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## Implication of *PIGA* genotype on erythrocytes phenotype in Paroxysmal Nocturnal Hemoglobinuria

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### To the Editor:

Somatic mutations of the X-linked phosphatidylinositol N-acetylglucosaminyltransferase subunit A (*PIGA*) gene in hematopoietic stem cells are the key molecular events in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH) [1]. *PIGA* mutations lead to impaired biosynthesis of glycosylphosphatidylinositol (GPI)-anchor [2]. Resultant lack of GPI-anchored proteins (GPI-AP), primarily CD55 and CD59, generates clonal selection of PNH clone(s) and increases sensitivity of PNH red cells (RBCs) to complement-mediated hemolysis [3]. Based on immuno-fluorescent staining, the grade of GPI-APs deficiency on the RBCs can distinguish: PNH type-1 (TI) with normal expression; PNH type-2 (TII) with partial reduction; and PNH type-3 (TIII) with total absence of two common GPI-APs (CD55 and CD59) [4]. Fractions of these RBC types are believed to be a result of the molecular spectrum of *PIGA* mutations, ranging from missense in various amino acids positions to

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#### Author contributions

CG and JPM generated and conceived the study design, figures, tables and manuscript. JPM and BJP collected and analyzed experimental patients' data. CG and SP performed statistical analyses. VV, SP, BJP, HA, SK, LT, MZ, SC, AEL, HEC, and AW kindly reviewed the clinical data, took a part in samples selection and helped edited the manuscript. All authors participated in data interpretation and critical review of the final paper and submission.

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truncations and locus deletions, which can be concurrent in some patients [5, 6]. Consequently, the presence of TII and TIII cells may indicate the overall severity of *PIGA* defect, and thus, individual phenotypic subtypes can be defined as predominantly TII or TIII according to the dominant clonal defect [7, 8].

We hypothesize that *PIGA* genotype may shape the PNH phenotype trajectory with regard to the severity of GPI-AP deficiency and clinical course. For instance, incomplete TII RBCs PNH phenotype should result in slower expansion due to incomplete selection advantage as compared to complete loss of *PIGA* function indicated by TIII RBCs, typical of hemolytic PNH. However, the process of “GPI-AP painting”, a passive transfer from normal to TIII RBC could result in the presence of pseudo-TII RBCs with an intermediate phenotype [9]. Here, we first used modern deep targeted DNA sequencing to characterize the molecular landscape of patients with PNH and then correlated the type of *PIGA* mutations with flow-cytometric phenotypes. For improved resolution, our study focused on patients with large clones and hemolytic PNH.

Among 295 diagnosed patients who had a PNH clone (>0.5% of PNH granulocytes), we identified 60 who fulfilled the criteria for fully blown hemolytic PNH (PNH clone size >20% and lactate dehydrogenase >2.5x upper limit of normal). Test for PNH was conducted before initiation of complement blockade therapy [10, 11]. Whole blood DNA was subjected to multi-amplicon deep next generation sequencing (NGS) using primers covering all coding exons of *PIGA* gene as previously shown [12]. Five-color flow cytometry strategy was conducted as previously described [13, 14] for detection of PNH granulocytes. In particular, fresh specimens were stained with CD15-V450, CD45-PC7, CD64-APC, CD157-PE, FLAER-Alexa 488, while CD59-PE and CD235a-FITC antibodies were used to determine PNH RBCs (see also Supplementary Appendix).

