RED CELLS, IRON, AND ERYTHROPOIESIS

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

Peter M. Krawitz,[1] Britta Höchsmann,[2] Yoshiko Murakami,[3] Britta Teubner,[1] Ulrike Krüger,[1] Eva Klopocki,[4] Heidemarie Neitzel,[1] Alexander Hoellein,[5] Christina Schneider,[2] Dmitri Parkhomchuk,[1] Jochen Hecht,[6] Peter N. Robinson,[1] Stefan Mundlos,[1] Taroh Kinoshita,[3] and Hubert Schrezenmeier[2]

[1]Institute for Medical Genetics and Human Genetics, Charité Universitätsmedizin, Berlin, Germany; [2]Institute for Clinical Transfusion Medicine and Immunogenetics, German Red Cross Blood Transfusion Service Baden Württemberg – Hessen, University of Ulm, Ulm, Germany; [3]Research Institute for Microbial Diseases and World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan; [4]Institute for Medical Genetics, University of Würzburg, Würzburg, Germany; [5]Medizinische Klinik III, Technische Universität München, Munich, Germany; and [6]Next-generation Sequencing Facility, Berlin-Brandenburg Center for Regenerative Therapies, Berlin, Germany

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.[1-5] 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 complex[8] 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.

Targeted genomic sequencing, array comparative genomic hybridization, and fluorescence in situ hybridization

For the targeted enrichment of exons of all known GPI anchor synthesis genes (supplemental Table 1 on the *Blood* website), we used a customized SureSelect library (Agilent) as previously described.[10] Genomic DNA of the patient and 9 controls was isolated from whole blood and enriched for GPI pathway exons according to the manufacturer's protocol, followed by single-read cluster generation on a Cluster Station (Illumina). The captured, purified, and clonally amplified library was then sequenced on an Illumina Genome Analyzer IIx and mapped to the human reference sequence GRCh37, resulting in a mean coverage of >300-fold for all exons and >10-fold coverage for >95% of the target region. Variants were detected with SAMtools,[11] annotated with ANNOVAR,[12] and further analyzed in GeneTalk.[13]

For the detection of exon deletions, we first counted the reads per exon and normalized this value for each sample by the total number of reads that were mapped to the target region. This normalized read count per exon was used to compute the mean and variance for the coverage per exon in all analyzed samples. Exons with a normalized coverage that was 2 standard deviations below the mean were classified as partially deleted in a subpopulation of cells and further analyzed.

Array comparative genomic hybridization (arrayCGH) was carried out on genomic DNA isolated from a peripheral blood draw using whole-genome 1 M Oligonucleotide-Array (Agilent) to confirm the deletion of *PIGT* and to characterize its extent. Analysis was performed with Feature Extraction and CGH Analytics software (Agilent) as described previously.[14] The copy number variants (CNV) involving *PIGT* was further analyzed with

Submitted January 28, 2013; accepted May 23, 2013. Prepublished online as *Blood* First Edition paper, June 3, 2013; DOI 10.1182/blood-2013-01-481499.

The online version of this article contains a data supplement.

