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Cooperating G6PD Mutations Associated with Severe Neonatal Hyperbilirubinemia and Cholestasis

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Abstract

We report a novel glucose-6-phosphate dehydrogenase (G6PD) mutation, which we propose to name G6PD Cincinnati (c.1037A>T, p.N346I), found in combination with G6PD Gastonia (c.637G>T, p.V213L) in an infant who presented with neonatal cholestasis. The G6PD Cincinnati mutation results in a non-conservative amino acid substitution at the tetramer interface disturbing its formation, as seen by native gel electrophoresis and immunoblotting. G6PD Gastonia disrupts dimerization of the enzyme and by itself causes chronic non-spherocytic hemolytic anemia. The G6PD Cincinnati mutation may have aggravated the clinical picture of G6PD Gastonia with the result of severe perinatal hemolysis causing cholestasis and associated liver injury.

Keywords

G6PD deficiency; neonatal jaundice; cholestasis

INTRODUCTION

The enzyme glucose-6-phosphate dehydrogenase (G6PD, OMIM #305900) catalyzes the first step in the pentose phosphate pathway, with concomitant reduction of the cofactor nicotinamide-adenine-dinucleotide-phosphate (NADP⁺) to NADPH. NADPH preserves the reduced form of glutathione and counterbalances oxidative stress in cells. G6PD deficiency is the most common erythrocyte enzyme disorder, estimated to affect more than 300 million people worldwide [1]. Insufficient NADPH production in the red blood cells (RBC) renders them prone to hemolysis, particularly in situations of oxidative stress from exposure to certain drugs, foods, or infectious agents.

The considerable heterogeneity of clinical phenotypes reflects the variability in enzyme deficiency resulting from greater than 160 different mutations identified in the G6PD gene on the X chromosome (Human Genome Mutation Database: <http://www.hgmd.cf.ac.uk/ac/index.php>). The G6PD variants are classified based on the level of RBC enzyme activity and the clinical manifestations [2]. Class I variants are

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associated with chronic non-spherocytic hemolytic anemia (CNSHA), class II have less than 10% of residual enzyme activity but without CNSHA, class III have 10-60% residual activity, class IV have normal activity, while in class V G6PD activity is increased. Some of the variants originally classified as distinct based on phenotype or geographical clustering were later found to be identical mutations upon gene sequencing [3]. Biochemically, the G6PD monomer is catalytically inactive [4] while the oligomers appear to have similar specific activity, i.e. the tetramer has twice the activity of the dimer [5]. The active enzyme exists in dimer-tetramer equilibrium, depending on pH and ionic strength: the tetramer predominates at low pH while high ionic strength favors the dimer; inactive monomers increase at high pH [6].

Here we describe an infant with G6PD deficiency, due to mutations affecting dimer and tetramer formation, who presented at birth with profound hyperbilirubinemia in association with cholestasis and liver injury as a result of severe perinatal hemolysis.

CASE REPORT

A Caucasian male infant was born at 37.5 weeks gestation via urgent c-section due to fetal distress and required mechanical ventilation. Physical examination revealed hepatosplenomegaly and profound jaundice. Laboratory investigation revealed total serum bilirubin 36.7 mg/dL, conjugated bilirubin 26.1 mg/dL, hemoglobin 8.2 g/dL; reticulocytes 30%, platelets 85,000/ μ L, AST 1846 units/L, and ALT 302 units/L. Infant blood type was A +, same as the mother's, with negative antibody screen and direct antiglobulin test (DAT). He underwent exchange transfusion at first day of life, with the total bilirubin level decreasing to 22.9 mg/dL. Complete workup for bacterial sepsis and congenital infections was negative. After three platelet transfusions within the first week of life, he maintained a normal platelet count. The third week, he was extubated and weaned to room air, and was started on ursodeoxycholic acid. Findings from percutaneous liver biopsy, performed at two weeks of life, included neonatal hepatitis, with multinucleated giant cell transformation and focal pericellular fibrosis, present bile ducts with no significant injury or proliferation and prominent extramedullary hematopoiesis.

