RED CELLS, IRON, AND ERYTHROPOIESIS

A case of paroxysmal nocturnal hemoglobinuria caused by a germline mutation and a somatic mutation in PIGT

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Key Points
- A carrier of a deleterious splice site mutation in PIGT acquired a second hit in PIGT and developed PNH.

To ascertain the genetic basis of a paroxysmal nocturnal hemoglobinuria (PNH) case without somatic mutations in PIGA, we performed deep next-generation sequencing on all exons of known genes of the glycosylphosphatidylinositol (GPI) anchor synthesis pathway. We identified a heterozygous germline splice site mutation in PIGT and a somatic 8-MB deletion in granulocytes affecting the other copy of PIGT. PIGA is essential for GPI anchor synthesis, whereas PIGT is essential for attachment of the preassembled GPI anchor to proteins. Although a single mutation event in the X-chromosomal gene PIGA is known to cause GPI-anchored protein deficiency, 2 such hits are required in the autosomal gene PIGT. Our data indicate that PNH can occur even in the presence of fully assembled GPI if its transfer to proteins is defective in hematopoietic stem cells. (Blood. 2013;122(7):1312-1315)

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia that results from the expansion of hematopoietic stem cells that are deficient for glycosylphosphatidylinositol (GPI), a glycolipid moiety that anchors >100 different proteins to the cell surface.1,2 PNH patients were reported to be deficient for an initial step in the GPI anchor synthesis that is catalyzed by the GPI-GlcNAc transferase,3,6,7 and somatic mutations in the X-chromosomal gene PIGA that encodes a subunit of this transferase complex8 are regarded as the causative event in the predominant number of PNH cases.2,5,7,9 However, in a small number of PNH cases with a clear GPI anchor deficiency, no mutations in PIGA have been found.

In this work, we report about 2 mutation events, a germline splice site mutation and a somatic deletion in PIGT, which is another gene of the GPI anchor synthesis pathway, that we identified performing next-generation sequencing in a PNH patient with wild-type PIGA.

Study design

Patient sample

This study was conducted in accordance with the Declaration of Helsinki. Genetic analysis was performed after approval by ethical committee and informed consent.


The online version of this article contains a data supplement.

There is an Inside Blood commentary on this article in this issue.

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proteins, CD55 and CD59, as previously described. Two days later, lysates of B lymphocytes did not express the GPI-AP CD48. The percentages of cells with reduced or absent GPI-AP, ie, PNH cells, and normal range is shown in the supplemental Materials. (B) PIGT-deficient Chinese Hamster Ovary cells were transiently transfected with wild-type or a mutant version skipping exon 11 of transcript NM_0015937. (Left) Restoration of the cell surface protein levels of wild-type PIGT and the mutant PIGT lacking 28 amino acids encoded by exon 11 was assessed by flow cytometry. Wild-type PIGT efficiently restored expression levels of CD59 and CD55 at the cell surface (dotted black lines), whereas the mutant PIGT did not rescue CD59 and only partially rescued CD55 expression (solid black lines). Dark shading, empty vector; light shading, isotype-matched control. (Right) Expression levels of transfected wild-type and the mutant HA-tagged PIGT. PIGT proteins were determined by western blotting with anti-HA; GAPDH, loading control. Normalized PIGT levels are shown at the bottom.

**Results and discussion**

We performed targeted enrichment of all exons of genes involved in GPI anchor synthesis followed by ultradepth sequencing in a female patient with classical hemolytic PNH that is negative for mutations in PIGA. The patient was diagnosed with hemolytic anemia with a negative direct antiglobulin test at the age of 44 years and experienced frequent hemolytic crises, abdominal pain, diarrhea, headache, arthralgia, dyspnea, and fatigue in the following years. At the age of 49 years, a flow cytometric analysis was performed that showed reduced expression of GPI-anchored proteins on blood cells (Figure 1A). DNA was isolated from blood at that time and subjected to ultradepth sequencing. The patient was started on eculizumab due to PNH-related symptoms soon after it became available 6 years ago and responded to this treatment (see supplemental Materials for a detailed clinical description of the patient).