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

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

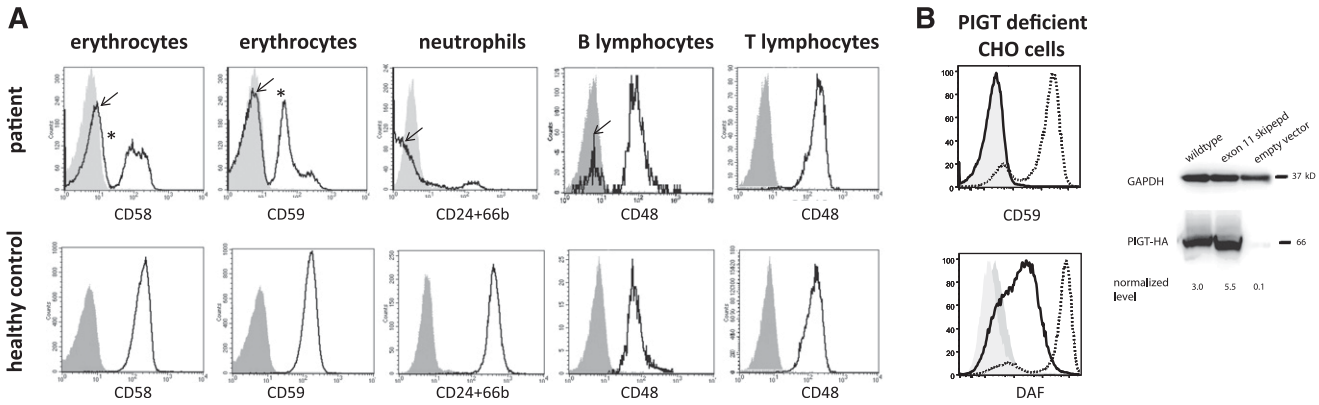

Figure 1. Expression of GPI-anchored proteins on patient's peripheral blood cells; and reduced activity of PIGT mutant in restoring surface expression of GPI-anchored proteins after transfection into *PIGT*-null cell lines. (A) Expression of (left) CD58 and CD59 on red cells, (center) CD24 and CD66b on neutrophil granulocytes, and (right) CD48 on B and T lymphocytes. The first row shows the expression of GPI-anchored protein (AP) at the time of ultradeep sequencing (4.5 years after start of eculizumab); the second row shows expression of GPI-AP in a healthy control. In healthy controls, CD58 and CD59 are expressed on >99.9% and >99.5% of red cells, respectively, and CD24/CD66b is expressed on >99.8% of neutrophil granulocytes (second row). In contrast, the patient shows a mosaic of cells with normal expression of GPI-anchored proteins and cells with reduced or completely missing expression of GPI-AP on (left) erythrocytes or (center) neutrophil granulocytes. The cell populations that completely lack expression of the respective GPI-AP are indicated by arrows; the populations with reduced GPI-AP expression are marked by asterisks. The patient did not receive any blood transfusions over a period of 3 months before this measurement. Expression of the GPI-AP CD48 on T lymphocytes was normal, whereas a subpopulation 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.

fluorescence in situ hybridization (FISH) using BAC clone RP3-337O18 in metaphases of phytohemagglutinin-stimulated T lymphocytes and granulocytes that were enriched by a Ficoll gradient.

Cell culture and fluorescence-activated cell sorter

We cloned a coding region of human *PIGT* (NM_015937) from a cDNA library derived from placenta,[15] tagged with FLAG at the *N* terminus, and subcloned it into plasmid mammalian expression.[16] A *PIGT* mutant with skipped exon 11 was generated by site-directed mutagenesis. Mutant and wild-type *PIGT* plasmids were transfected by electroporation into PIGT-deficient Chinese Hamster Ovary cells expressing human GPI-anchored proteins, CD55 and CD59, as previously described.[17] Two days later, lysates were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting against anti-FLAG antibody to determine levels of expressed PIGT. The levels of CD55 and CD59 restored at the cell surface were determined by fluorescence-activated cell sorter.

Results and discussion

We performed targeted enrichment of all exons of genes involved in GPI anchor synthesis followed by ultradeep 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 ultradeep 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 (RP3-337O18) 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 RP3-337O18, 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 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).

