Red Cell Disorders

Two new glucose-6-phosphate dehydrogenase mutations causing chronic hemolysis

We describe two new missense mutations in the glucose-6-phosphate dehydrogenase (G6PD) gene associated with chronic hemolytic anemia: mutation 1205C→A in exon 10 predicts the amino acid change 402Thr→Asn in the β-sheet M of the polypeptide chain, within the dimer interface (G6PD *Covão do Lobo*); mutation 1366G→A in exon 12 predicts the amino acid substitution 456Asp→His in the α-helix N, at the protein surface (G6PD *Figueira da Foz*).

haematologica 2005; 90:1135-1136
(*http://www.haematologica.org/journal/2005/8/1135.html*)

The majority of individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency are usually asymptomatic, with normal hematologic parameters, but may develop acute hemolysis in response to oxidative stress induced by several drugs, infection or fava bean ingestion (classes II to IV G6PD variants).[1] A small number of G6PD-deficient patients have rare class I molecular variants responsible for chronic non-spherocytic hemolytic anemia of heterogeneous severity; this, too, can be further exacerbated by oxidative stress.[2] To date more than 60 G6PD class I mutations have been identified, the great majority being missense mutations present in and around exon 10, with the corresponding amino acids located within or close to the subunit interface of the G6PD dimer.[3,4] Structure-function studies have verified that class I mutations mainly occur at conserved amino acids.[5]

In this report we present the molecular characterization of three severe G6PD-deficient Portuguese patients with chronic hemolysis from two unrelated families.

Case #1. A 25-year old Portuguese male presented at the emergency room complaining of weakness, jaundice and dark urine for the last 3 days. His spleen was palpable 15 cm below the left costal margin. He had had neonatal jaundice, requiring phototherapy, had chronically yellowish sclera and had had one severe hemolytic episode in infancy. His hemoglobin concentration was 70 g/L, his mean cell volume 101 fL, reticulocytes 12%, unconjugated serum bilirubin 58.1 *mol/L (normal range: 3-20); a peripheral blood smear showed some erythrocytes with oxidative stress. Out of the hemolytic episode, he has a just palpable spleen and moderate macrocytic anemia (Table 1).

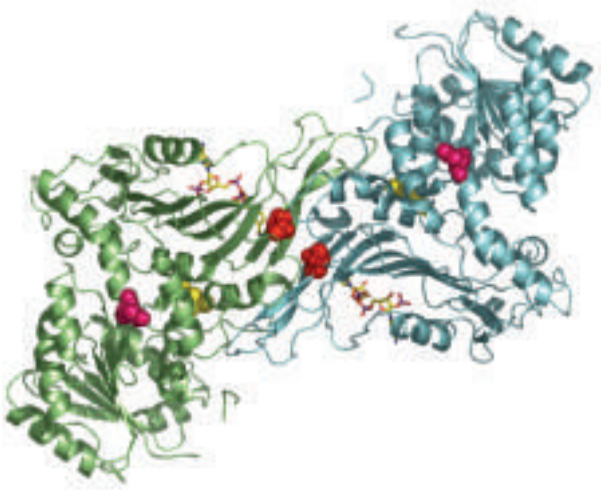

Figure 1. Human G6PD dimer showing mutated residues Thr402 (shown as red spheres) within the dimer interface, and Asp456 (shown as pink spheres) located within alpha helix N. The structural $NADP^+$ molecules are shown as stick models and the catalytic Lys205 is highlighted in yellow. G6PD subunits are colored in green and blue. The figure was prepared with PyMOL[10] using the co-ordinates of the human G6PD Canton mutant 1QKI[4] (Protein Data Bank (*http://www.rcsb.org/pdb/*).

Case #2. At the age of 3 years old, a Portuguese boy presented with a febrile episode and jaundice. His spleen was not palpable. His hemoglobin concentration was 86 g/L and unconjugated serum bilirubin: 51 µmol/L. He had a history of neonatal jaundice, requiring phototherapy, but no other important hemolytic episodes. A family study revealed a maternal uncle (*Case 3*) with chronic jaundice and a palpable spleen who had had several episodes of hemolytic anemia (data on Table 1). To exclude other causes of chronic non-spherocytic hemolytic anemia, the samples were screened for abnormal hemoglobins and common glycolytic enzyme deficiencies.

Table 1. Hematologic parameters, G6PD activity out of the hemolytic crisis and molecular data.