The infant was transferred to our institution at seven weeks of life for further evaluation of neonatal hepatitis [7]. At that time, serum AST was 465 units/L, ALT 238 units/L, conjugated bilirubin 13.1 mg/dL, unconjugated bilirubin 2.6 mg/dL, and LDH 1763 units/L. Anemia was noted (Hb 8.9 g/dL, MCV 100, RDW 24.2%, absolute reticulocyte count (ARC) 663,000/ μ L) and hematology was consulted for further evaluation. Blood smear review revealed significant anisocytosis, poikilocytosis, polychromasia and presence of nucleated RBCs suggesting a hemolytic process (Fig. 1A).

The family reported a number of maternal relatives with history of cholelithiasis, including the infant's mother who underwent cholecystectomy at the age of 6. The maternal grandfather and uncle suffered of chronic hemolytic anemia and had been diagnosed with G6PD deficiency. Neither consented to further testing. The patient's G6PD level was 0.8 units/g hemoglobin (normal range 6.9–13.9 units/g Hb). The patient had complete resolution of jaundice and normalization of serum ALT/AST by four months of age. He continues to have chronic hemolytic anemia, with Hb 8.5-10 g/dL and ARC 340,000-440,000/ μ L. At 6 months of age, he developed an acute hemolytic episode with hemoglobin decrease to 6.2 g/dL and ARC up to 736,000/ μ L, likely precipitated by a viral infection. During this episode his bilirubin level was only 0.9 mg/dL, indicating improved clearance by the liver at this time.

MATERIALS AND METHODS

G6PD Gene Sequencing

was performed by the Emory Genetics Laboratory after PCR amplification of the 12 coding exons and immediate flanking regions of the G6PD gene. The PCR products were sequenced in the forward and reverse directions. Nucleotide numbering is based on GenBank accession number NM_000402; nucleotide 1 corresponds to A of start codon ATG.

G6PD Activity Assay

was performed using quantitative ultraviolet kinetic determination with commercial reagents (Trinity Biotech, St. Louis, MO).

Native Gel Electrophoresis and Immunoblotting

Blood samples from the patient and control subjects were lysed and depleted of hemoglobin using HemoVoid (Biotech Support Group, North Brunswick, NJ). Non-bound protein was eluted at pH 9.8 and placed in native sample buffer, pH 6.8 (Bio-Rad Laboratories, Inc., Hercules, CA) and analyzed by native gel electrophoresis in polyacrylamide gradient gels of 4-15%, in the absence of SDS, followed by western blotting and immunoblotting for G6PD, using a rabbit anti-human G6PD polyclonal antibody (Bethyl Laboratories, Inc. Montgomery, TX).

RESULTS AND DISCUSSION

G6PD gene sequencing revealed the mutation c.637G>T (p.V213L) which is known as the Gastonia, Marion, or Minnesota variant and is clinically associated with CNSHA [8]. This mutation is located at the dimer interface and disrupts dimerization causing decreased enzyme activity and a marked decline in intravascular erythrocyte survival [8,9]. Another mutation, not previously reported in the literature or the Human Genome Mutation Database, c.1037A>T (p.N346I) was also identified in exon 9 of the G6PD gene, located in a hydrophilic loop at the tetramer interface (Fig. 1B). In our patient, the hydrophobic side chain of isoleucine substitutes the hydrophilic side chain of asparagine, potentially disrupting the tetramer formation. This mutation, which we propose to name G6PD Cincinnati, affects a highly conserved amino acid residue of the enzyme and therefore it is unlikely to be an asymptomatic polymorphism (Fig. 2A). Search in NCI human SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_gf.cgi) querying genotypes from a population of 870 individuals revealed no polymorphisms at this location. Of interest, the glutamic acid at the position 347 (E347), just next to N346 (Fig. 2A), participates in one of the salt bridges between dimers to stabilize the tetramer, as deduced by crystallography studies [10]. While several of the class I mutations are located at the dimer interface, there is one more class I mutation at the tetramer interface (E274K named G6PD Cleveland) that has been described so far [10,11].

