Dear Editor,

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder caused by mutation of the X-linked PIGA gene, resulting in a deficient expression of glycosylphosphatidylinositol-anchored proteins, such as CD55 and CD59. Patients with PNH may present with hemolytic anemia, thrombosis, and bone marrow failure. The loss of CD55 and CD59 renders PNH erythrocytes susceptible to intravascular hemolysis and thrombosis.

There is a close relationship between PNH and aplastic anemia (AA). The clinical picture may shift from one to the other during the course of disease. Genes commonly mutated in myeloid neoplasms have been tested in patients with AA, and some carry prognostic significance. For instance, mutations in PIGA, BCOR, and BCORL1 correlate with a better response to immunosuppressive therapy and a longer duration of overall survival and progression-free survival in patients with AA, whereas mutations in DNMT3A, RUNX1, JAK2, JAK3, and CSMD1 are associated with a worse prognosis. However, studies on the mutations of myeloid cancer-related genes in PNH and on the mechanism of PNH clonal expansion are limited or inconclusive. On the other hand, thrombosis is the most common complication in patients with PNH. Although the risk of thrombosis correlates with the PNH clone size, thrombotic events do occur in patients with small PNH clones. Recent studies have uncovered that mutations rather than PIGA may function as additional risk factors for thrombosis, but the results vary among different studies.

In this study, we investigated the mutational profiles of 41 patients with newly diagnosed PNH as well as the CD59+ and CD59- cell fractions of peripheral blood from 6 PNH patients by whole-exome sequencing. We further examined the relations between these mutations and patients’ clinical and laboratory parameters, in particular, we examined the roles of these mutations in the expansion of PNH clones and thrombosis.

The study cohort included 12 patients with PNH and 29 with PNH/AA. There were 29 men and 12 women with a median age of 35 years (range, 15–72). Thirty-nine patients had anemia (Hgb median 78 g/L, range 36–140 g/L), 20 had leukopenia (WBC median 4.11 × 10^9/L, range 1.5–10.9 × 10^9/L), and 20 had thrombocytopenia (PLT median 110 × 10^9/L, range 11–349 × 10^9/L). Fifteen patients had pancytopenia. Twenty-three patients had increased unconjugated bilirubin (UCB) and 39 had increased lactate dehydrogenase (LDH). The median PNH clone sizes were 83% (range, 10–98%), 82% (range, 10–98%), and 48% (range, 0–97%) by the proportions of FLAER- granulocytes, CD59- granulocytes, and CD59-RBCs, respectively.

Thirteen (31.7%) patients had a history of thrombosis. The median PNH clone size (FLAER negative granulocytes) was 83% (range, 18–95%) in those with thrombosis and 83% (range, 10–98%) in those without (p = 0.688). Coronary artery was the most common site of thrombosis (23.1% of events), followed by visceral vein and/or deep vein (15.4% of events each). Four patients had thrombosis in multiple sites. Common inherited hypercoagulable states (factor V Leiden mutation, prothrombin gene mutation, deficiency of protein S, protein C, or antithrombin) were not detected in those PNH patients. There was no difference in the baseline characters between patients with or without thrombosis, except the value of D-dimer (Supplementary Table S1).

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Of the 178 genes frequently mutated in myeloid neoplasms (Supplementary Methods), 158 were mutated in our cohort (Fig. 1A). All 41 patients had mutations and 39 (95%) had multiple mutations. The average mutation load was 3.85 genes per patient (range, 1–9). As expected, PIGA was most commonly
mutated and detected in 22 patients (53.7%). The types of PIGA mutations included truncation (n = 3), splicing-site mutations (n = 2), frame-shift deletion (n = 2), and missense mutation (n = 1); additional mutations located in intronic sites (n = 11) or in the 3’ untranslated region (n = 3). PIGT was mutated in one (2.4%) patient. Following PIGA gene, the most commonly mutated genes included MAP3K4 and CSMD1, detected in 5 patients (12.2%) each. Genes mutated in 4 patients (9.8%) each included NOTCH1, FANCED2, RUNX1TI, PEG3, DIS3, BCORL1 and SETBP1. Genes mutated in 3 patients (7.3%) each included FANCG, RAD50, FANCA, CDH23, UMODL1, BRAF, and NCOR2. In addition, 24 genes mutated in 2 patients and 43 in 1 patient each (Supplementary Table S2).