Of 60 patients (median age 37 years, range 5–84, see also Table 1 and Supplementary Table 1), 13/60 (22%) were classified as having TII and 47/60 (78%) TIII PNH. Overall, median PNH granulocyte clone size was 71.4% (21.5–99.4) with no differences between TII/TIII subgroups ( $p = 0.872$ ). Median TII RBC clone was 40.9% (6.8–84.1) and 1.9% (0.54–30) in TII and TIII PNH patients while median TIII RBC clone was 3.2% (1.2–21.8) and 22.1% (11.4–93.4), respectively. No differences were found at baseline between the two subgroups for male:female ratio, blood counts, and previous history of aplastic anemia (AA) while TIII patients had higher lactate dehydrogenase (LDH) levels (571 vs. 346 U/L in TII,  $p = 0.015$ ), probably expression of the increased complement sensitivity resulting from total loss of GPI-APs. Thrombotic events occurred in 17/60 (28%) of patients with no differences in TII vs. TIII cases (46% vs. 23%,  $p = 0.162$ ). Majority of cases 15/17 (88%) were registered before anti-complement treatment start and typically involved venous districts (16/17, 94%) with no fatal events. (See also Supplementary Table 2).

A total of 137 *PIGA* mutations were found with 27 in TII and 110 in TIII dominant patients (Fig. 1a–c, Fig. S1). Overall, >1 and >2 mutations (clonal mosaicism) were found in 85% and 57% of patients, respectively. Frameshift ( $n = 54$ , 39%) and missense ( $n = 41$ , 30%) mutations were most common, followed by splice site ( $n = 22$ , 16%), nonsense ( $n = 15$ , 11%) and non-frameshift insertion/deletion ( $n = 5$ , 4%). Although *PIGA* mutations are

distributed throughout the entire coding region, TIII patients harbouring non-frameshift hits more often had truncating mutations mapping within the first 200 bps across the *PIGA* domain. Median total variant allele frequency (VAF) adjusted for X-chromosome clonality burden was 17.9% (range, 1.2–63%) and correlated with number of mutation ( $p = 0.0007$ ) but without differences between TII/TIII patients ( $p = 0.345$ ). Overall, *PIGA* mutations VAF correlated with PNH granulocytes clone size in both the entire cohort ( $p < 0.0001$ ) and in the two subgroups of patients (TII,  $p = 0.02$ ; and TIII,  $p < 0.0001$ ; Fig. S2).

In cases negative for *PIGA* mutations by NGS (9/60, of which 8/9 presenting TIII-dominant PNH RBC clones), no mutations were found even after sorting to enrich for GPI (–) cells. This is likely due to inability to detect deletions larger than 250 bp by targeted sequencing of *PIGA* gene [6, 12]. To further understand the molecular basis of cases with wild type *PIGA*, we collected results of single-nucleotide polymorphism (SNP) array-based karyotyping to detect microdeletions of Xp22.2 encompassing the *PIGA* locus, an alternative mechanism that ultimately leads to the same selective clonal advantage as hypomorphic mutation of the *PIGA* gene as we previously showed [6]. Of the 9 negative cases, one of the patients (UPN 3) harboured a submicroscopic chromosomal Xp22.2 deletion. Furthermore, we investigated the presence of mutations in other genes involved in GPI biosynthetic pathway *e.g.*, *PIGT*, previously shown to be responsible for atypical PNH cases without *PIGA* mutations and with a clinical picture characterized by the presence of inflammatory symptoms. Of note, our cases were negative for *PIGT* and did not show any of these symptoms [15].

Our results demonstrate that missense *PIGA* variants were enriched in TII PNH, while frameshift deletions in TIII dominant patients ( $p = 0.0021$  and  $0.0201$ , respectively). Moreover, when only the dominant clone ( $>2\times$  the remaining VAF) was analysed, missense and frameshift mutations were strong predictors of PNH RBC phenotype, defining TII RBC ( $p = 0.0002$ ) and TIII RBC patients ( $p = 0.02$ ), respectively. Of note, we also found higher LDH levels in patients carrying truncating vs. missense mutations (median LDH 900 vs 378 U/L,  $p = 0.04$ ), further emphasizing the higher hemolysis tendency of completely GPI-deficient TIII cases deriving from disruptive genomic lesions. When patients harbouring one unique somatic hit were studied (16/60, 27%), missense mutations accounted only for 19% of cases. Finally, analysing an internal cohort of 89 AA patients with small PNH granulocytes clones ( $<20\%$ ), we found that missense mutations accounted for 43% of cases indicating incomplete immune privilege.