Immunoblotting for G6PD after native gel electrophoresis was performed in the patient's blood sample and control specimens (Fig. 2B). Bands at the expected molecular weights for the G6PD monomer, dimer, and tetramer were seen in the control specimen. The quantity of monomer was similar between patient and control, while the patient's G6PD dimer and tetramer were significantly decreased. Although it is considered unlikely that a substantial amount of monomer exists normally, because NADP in the RBCs induces dimerization [5], about one-fifth of the total enzyme in the control sample was in monomeric form. This may have been caused by processing of the RBC lysate to achieve hemoglobin depletion, which was necessary to allow immunoblotting at an area otherwise overwhelmed by an excessive

amount of hemoglobin tetramer (M.W. 68 kDa). G6PD deficiency can be caused either by a reduced number of G6PD molecules with normal catalytic activity or by a normal number of molecules with decreased catalytic activity or by a combination of these two mechanisms [5]. The number of G6PD molecules declines due to accelerated breakdown rather than decreased rate of synthesis [12]. From the immunoblot in Fig. 2B, we conclude that decreased amount of the enzyme along with decreased activity due to impaired oligomer formation contribute to the phenotype of class I G6PD deficiency in our patient.

We cannot exclude the possibility that the severe presentation of our patient could have been caused by the G6PD Gastonia mutation alone causing overwhelming hemolysis due to oxidative stress caused by perinatal complications. However, the concurrent G6PD Cincinnati mutation may have aggravated the phenotype, as it has been previously described with multiple G6PD mutations [13]. Neonatal hemolysis causing progressive cholestasis, hepatosplenomegaly, and liver dysfunction has been previously reported in association with hemolytic conditions, such as pyruvate kinase deficiency [14], Rh incompatibility [15,16], hereditary pyropoikilocytosis [16], and recently G6PD deficiency [17]. The common pathophysiology proposed in these clinical disorders is bilirubin-load out of proportion to the rate of cholestasis in the neonatal liver, leading to cholestasis and hepatitis. Complete work-up needs to be performed in a case of neonatal obstructive jaundice to rule-out sepsis, congenital infections, inborn errors of metabolism, and primary liver disease. However, when serum AST is disproportionately higher than serum ALT, the possibility of extensive hemolysis needs to be considered. And as it is frequently true in pediatrics, a complete family history can be very helpful.

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REFERENCES

1. Beutler E. Glucose-6-phosphate dehydrogenase deficiency: a historical perspective. *Blood* 2008;111(1):16–24. [PubMed: 18156501]
2. WHO gw. Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group. *Bull World Health Organ* 1989;67(6):601–611. [PubMed: 2633878]
3. Bulliamy T, Luzzatto L, Hirono A, et al. Hematologically important mutations: glucose-6-phosphate dehydrogenase. *Blood Cells Mol Dis* 1997;23(2):302–313. [PubMed: 9410474]
4. Cancedda R, Ogunmola G, Luzzatto L. Genetic variants of human erythrocyte glucose-6-phosphate dehydrogenase. Discrete conformational states stabilized by NADP + and NADPH. *Eur J Biochem* 1973;34(1):199–204. [PubMed: 4144831]
5. Luzzatto L, Testa U. Human erythrocyte glucose 6-phosphate dehydrogenase: structure and function in normal and mutant subjects. *Curr Top Hematol* 1978;1:1–70. [PubMed: 45418]
6. Cohen P, Rosemeyer MA. Subunit interactions of glucose-6-phosphate dehydrogenase from human erythrocytes. *Eur J Biochem* 1969;8(1):8–15. [PubMed: 5781270]
7. Balistreri WF, Bezerra JA. Whatever happened to “neonatal hepatitis”? *Clin Liver Dis* 2006;10(1):27–53. v. [PubMed: 16376793]
8. Beutler E, Kuhl W, Gelbart T, et al. DNA sequence abnormalities of human glucose-6-phosphate dehydrogenase variants. *J Biol Chem* 1991;266(7):4145–4150. [PubMed: 1999409]
9. Kiani F, Schwarzl S, Fischer S, et al. Three-dimensional modeling of glucose-6-phosphate dehydrogenase-deficient variants from German ancestry. *PLoS One* 2007;2(7):e625. [PubMed: 17637841]
10. Au SW, Gover S, Lam VM, et al. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. *Structure* 2000;8(3):293–303. [PubMed: 10745013]