We then examined whether these mutations correlated with clinical and laboratory parameters. We focused on 10 genes with the highest frequency of mutation, including PIGA, BCORL1, RUNX1TI, MAP3K, CSMD1, NOTCH1, FANCED2, PEG3, DIS3, and SETBP1 (Table 1). Patients with PIGA mutation had a larger PNH clone size than those without (90.6 ± 7.4% vs 64.4 ± 32.1%, p < 0.001), and were predominantly females (58.3% vs 3.6%, p < 0.001). Patients with BCORL1 mutation were 20 years older (57 ± 19 vs 37 ± 13 year-old, p = 0.007). Patients with RUNX1TI mutation had a larger PNH clone of granulocytes (92.8 ± 5.4% vs 67.1 ± 31.3%, p < 0.001), a lower hemoglobin level (55.5 ± 13.3 g/L vs 83.7 ± 22.6 g/L, p = 0.020), and a higher level of unconjugated bilirubin (30.9 ± 16.2 μmol/L vs 15.6 ± 9.8 μmol/L, p = 0.008), suggestive of a higher tendency of hemolysis. No difference in clinical and laboratory parameters was observed between patients with and patients without these uncommonly mutated genes (Supplementary Table S2).

To further explore the correlation between the PNH clone size and the mutational frequency of genes indicating worse outcome in AA, we divided the patients into 3 groups according to their clone size: ≤40% (n = 9), 41–80% (n = 10), and >80% (n = 22). The mutational frequency of those genes was 44% (4/9) in patients with PNH clone size ≤40%, 30% (3/10) in those with clone size of 41–80%, and 9% (2/22) in patients with clone size >80%. Logistic regression analysis indicated that the mutational frequency declined with the increase of PNH clone size (p = 0.026) (Fig. 1B).

Next we investigated the potential role of mutations in clonal expansion in sorted CD59+ and CD59− cells of peripheral blood from 6 patients with a relatively large PNH clone. Overall the mutation frequencies were similar in CD59− and CD59+ population, except for PIGA mutation, which was detected only in CD59− population. We then searched for those associated with cell proliferation according to the criteria in Supplementary Methods and compared them between sorted CD59− and CD59+ cells. Of the 723 genes associated with cell proliferation, 210 were found mutated in the 6 patients. The mutational loads of those 210 genes were 46.7 (range, 41–53) and 48 (range, 42–52) in the CD59− and CD59− cells, respectively. Mutated genes unique in CD59− cells included MUC16, NCOR2, PTPN11, CIC, MAML2, BCR, RGPD3, ARID1A, KMT2C, MSH2, NCOR2, and TCL1A, and mutations found in CD59+ cells only included ROBO2, SF3B1, H2B3, and BCR. Overall, there was a strong trend toward more cell proliferation or clone expansion associated mutations in CD59− cells than in CD59+ cells (p = 0.062) (Supplementary Table S3).
Then we examined the potential role of mutations in thrombosis. A total of 55 thrombosis-related genes (Supplementary Table S4) were selected as the candidate genes according to the criteria in Supplementary Methods. No difference in mutation rate was found in those candidate genes between CD59+ and CD59- cells. In addition, mutation in SRRD gene was more common in patients with visceral thrombosis than those with thrombosis in other sites (80% vs 12.5%, \( p = 0.032 \)), whereas EGR4 mutation was more common in patients with myocardial infarction (100% vs 11.1%, \( p = 0.007 \)) (Fig. 1C). In patients with thrombosis, the number of those candidate mutations was negatively correlated with the PNH clone size (\( p = 0.036, R^2 = 0.341 \)) (Fig. 1D).

Table 1 Correlation of common mutations with clinical and laboratory features in PNH.