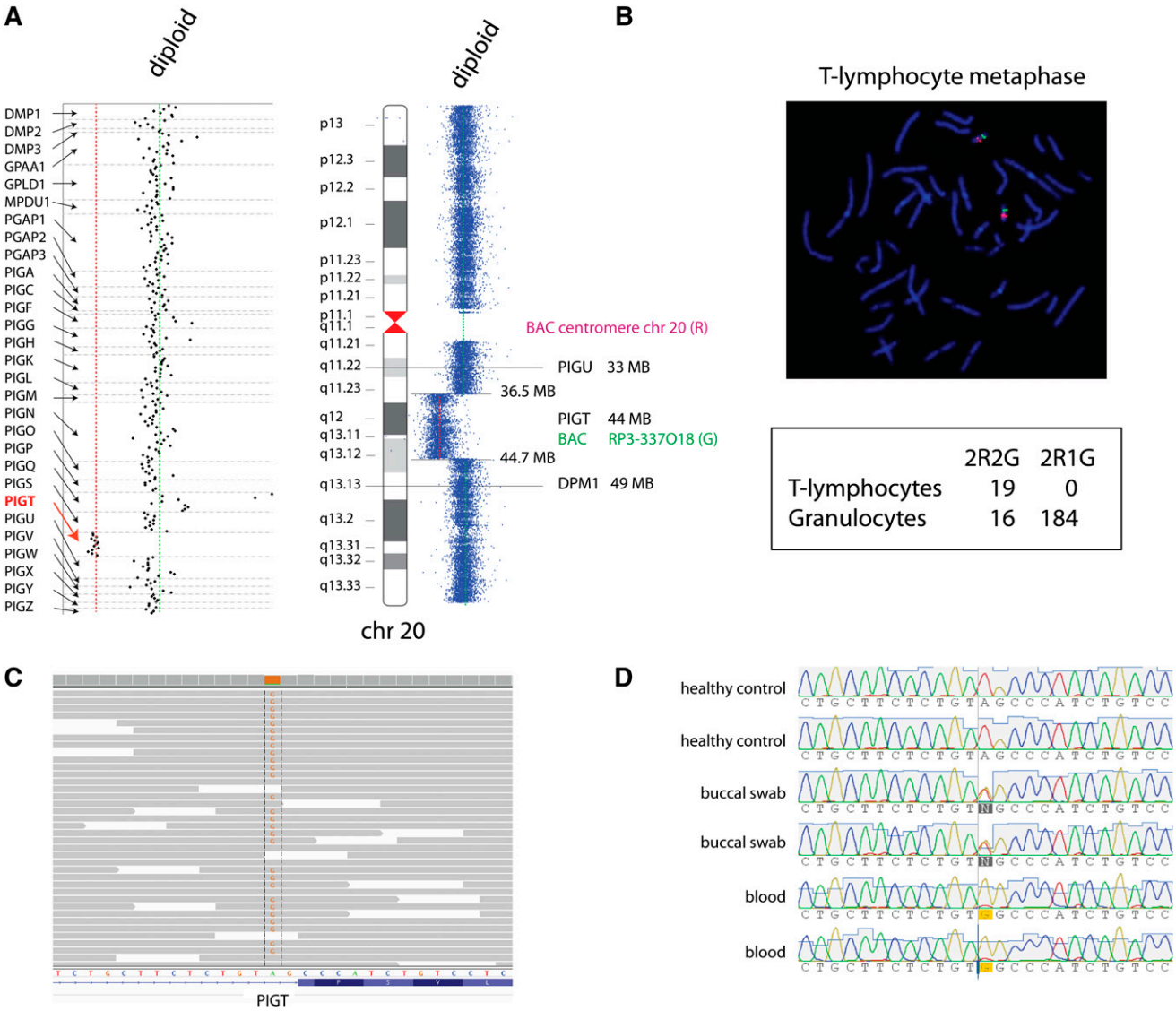

Figure 2. Ultradeep sequencing of all exons of genes involved in GPI anchor synthesis reveals two mutation events in *PIGT*: a germline splice site mutation and a somatic deletion. (A) DNA was isolated from whole blood and enriched for all exons of genes involved in GPI anchor synthesis and subjected to ultradeep sequencing. The coverage of *PIGT* exons was significantly reduced compared with exons of all other GPI anchor synthesis genes, suggesting a deletion involving *PIGT*. The extent of the deletion was further characterized by arrayCGH comprising in total 8 MB, arr20q11.23q13.12. (B) FISH with BAC clone RP3-337O18 (G) and a probe targeting the centromere of chromosome 20 (R) was used to analyze the deletion in T lymphocytes and granulocytes. Although 2 signals of RP3-337O18 were present in all complete metaphases of T lymphocytes, the majority of granulocytes showed only 1 signal for RP3-337O18, indicating a somatic deletion in a myeloid lineage. (C) A single nucleotide substitution in *PIGT* affecting the splice acceptor site of intron 10, NM_015937:c.1401-2A>G, was observed in the ultradeep sequencing data of DNA extracted from whole blood. In total, 1463 sequence reads covered the canonical splice site, and 85% of these reads showed the alternate base, indicating that the mutation is present on the undeleted haplotype of *PIGT*. (D) The splice site mutation was validated by ABI Sanger sequencing and shown to be heterozygous in DNA extracted from epithelial cells of a buccal swap, confirming its presence in different tissues.

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*.[18] 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,[8] PIGT is a component of the transamidase complex that is required for attachment of preassembled GPI to proteins.[15] 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*,[19] *PIGM*,[20] *PIGO*,[21] *PIGL*,[22] *PIGV*,[23] and even *PIGA*[24] and *PIGT*,[25] chronic hemolysis was described in patients with a congenital CD59

deficiency[26] that responds to eculizumab therapy.[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.

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.

Correspondence: Peter Krawitz, Institute for Medical Genetics and Human Genetics, Augustenburger Platz 1, 13353 Berlin, Germany; e-mail: peter.krawitz@gmail.com.