Patient	*Age (Yr)*	*Hb (g/L)*	*MCV (fL)*	*Retics (% of RBC)*	*Unc Bilir (µmol/L)*	*LDH (U/L)*	*Serum Ferritin (ng/mL)*	*G6PD (% of N)*	*(TA)n in UGT1*	*G6PD Mutation*	*Amino acid substitution*
Patient #1	26	112	108	7	35.3	416	1290	0.9	TA_6/ TA_6	1205C→A	402Thr→Asn
Mother		120	92	0.9	ND	ND	ND	76	ND	1205C→A/NF	
Patient #2	10	131	101	5.2	57.3	512	120	2.9	TA_7/TA_7	1366G→A	456Asp→His
Mother		131	97	ND	ND	ND	ND	97	ND	1366G→A/NF	
Patient #3 (Maternal uncle) 456Asp→His		41	137	110	4.9	39.1	ND	ND	1.2	TA_7/TA_6	1366G→A

Hb: hemoglobin (normal values: adult females 120-150 g/L; adult males 135-170 g/L; 6-12 years 115-150 g/L); MCV: mean cell volume (normal values adults 92±9 fL; 6-12 years 75-95 fL); Retics: Reticulocytes (normal values: 0-2%); Unc Bilir: unconjugated bilirubin (normal values: 3-20 µmol/L); LDH: lactate dehydrogenase (normal values: 100-190 U/L); Serum Ferritin (normal values: age 4 months to 16 years 20-200 ng/mL; adult males 30-400 ng/mL); ND: not determined; NF: not found. Screening for hemoglobinopathies detected a heterozygosity for the Hb variant City of Hope (CD.69 GGT→AGT) in patient 1; this variant has not been associated with any phenotypic changes. None of the patients has detectable hemosiderinuria between the hemolytic episodes.

After patients and relatives had given informed consent, genomic DNA was extracted from EDTA peripheral blood using standard methods, and G6PD gene exons 2 to 13 were amplified by polymerase chain reaction (PCR) as described elsewhere.[6] PCR products were screened by single strand conformation polymorphism (SSCP) analysis and sequenced with the automatic genetic analyzer ABI Prism 310.

The G6PD gene from patient 1 showed a SSCP mobility shift in the fragment spanning exon 10 and sequencing revealed a previously undescribed mutation 1205C→A, predicting the amino acid change 402 Thr→Asn. T402 is a poorly conserved residue, located at the β-sheet M of the polypeptide chain; crystal structure analysis of human G6PD protein[4] showed that T402 is located at the dimer interface (Figure 1). The T402 side chains of the two monomers do not make any intersubunit contacts and are 5.4Å apart, across the dimer interface. Instead, they are within a distance compatible with a van der Waals effect of the L420 side chain in β strand N within the same monomer. Furthermore, β strand N harbors D421, a residue interacting with the nicotinamide ring of the structural $NADP^+$. The T402N mutation would introduce steric hindrance forcing the two dimers apart. In summary, as suggested for amino acid substitutions mapping close to this region,[7-9] T402N will both affect the dimer interface and interfere with the structural $NADP^+$ binding site,[4] severely affecting protein stability. DNA sequencing of the G6PD gene from patients 2 and 3 revealed a previously undescribed mutation in exon 12, a 1366G→A substitution, predicting the amino acid change 456Asp→His. D456 is located in the beginning of the α-helix N, further down the polypeptide carboxylic terminus. Structural analysis showed D456 at the protein surface, far away from the dimer interface (Figure 1). D456 forms a strong salt bridge with R454 within helix N which is further stabilized by interactions with D282 and D286 in helix J. Replacing D456 by His would partially disrupt this network of salt bridges. Furthermore, the positively charged His side chain at this position will certainly introduce unfavorable electrostatic interactions with R459, R454 and K293, affecting protein structure and stability. Although not located in the immediate vicinity of the active site, D456 is highly conserved from bacteria to humans reflecting important functional and/or structural features; hence D456H is in accordance with the general concept that the clinically more severe G6PD deficiencies are mainly associated with mutations at conserved amino acids.[5]

Both mutations were confirmed by restriction enzyme digestion and no other mutations were detected in the remaining exons or adjacent regions of the G6PD gene. The screening of 100 alleles from a control group failed to detect these mutations. The new variants T402N and D456H were named G6PD *Covão do Lobo* and G6PD *Figueira da Foz*, respectively, after the patients' place of birth.