*PIGA* missense and truncating mutations dictate the susceptibility of PNH RBCs to complement. In sum, we show that while missense mutations may still lead to a deficient but not totally absent GPI-APs synthesis, truncating mutations seem to be responsible for an entirely disruptive phenotype, causing the complete absence of GPI-APs on RBC membrane surface. Moreover, the non-missense TII dominant patients (pseudo-TII) may be an epiphenomenon of the “painting” process [9, 15] (Fig. 1d). Our results suggest also that complete molecular screening and results of SNP array-based karyotyping to detect mutations or microdeletions of *PIGA* locus and other genes of the GPI synthetic pathway may help to rule out other causes explaining the PNH phenotype of cases lacking *PIGA* mutations. Finally, to overcome the limits of conventional targeted sequencing strategies,

whole genome scanning approaches may also better magnify the diagnostic power in selected cases resulting negative for the aforementioned classical approaches.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

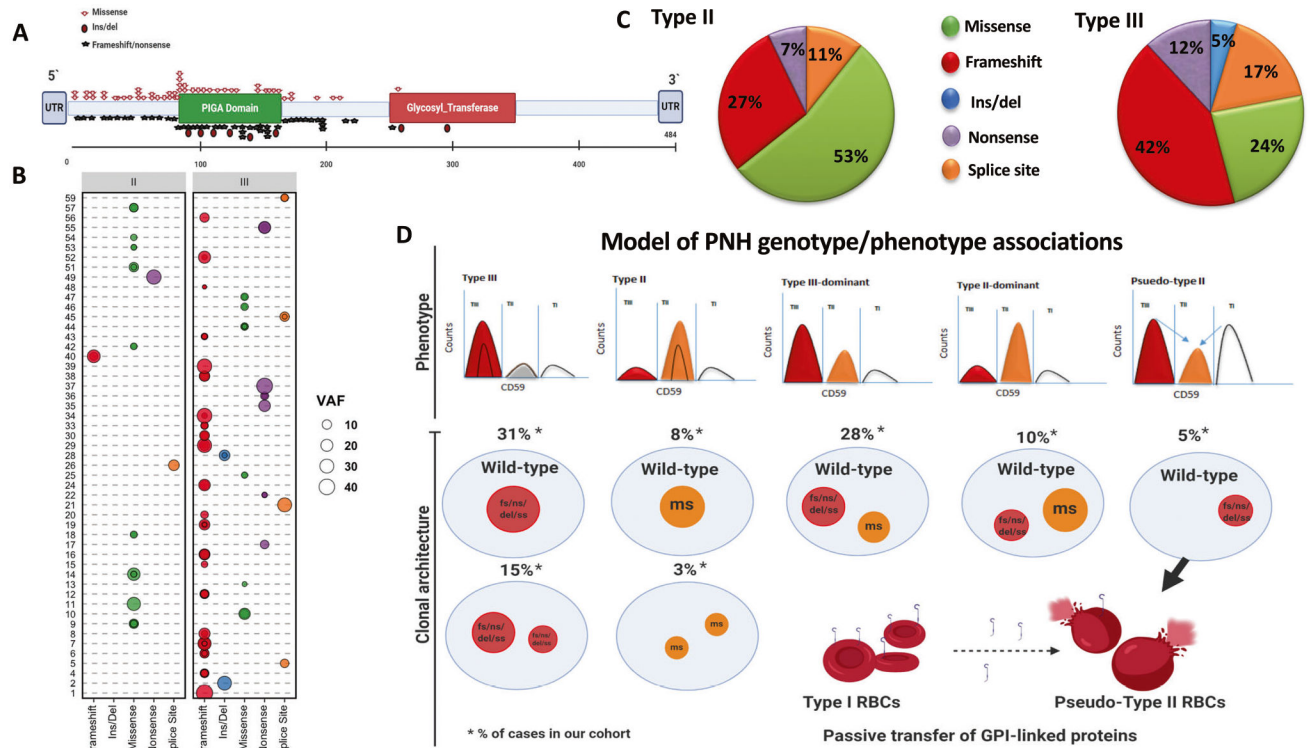
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**Fig. 1. PIGA mutational landscape of hemolytic PNH.**