References

1. Brodsky RA. Advances in the diagnosis and therapy of paroxysmal nocturnal hemoglobinuria. *Blood Rev.* 2008;22(2):65-74.
2. Bessler M, Mason P, Hillmen P, Luzzatto L. Somatic mutations and cellular selection in paroxysmal nocturnal haemoglobinuria. *Lancet.* 1994;343(8903):951-953.
3. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. *N Engl J Med.* 1995;333(19): 1253-1258.
4. Rosse WF. Epidemiology of PNH. *Lancet.* 1996; 348(9027):560.
5. Socié G, Mary JY, de Gramont A, et al; French Society of Haematology. Paroxysmal nocturnal haemoglobinuria: long-term follow-up and prognostic factors. *Lancet.* 1996;348(9027): 573-577.
6. Takahashi M, Takeda J, Hirose S, et al. Deficient biosynthesis of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. *J Exp Med.* 1993;177(2):517-521.
7. Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell.* 1993;73(4):703-711.
8. Miyata T, Takeda J, Iida Y, et al. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. *Science.* 1993; 259(5099):1318-1320.
9. Nafa K, Bessler M, Castro-Malaspina H, Jhanwar S, Luzzatto L. The spectrum of somatic mutations in the PIG-A gene in paroxysmal nocturnal hemoglobinuria includes large deletions and small duplications. *Blood Cells Mol Dis.* 1998;24(3): 370-384.
10. Krawitz PM, Murakami Y, Rieß A, et al. PGAP2 mutations, affecting the GPI-anchor-synthesis pathway, cause hyperphosphatasia with mental retardation syndrome. *Am J Hum Genet.* 2013; 92(4):584-589.
11. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics.* 2011;27(21): 2987-2993.
12. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 2010;38(16):e164.
13. Kamphans T, Krawitz PM. GeneTalk: an expert exchange platform for assessing rare sequence variants in personal genomes. *Bioinformatics.* 2012;28(19):2515-2516.
14. Klopocki E, Lohan S, Brancati F, et al. Copy-number variations involving the IHH locus are associated with syndactyly and craniosynostosis. *Am J Hum Genet.* 2011;88(1): 70-75.
15. Ohishi K, Inoue N, Kinoshita T. PIG-S and PIG-T, essential for GPI anchor attachment to proteins, form a complex with GAA1 and GPI8. *EMBO J.* 2001;20(15):4088-4098.
16. Takebe Y, Seiki M, Fujisawa J, et al. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol Cell Biol.* 1988;8(1):466-472.
17. Hong Y, Ohishi K, Inoue N, et al. Requirement of N-glycan on GPI-anchored proteins for efficient binding of aerolysin but not Clostridium septicum alpha-toxin. *EMBO J.* 2002;21(19):5047-5056.
18. Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA.* 1971;68(4):820-823.
19. Maydan G, Noyman I, Har-Zahav A, et al. Multiple congenital anomalies-hypotonia-seizures syndrome is caused by a mutation in PIGN. *J Med Genet.* 2011;48(6):383-389.
20. Almeida AM, Murakami Y, Layton DM, et al. Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency. *Nat Med.* 2006;12(7):846-851.
21. Krawitz PM, Murakami Y, Hecht J, et al. Mutations in PIGO, a member of the GPI-anchor-synthesis pathway, cause hyperphosphatasia with mental retardation. *Am J Hum Genet.* 2012;91(1): 146-151.
22. Ng BG, Hackmann K, Jones MA, et al. Mutations in the glycosylphosphatidylinositol gene PIGL cause CHIME syndrome. *Am J Hum Genet.* 2012; 90(4):685-688.
23. Krawitz PM, Schweiger MR, Rödelsperger C, et al. Identity-by-descent filtering of exome sequence data identifies PIGV mutations in hyperphosphatasia mental retardation syndrome. *Nat Genet.* 2010;42(10):827-829.
24. Johnston JJ, Gropman AL, Sapp JC, et al. The phenotype of a germline mutation in PIGA: the gene somatically mutated in paroxysmal nocturnal hemoglobinuria. *Am J Hum Genet.* 2012;90(2): 295-300.
25. Kvarnung M, Nilsson D, Lindstrand A, et al. A novel intellectual disability syndrome caused by GPI anchor deficiency due to homozygous mutations in PIGT [published online ahead of print May 1, 2013]. *J Med Genet.*
26. Yamashina M, Ueda E, Kinoshita T, et al. Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. *N Engl J Med.* 1990;323(17):1184-1189.
27. Höchsmann B, Dohna-Schwake C, Rojewski M, et al. Targeted Therapy with Eculizumab for inherited CD59 Deficiency, submitted