break in the 3' untranslated region of the HMGA2 gene has been reported.9 Overexpression of an HMGA2 truncated protein recapitulates an myeloproliferative neoplasms (MPN)-like phenotype in a murine model and could theoretically contribute to clonal expansion in PNH.10

Interestingly, literature from the 1970’s has reported several cases of PNH in association with myelofibrosis and other MPNs.11,12 Here we report on three index cases of PNH with myeloproliferative features harboring a JAK2V617F mutation, which is now understood to drive clonal expansion in many MPNs.13

The first case is a 51-year-old male, presenting with right hemiparesis and dysarthria secondary to a stroke, followed by multiple thrombotic events, including the Budd Chiari Syndrome (BCS). A hypercoagulable workup revealed the presence of the JAK2V617F mutation in the peripheral blood. Upon referral to us, a complete

Figure 1. Morphological features of the bone marrow in patients with PNH and the JAK2V617F mutation. Patient 1: (i) hematoxilin–eosin-stained section showing hyperplasia and mild dysplasia. (ii) Anti-factor VIII section showing increased number of megakaryocytes (stained in brown) with mild dysplasia. (iii) Reticulin stain showing moderately increased reticulin staining (grade 2), indicative of mild fibrosis. Patient 2: (i) hematoxilin–eosin-stained section, showing hypercellularity and an increase in small hypolobated megakaryocytes. The myeloperoxidase stain (data not shown) is strongly positive in most of the cells. (ii) Wright stain. There were no spicules present, but the aspirate is richly cellular and demonstrates predominance of myeloid cells with an M:E ratio of ~ 8:1. (iii) Reticulin stain showing diffuse increase in reticulin. Trichome stain was negative. Patient 3: (i) hematoxilin–eosin-stained section, 2006, showing normocellularity; megakaryocytes were present but not increased; (ii) repeat marrow examination in 2010, demonstrating marked distortion of the architecture by fibrosis; reticulin stain was 4+–positive at this time. (iii) peripheral smear findings in 2011 typical of a myeloproliferative syndrome: nucleated red blood cells, giant PLTs and occasional teardrops.
blood count noted an Hgb of 5 g/dl, platelets (PLTs) of 492 x 10^9/l and a white blood cell (WBC) 8.90 x 10^9/l. Bone marrow biopsy revealed a hypercellular marrow (80-100%), dysmegakaryopoiesis, a 4% myeloblast population and normal cytogenetics (Figure 1). The patient later presented with an elevated lactate dehydrogenase (LDH) and undetectable haptoglobin. Flow cytometry

Figure 2. Cellular origin of PNH and JAK2V617F mutation. (a) To clarify the cellular origin of JAK2V617F mutation, GPI (-) cells and GPI (+) cells were isolated with fluorescence-activated cell-sorting method using a FACSAria (BD Biosciences, San Jose, CA, USA). CD11b+ FLAER+ (GPI-) granulocytes, CD11b+ FLAER+ (GPI+) granulocytes and CD3+ FLAER+ (GPI+) lymphocytes were isolated from patient no.1, whereas CD11b+ FLAER- (GPI-) granulocytes and CD3+ FLAER- (GPI+) lymphocytes were isolated from patient no.2. Purity of sorted CD11b+ FLAER- (GPI-) granulocytes from patient no.1 exceeded 99%. CD11b+ FLAER+ (GPI+) granulocytes were 83% pure. From each population, genomic DNA was isolated using the PureLink DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The DNA tetra-primer ARMS was performed to detect the JAK2V617F mutation using a mutant and a specific forward primer with a common reverse primer. Presence of the mutation resulted in the amplification of a 203-bp DNA product; wild-type primers generated a 364-bp product. HEL and K562 cells were included as positive and negative controls for the JAK2V617F mutation, respectively. This analysis was non-quantitative, but demonstrated the presence and absence of JAK2V617F as shown (+ vs -) in different cell populations. (b) Waterfall plot displaying clinical characteristics of JAK2V617F-positive mutants PNH patients (red) compared with JAK2V617F-negative mutants (black). LDH (IU/l), % GPI (+) granulocytes, % GPI (-) erythrocytes, WBC, (cells x 10^9/l), hemoglobin (g/l) and PLT (cells x 10^9/l) count. The percentage of GPI (-) granulocytes, WBC and PLT are all statistically higher (Mann-Whitney) in JAK2 mutant patients compared with controls, whereas LDH and GPI (-) erythrocytes are not. Analyses were performed on samples obtained from patients after provision of informed consent as per institutional protocols.
Serum LDH levels were elevated in most patients (mean 1204 IU/l, 72.3% (median 32.4%) by FLAER staining and flow cytometry. (median 71.6%) and GPI (C0 and 7 (23%) had antecedent aplastic anemia. The patient developed transfusion-dependent anemia secondary to progressive liver failure due to iron overload.