We detected a significant reduction in the coverage of all PIGT exons in the DNA extracted from blood compared with other genes of the GPI anchor synthesis pathway, which suggested a deletion of this gene in a subpopulation of cells (Figure 2A). We performed array CGH to measure the full extent of the CNV and detected an 8-MB deletion, arr20q11.23q13.12 (Figure 2A). To clarify which subpopulation was affected by the deletion, we used a FISH probe (RP3-337018) targeting the CNV interval in T lymphocytes and granulocytes. Although we did not observe any deletion in full metaphases of T lymphocytes, 92% of the evaluated granulocyte subpopulation was affected by the deletion, we used a FISH probe (RP3-337018) targeting the CNV interval in T lymphocytes and granulocytes. Although we did not observe any deletion in full metaphases of T lymphocytes, 92% of the evaluated granulocyte interphase nuclei showed only a single signal for RP3-337018, suggesting a heterozygous deletion including PIGT in a myeloid stem cell that occurred as a somatic event (Figure 2B).

The mutation analysis of the deep sequencing data revealed a single nucleotide substitution in PIGT affecting the splice acceptor site of intron 10: NM_015937:c.1401-2A>G (Figure 2C). From 1463 sequence reads that cover the splice site, 1239 showed the base substitution, suggesting that the mutation is present on the chromosome without the somatic deletion involving PIGT. We also measured the splice site mutation in a heterozygous state in ABI Sanger sequences of DNA that was extracted from epithelial cells of a buccal swab providing further evidence that c.1401-2A>G is the germline event (Figure 2D). Based on these findings, we hypothesized that the somatic deletion of the wild-type allele of PIGT occurred in a myeloid stem cell and resulted in a clone that is hemizygous for PIGT. In this clone, the single remaining copy of PIGT is functionally impaired due to the splice site mutation that results in skipping 84 bp of exon 11 and deleting 28 highly conserved amino acids in PIGT.

We analyzed the functional effect of the germline splice site mutation in PIGT-null Chinese Hamster Ovary cells. Although the transfection of wild-type PIGT into these cells restored the levels of wild-type GPI-linked proteins CD55 and CD59 at the cell surface, the transfection of the mutant only leads to a minor increase of CD55 surface expression but almost no CD59 expression at comparable PIGT protein levels (Figure 1B).
In contrast to the X-chromosomal PIGA, all other known genes involved in the GPI anchor synthesis pathway, including PIGT, are found on autosomes, and inactivating mutations in these genes have to occur on both alleles in the same cell to result in a GPI anchor deficiency. The co-occurrence of 2 mutations in the same gene is a situation that is similar to hereditary cases of retinoblastoma that have been explained by a 2-hit model of 1 inherited mutation and 1 somatic mutation in RB1. Therefore, individuals that are heterozygous for mutations in autosomal genes that impair GPI anchor synthesis, such as the reported splice site mutation in PIGT, might have an increased risk to develop PNH.

Although PIGA catalyzes the first step of the GPI anchor synthesis, PIGT is a component of the transamidase complex that is required for attachment of preassembled GPI to proteins. Therefore, even in the presence of fully assembled GPI anchors, PNH can occur. This suggests that not only the specific defect in the GPI anchor synthesis that is caused by PIGA mutations but also a GPI-anchored protein deficiency that is due to mutations in other genes of the pathway may predispose for PNH. Interestingly, a deletion on 20q is also a recurrent somatic abnormality in myelodysplastic syndrome; however, it is currently not clear whether the loss of heterozygosity of other genes in this region besides PIGT, contributes to the clonal expansion.

Recent findings of congenital GPI deficiencies also shed new light on the clinical feature of hemoglobinuria. Although no hemolysis was reported for patients with germline mutations in PIGN, PIGM, PIGO, PIGL, PIGV, and even PIGA, chronic hemolysis was described in patients with a congenital CD59...
deficiency\textsuperscript{26} that responds to eculizumab therapy.\textsuperscript{27} Further studies are therefore required to elucidate how mutations in GPI pathway genes contribute to the different phenotypic features and to what extent additional somatic events occur.

Acknowledgments

The authors thank the reviewers for valuable comments and Seval Turkmen for helpful discussions.

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (0313911), Deutsche Forschungsgemeinschaft grants KR 3985/1-1 (to P.M.K.) and SFB 665 (to S.M.), and grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labour and Welfare of Japan.

References


Authorship

Contribution: P.M.K. and D.P. performed research and analyzed the data; U.K., J.H., and C.S. performed sequencing studies; B.H. and H.S. provided patient samples and characterized the patient; A.H. provided patient samples, performed research, and analyzed data; E.K. performed arrayCGH; B.T. performed the FISH analysis; Y.M. performed cell culture experiments; H.S., B.H., and P.M.K. designed the study; and E.K., H.N., P.N.R., Y.M., J.H., T.K., and S.M. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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