Licínio Manco,° Paula Gonçalves,* Sandra Macedo-Ribeiro,# Carlos Seabra,@ Paula Melo,^ Maria Letícia Ribeiro**

**Unidade de Hematologia Molecular, Centro Hospitalar de Coimbra, Portugal; °Departamento de Antropologia, Universidade de Coimbra, Portugal; # Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Portugal; @Hospital Infante D. Pedro, Aveiro, Portugal; ^Laboratório de Patologia Clínica, Hospital Distrital da Figueira da Foz, Portugal*

Funding: this work was performed in part with the support of Forum Hematológico, Serviço de Hematologia, Centro Hospitalar de Coimbra, and Centro de Investigação em Antropologia - Universidade de Coimbra, Portugal.

Key words: G6PD deficiency, Portugal, G6PD class I mutations, dimer interface, G6PD structure and amino acid conservation.

Correspondence: Maria Letícia Ribeiro, MD, PhD, Unidade de Hematologia Molecular, Hospital Pediátrico, Centro Hospitalar de Coimbra, 3000 Coimbra, Portugal. Phone: international +351.239.480347. Fax: international +351.239.717216. E-mail: leticia.ribeiro@hpc.chc.min-saude.pt

References

1. Mehta A, Mason PJ, Vulliamy TJ. Glucose-6-phosphate dehydrogenase deficiency. Baillieres Best Pract Res Clin Haematol 2000;13:21-38.
2. Fiorelli G, Martinez di Montemuros F, Cappellini MD. Chronic non-spherocytic haemolytic disorders associated with glucose-6-phosphate dehydrogenase variants. Baillieres Best Pract Res Clin Haematol 2000;13:39-55.
3. Naylor CE, Rowland P, Basak AK, Gover S, Mason PJ, Bautista JM, et al. Glucose 6-phosphate dehydrogenase mutations causing enzyme deficiency in a model of the tertiary structure of the human enzyme. Blood 1996;87:2974-82.
4. Au SWN, Gover S, Lam VMS, Adams MJ. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. Structure Fold Des 2000;8:293-303.
5. Cheng YS, Tang TK, Hwang M. Amino acid conservation and clinical severity of human glucose-6-phosphate dehydrogenase mutations. J Biomed Sci 1999;6:106-14.
6. Calabro V, Mason PJ, Filosa S, Civitelli D, Cittadella R, Tagarelli G, et al. Genetic heterogeneity of glucose-6-phosphate dehydrogenase deficiency revealed by single-strand conformation and sequence analysis. Am J Hum Genet 1993; 52:527-36.
7. Costa E, Cabeda JM, Vieira E, Pinto R, Pereira SA, Ferraz L, et al. Glucose-6-phosphate dehydrogenase aveiro: a de novo mutation associated with chronic nonspherocytic hemolytic anemia. Blood 2000;95:1499-501.
8. Grabowska D, Jablonska-Skwiecinska E, Plochocka D, Chelstowska A, Lewandowska I, Witos I, et al. A novel mutation in the glucose-6-phosphate dehydrogenase gene in a subject with chronic nonspherocytic hemolytic anemia-characterization of enzyme using yeast expression system and molecular modeling. Blood Cells Mol Dis 2004;32:124-30.
9. van Wijk R, Huizinga EG, Prins I, Kors A, Rijksen G, Bierings M, et al. Distinct phenotypic expression of two de novo missense mutations affecting the dimer interface of glucose-6-phosphate dehydrogenase. Blood Cells Mol Dis 2004;32:112-7.
10. De Lano WL. The PyMOL Molecular Graphics System (2002) on World Wide Web (*http://www.pymol.org*).

Red Cell Disorders

Prevalence and severity of liver disease in patients with β thalassemia major. A single-institution fifteen-year experience

During the last years, liver disease has emerged as a major cause of mortality in patients with β thalassemia major (TM).[1,2] In spite of its clinical relevance, TM-associated liver damage has been insufficiently characterized.[1,2-5] We therefore retrospectively analyzed all TM patients of our Department who underwent liver biopsy since 1990.

haematologica 2005; 90:1136-1138
(*http://www.haematologica.org/journal/2005/8/1136.html*)

All patients were being regularly transfused in order to maintain the pretransfusion hemoglobin level at approximately 9.5 g/dL. Chelation therapy with desferrioxamine 40–60 mg/kg/day for 5–6 days/week was initiated as soon