The third patient is a 78-year-old male with a history of prostate cancer in 2004, treated with brachytherapy. He also had a history of arthritis, pulmonary hypertension and cutaneous melanoma. In 2003, he was mildly anemic, and in 2006, a marrow examination revealed hypercellularity without excess blasts. In 2009, he noticed arthritis, pulmonary hypertension and cutaneous melanoma. In 2003, he was mildly anemic, and in 2006, a marrow examination revealed hypercellularity without excess blasts. In 2009, he noticed dark urine; the Hgb was 102 g/l with 4.5% reticulocytes, PLTs 648 × 10^9/l, WBC 10.1 × 10^9/l, 77% polys, 15% lymphocytes and 7% monocytes. The LDH was 1367 IU/L (normal < 243), and flow cytometry demonstrated 73% CD59-negative granulocytes and 53% CD59-negative red cells. Repeat marrow examination in 2010 was notable for hypercellularity and severe fibrosis (Figure 1). The karyotype was normal (20/20), and the JAK2V617F mutation was identified. The patient developed transfusion-dependent anemia not responding to eculizumab, steroids or danazol. At the time of referral in 2011, the WBC was 12.1 x 10^9/l with 71% polys, 3% metamyelocytes, 2% myelocytes, 1% blasts, 10% lymphocytes, 8% monocytes, 3% basophils and 1% nucleated red blood cells; Hgb was 79 g/l and the absolute reticulocyte count was 97 500/μl. The peripheral blood smear demonstrated poikilocytosis, rare teardrops, some giant PLTs and giant early myeloid precursors. The spleen was not palpable. Repeat analysis demonstrated 43% CD59-negative red cells and 99.7% FLAER-negative, CD24-negative granulocytes. Molecular testing again revealed the JAK2V617F mutation. He was treated with eculizumab, aspirin and fondaparinux. The patient had several bouts of pneumonia and eventually succumbed in September 2011 to a severe Clostridium difficile infection.

A total of 26 subsequent patients (mean age, 36 years; range, 15–65) with PNH were tested for the JAK2V617F mutation by the DNA tetra-primer amplification refractory mutation system (ARMS). All patients had intravascular hemolysis and were considered to have classic PNH. A total of 23 cases (77%) were de novo and 7 (23%) had antecedent aplastic anemia. The percentage of GPI (–) granulocytes ranged from 22 to 99% (median 71.6%) and GPI (–) erythrocytes ranged from 0.86 to 72.3% (median 32.4%) by FLAER staining and flow cytometry. Serum LDH levels were elevated in most patients (mean 1204 IU/l, range 129–4393 IU/l, normal < 618 IU/l). The median WBC was 4.48 × 10^9/l (range, 2.44–8.90), median Hgb level was 117 g/l (range, 64–14.0) and median PLT count was 108 × 10^9/l (range, 28–648). Hepatic, cerebral or mesenteric thrombosis was found in 6 of the 29 patients (21%), and 3 had BCS. On the basis of the ARMS analysis, the JAK2V617F mutation was not detected in any of these 26 patients.

In both case 1 and 2, the molecular lesion in the PIG-A gene was identified to be a 500-kb deletion at Xp22.2 by single-nucleotide polymorphism array, as we have published previously. To determine the cellular origin of the JAK2V617F mutation in these two patients, DNA was extracted from GPI (–) CD11b+ granulocytes, GPI (+) CD11b+ granulocytes and CD3+ T cells after FLAER staining and flow cytometry sorting. JAK2V617F was selectively detected in GPI (–) granulocytes, but not in GPI (+) granulocytes or T cells, clearly showing that the JAK2V617F mutation is not in the germline and that it co-exists within in the PNH clone (Figure 2).

The occurrence of PNH clones in MDS and aplastic anemia is well documented and routinely evaluated in clinical practice. This series now documents the coexistence of PNH and JAK2V617F, associated MPNs in three index cases with a higher PLT count and WBC compared with PNH patients overall (Figure 2b). Two of the three cases were shown to harbor the JAK2V617F mutation within the PNH clone. In the third patient, 99% of the granulocytes were GPI (–), suggesting that JAK2V617F mutation also occurred within the PNH clone. Apart from HMGA2, this now represents the second mutation coexisting with PNH that could explain clonal expansion. The prevalence of PNH clones among MPN patients is unknown, because these three index patients were ascertained by referral. In rare patients, even before the era of flow cytometry and molecular testing, a MPN/PNH overlap syndrome was identified. Of note, no JAK2V617F mutations were detected in 26 subsequently screened PNH patients, suggesting that the prevalence of this mutation is not high in classic PNH, consistent with an earlier report.

On the basis of these findings, we believe that JAK2 mutation testing should be performed on patients with PNH, who have elevated peripheral blood counts. Considering that most patients with PNH are mildly cytopenic (Figure 2b), this recommendation might extend to those with blood counts towards the upper limit of normal. Unexplained splenomegaly or a fibrotic marrow would also represent an indication for JAK2 mutation analysis. Conversely, patients with an MPN and signs of hemolysis should be tested for PNH by flow cytometry.

CONFLICT OF INTEREST
Dr Epling-Burnette received a grant from Genzyme Corporation for the development of the PNH test. Dr Araten is on the Scientific Advisory Board of Alexion, Inc. Dr Lee has attended advisory meetings for Alexion, Millenium and Novartis. All other authors have no potential conflicts of interest.

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C Sugimori1,2, E Padron3,7, G Caceres2,7, K Shain2, L Sokol2, L Zhang3, R Tiu4, CL O’Keefe4, M Afable4, M Clemente4, JM Lee5, C Sugimori1,7, E Padron2,7, G Caceres2,7, K Shain2, L Sokol2, L Zhang3, R Tiu4, CL O’Keefe4, M Afable4, M Clemente4, JM Lee5, JP Maciejewski7, AF List5, PK Epling-Burnette1,8 and DJ Araten6,8
1Department of Immunology, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA; 2Malignant Hematology Division, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA;
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