11. Xu W, Westwood B, Bartsocas CS, et al. Glucose-6 phosphate dehydrogenase mutations and haplotypes in various ethnic groups. *Blood* 1995;85(1):257–263. [PubMed: 7803800]
12. Mason PJ, Bautista JM, Gilsanz F. G6PD deficiency: the genotype-phenotype association. *Blood Rev* 2007;21(5):267–283. [PubMed: 17611006]
13. McDade J, Abramova T, Mortier N, et al. A novel G6PD mutation leading to chronic hemolytic anemia. *Pediatr Blood Cancer* 2008;51(6):816–819. [PubMed: 18677765]
14. Raphael MF, Van Wijk R, Schweizer JJ, et al. Pyruvate kinase deficiency associated with severe liver dysfunction in the newborn. *Am J Hematol* 2007;82(11):1025–1028. [PubMed: 17654506]
15. Dunn PM. Rh Haemolytic Disease of the Newborn, 1960-1961. *Arch Dis Child* 1963;38:596–599.
16. Allgood C, Bolisetty S. Severe conjugated hyperbilirubinaemia and neonatal haemolysis. *Int J Clin Pract* 2006;60(11):513–514.
17. Kordes U, Richter A, Santer R, et al. Neonatal cholestasis and glucose-6-P-dehydrogenase deficiency. *Pediatr Blood Cancer* 2010;54(5):758–760. [PubMed: 20052779]
18. Bain BJ. A ghostly presence-G6PD deficiency. *Am J Hematol* 2010;85(4):271. [PubMed: 20196170]
19. Moreland JL, Gramada A, Buzko OV, et al. The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications. *BMC Bioinformatics* 2005;6:21. [PubMed: 15694009]

Figure 1.

A. Blood smear demonstrating poikilocytosis and polychromasia as well as irregularly contracted cells (1) and a “ghost” cell (2), suggesting hemolysis due to G6PD deficiency [18]. **B.** Hydrophobicity map of the G6PD tetramer visualized by the 3D-structure viewer application Protein Workshop (<http://www.pdb.org/robohelp/viewers/proteinworkshop.htm>) [19], with the arrows pointing to the two amino-acid sites mutated in the case discussed. Inset shows enlarged the site of asparagine (N) at the position 346 in a hydrophilic loop at the dimer interface, which in our patient has been substituted by a hydrophobic isoleucine (I).

Figure 2.

A. G6PD protein sequences for the species shown were obtained from the NCBI nucleotide database (accession numbers NP_001035810, NP_032088, NP_001080019, XP_699168, P12646, AAT93017, and NP_416366 respectively). Sequence alignment was performed using the ClustalW protein sequence alignment tool (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>); the N346 position is highlighted. Overall sequence homology to the human protein sequence was 93% for *M.musculus*, 77% for *X.laevis*, 75% for *D.rerio*, 61% for *D.melanogaster*, 44% for *S.cerevisiae*, and 34% for *E.coli*. **B.** Immunoblotting for G6PD after running hemoglobin-depleted blood samples from the patient (P) and a control (C) in native gel electrophoresis. Bands at the expected molecular weights for the monomer (52 kDa), dimer (105 kDa), and tetramer (210 kDa) are seen in the control specimen. Densitometry was performed using the ImageJ program (<http://rsbweb.nih.gov/ij>) and the relative quantitation of the bands is shown. The tetrameric form consists approximately 52% of the total enzyme in the control sample and 37% of the total enzyme in the patient's sample. GAPDH at the molecular weight of a tetramer (145 kDa) as expected in a native gel was detected in the same two lanes as loading control.