| Gene  | Patients with thrombosis (%) | FLAER-proportion of fluorescent aerolysin-negative granulocytes | UCB (μmol/L, SD) | HGB (g/L, SD) | RET (10⁹/L, SD) | LDH (U/L, SD) |
|-------|-------------------------------|---------------------------------------------------------------|------------------|---------------|---------------|---------------|
| PIGA  | Mutated 39 (12) 13*** 50 50 | 90.6 (7.3)*** 15.8 (10.0) 79.9 (15.1) 21.0 (12.6) 1311 (899)  |
|       | Unmutated 39 (14) 85*** 45 27 | 64.4 (32.1)*** 17.4 (11.7) 81.2 (25.1) 30.5 (63.7) 1159 (734)  |
| MAP3K4| Mutated 34 (14) 60 20 20 | 72.4 (35.4) 14.2 (13.2) 72.8 (18.8) 10.8 (10.8) 1189 (1057)  |
|       | Unmutated 40 (15) 72 50 33 | 68.2 (30.6) 17.5 (11.1) 82.1 (23.9) 30.8 (60.8) 1189 (729)  |
| CSMD1 | Mutated 37 (19) 60 20 0 | 56.6 (42.9) 12.6 (8.8) 79.8 (27.1) 9.9 (6.8) 779 (565)  |
|       | Unmutated 40 (14) 72 50 33 | 71.4 (29.0) 17.7 (11.5) 81.1 (23.2) 31.5 (61.6) 1246 (771)  |
| NOTCH1| Mutated 41 (9) 50 75 50 | 63.0 (37.4) 9.8 (4.8) 94.5 (26.1) 15.0 (8.4) 787 (404)  |
|       | Unmutated 39 (15) 73 43 30 | 70.3 (30.5) 17.8 (11.5) 79.5 (22.9) 30.3 (61.0) 1232 (779)  |
| FANCD2| Mutated 37 (16) 20 25 25 | 67.7 (35.6) 11.1 (8.9) 72.5 (20.4) 18.8 (19.0) 1165 (1323)  |
|       | Unmutated 40 (14) 76 49 32 | 69.8 (30.7) 17.7 (11.4) 81.9 (23.7) 29.9 (60.9) 1191 (703)  |
| RUNX1T1| Mutated 36 (4) 100 75 0 | 92.8 (5.4)*** 30.9 (16.2) 55.5 (13.3)* 31.6 (30.4) 1301 (565)  |
|       | Unmutated 40 (15) 68 43 32 | 67.0 (31.3)*** 15.6 (9.8)** 83.7 (22.6)* 28.4 (60.5) 1177 (783)  |
| PEG3  | Mutated 50 (22) 75 0 25 | 59.5 (33.7) 16.7 (11.7) 81.3 (23.0) 54.6 (85.4) 1355 (1141)  |
|       | Unmutated 38 (13) 70 51 32 | 70.6 (30.7) 17.1 (11.4) 81.0 (23.7) 25.8 (54.9) 1171 (727)  |
| DIS3  | Mutated 40 (19) 100 75 25 | 75.7 (15.0) 13.9 (6.7) 92.5 (34.3) 13.7 (10.1) 970 (502)  |
|       | Unmutated 39 (14) 68 43 32 | 68.9 (32.0) 17.4 (11.7) 79.7 (22.2) 30.5 (60.9) 1212 (783)  |
| BCORL1| Mutated 57 (19)** 50 0 25 | 53.2 (28.6) 17.9 (11.1) 65.3 (17.3) 53.8 (85.4) 1316 (1069)  |
|       | Unmutated 37 (13)** 73 51 32 | 71.3 (30.8) 17.0 (11.4) 82.7 (23.5) 25.9 (55.0) 1175 (737)  |
| SETBP1| Mutated 47 (13) 100 75 0 | 69.0 (35.7) 16.9 (8.9) 76.3 (28.2) 13.1 (10.4) 1050 (639)  |
|       | Unmutated 38 (15) 68 43 32 | 69.6 (30.7) 17.1 (11.6) 81.5 (23.2) 30.6 (60.9) 1204 (777)  |

Measurement data were presented as average (standard deviation).

* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
those with relatively smaller PNH clone sizes tended to have more thrombosis-related mutations, especially mutations of PADI1 (p = 0.001), SLC2A9 (p = 0.002) and TCF3 (p = 0.011) gene.

In summary, by whole-exome sequencing 10 most frequently mutated genes in PNH included PIGA, BCORL1, RUNX1T1, MAP3K4, CSMD1, NOTCH1, FANCD2, PEG3, DIS3, and SETBP1. PIGA mutation was associated with a larger PNH clone size and female sex, BCORL1 mutation was associated with a younger age, and RUNX1T1 mutation correlated with a larger PNH clone size, a lower hemoglobin level, and a higher level of unconjugated bilirubin. Mutations indicating an unfavorable outcome in AA were uncommon in PNH and as one group associated with a smaller PNH clone size, a lower level of LDH, and a lower level of unconjugated bilirubin. CD59- fraction tended to have more mutations in proliferation-related genes compared with CD59+ fraction. Thrombosis in different sites demonstrated different gene mutations. SRRD mutation was associated with visceral thrombosis and EGR4 mutation was associated with myocardial infarction. For the first time, we demonstrated the clinical significance of mutation profile in PNH, particularly, in PNH clonal expansion and thrombosis.

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Conflict of interest
The authors declare no competing interests.

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