**A** Lollipop plot for amino acid changes resulting from *PIGA* mutations. **B** Bubble chart outlining PNH patients and their mutations according to the type and the size (VAF) of *PIGA* somatic hits (the area of the bubble is proportional to *PIGA* VAF). **C** Pie charts showing the differences in mutation types between patients with type II and type III RBCs. **D** Model depicting the various possibilities of *PIGA* mutations and the resultant phenotypes: TII RBCs phenotype in the presence of a dominant missense mutation (8% of cases in our cohort) or multiple missense mutations (3%); TIII RBCs phenotype in the presence of a truncating mutation (31%) or in the presence of multiple truncating mutations (15%); T-III dominant in case of a dominant truncating and a subclonal missense mutation (28%); TII-dominant in case of a dominant missense and a subclonal truncating mutation (10%); pseudo-TII RBCs phenotype in case of patients with TII RBCs phenotype harboring truncating mutations (5%). Pseudo-TII RBC may result from the passive transfer of GPI-linked proteins from normal RBCs (type I) to the GPI-deficient TIII RBCs generated by non-missense mutations in TII dominant patients (pseudo-TII), a phenomenon called “painting” process. fs frameshift, del/ins deletion/insertion, ss splice site, ns nonsense, ms missense, VAF variant allelic frequency calculated as the sum of all mutations found in an individual patient (\*adjusted for X-chromosome zigosity).

Table 1

Patients' characteristics.

	ALL	Type-II dominant	Type-III dominant	<i>p</i> value
Overall	N 60	13 (21%)	47 (79%)	
Gender	F 33 (55%) M 27 (45%)	9 (69%) 4 (31%)	24 (51%) 23 (49%)	0.348
Age	37 [5–84]	39 [15–80]	36 [5–84]	0.501
Diagnosis	pPNH 28 (46%) sPNH 32 (53%)	4 (30%) 9 (70%)	24 (51%) 23 (49%)	0.348
Granulocytes clone size (%) <sup>a</sup>	71.49 (21.5–99.9)	69.5 (21.5–99.5)	78.2 (22.1–99.9)	0.872
Type II RBC clone size (%) <sup>a</sup>	2.41 (0–84.6)	40.9 (6.8–84.1)	1.9 (0.54–30)	
Type III RBC clone size (%) <sup>a</sup>	14.75 (0–93.4)	3.2 (1.2–21.8)	22.1 (11.4–93.4)	
Hb (gr/dl) at diagnosis <sup>a</sup>	9.9 [8–11]	9.3 (8.3–10.45)	10(8.1–11.4)	0.452
ANC (10 <sup>9</sup> /L) diagnosis <sup>a</sup>	2 (1.19–3)	2.4 (1.17–2.85)	2 (1.27–3.05)	0.98
PLT (10 <sup>9</sup> /L) at diagnosis <sup>a</sup>	68 (5–241)	37 (15–148)	87 (5–241)	0.085
LDH (U/L) at diagnosis <sup>a</sup>	498 (170–3666)	346 (170–939)	571 (196–3666)	0.015
Median Follow-up (months)	102.7 (0.5–375)	82.3 (11–328)	109 (0.5–375)	

<sup>a</sup> All values are expressed in median (ranges).

F female, M male, RBC red blood cells, Hb hemoglobin, ANC absolute neutrophils count, PLT platelets, pPNH primary paroxysmal nocturnal hemoglobinuria, sPNH secondary paroxysmal nocturnal hemoglobinuria, LDH lactate dehydrogenase.