Blood Cells, Molecules and Diseases 60 (2016) 18–23

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Hereditary nonspherocytic hemolytic anemia caused by red cell glucose-6-phosphate isomerase (GPI) deficiency in two Portuguese patients: Clinical features and molecular study

Licínio Manco [a,b,*], Celeste Bento [a,b], Bruno L. Victor [c], Janet Pereira [b], Luís Relvas [b], Rui M. Brito [c,d], Carlos Seabra [e], Tabita M. Maia [a], M. Letícia Ribeiro [a,b]

[a] Department of Hematology, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal
[b] Research Centre for Anthropology and Health (CIAS), Department of Life Sciences, University of Coimbra, Coimbra, Portugal
[c] Chemistry Department, University of Coimbra, Coimbra, Portugal
[d] Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[e] Serviço de Patologia Clínica, Hospital Infante D. Pedro, Aveiro, Portugal

ARTICLE INFO

Article history:
Submitted 30 March 2016
Revised 7 June 2016
Accepted 10 June 2016
Available online 11 June 2016

Keywords:
Glucose-6-phosphate isomerase (GPI)
GPI deficiency
Hemolytic anemia
Glycolysis
Portugal

ABSTRACT

Glucose-6-phosphate isomerase (GPI) deficiency cause hereditary nonspherocytic hemolytic anemia (HNSHA) of variable severity in individuals homozygous or compound heterozygous for mutations in *GPI* gene. This work presents clinical features and genotypic results of two patients of Portuguese origin with GPI deficiency. The patients suffer from a mild hemolytic anemia (Hb levels ranging from 10 to 12.7 g/mL) associated with macrocytosis, reticulocytosis, hyperbilirubinemia, hyperferritinemia and slight splenomegaly. Genomic DNA sequencing revealed in one patient homozygosity for a new missense mutation in exon 3, c.260G > C (p.Gly87Ala), and in the second patient compound heterozygosity for the same missense mutation (p.Gly87Ala), along with a frameshift mutation resulting from a single nucleotide deletion in exon 14, c.1238delA (p.Gln413Arg fs*24). Mutation p.Gln413Arg fs*24 is the first frameshift null mutation to be described in GPI deficiency. Molecular modeling suggests that the structural change induced by the p.Gly87Ala pathogenic variant has direct impact in the structural arrangement of the region close to the active site of the enzyme.

1. Introduction

Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) is a homodimeric enzyme that catalyses the interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), in the second reaction step of the Embden–Meyerhof glycolytic pathway [1].

The enzyme is present ubiquitously in most organisms and expressed in all tissues, which has several physiological consequences in addition to its essential role in carbohydrate metabolism. GPI belongs to the moonlighting family of proteins having multiple functions/activities [2]. Molecular cloning and sequencing revealed that GPI share the same sequence to the protein known as neuroleukin (NLK), a neurotrophic factor that mediates differentiation and survival of embryonic spinal and sensory neurons [3,4]. GPI has been shown to work as autocrine motility factor (AMF), a tumor-secreted cytokine which stimulates cell migration and metastasis in an autocrine manner in various tumor cells [5–7]. GPI also shares sequence homology with the differentiation and maturation inducer for human myeloid leukemia HL-60 cells to terminal monocytic cells which indicates that GPI and maturation factor (MF) also share a common biological function, regulating differentiation and proliferation of human myeloid leukemic cells [2,8]. It was also found that chronic arthritis spontaneously developed by the K/BxN T cell receptor-transgenic mouse, with many features of human rheumatoid arthritis disease, is initiated by T cell recognition of GPI enzyme [9]. Moreover, immunization with human recombinant GPI protein induced arthritis in several mice models [10–12]. Recent literature also suggests a positive correlation between anti-GPI autoantibody and the arthritis disease in humans [13].

GPI deficiency (OMIM: 172400) is the second most frequent erythroenzymopathy in glycolysis and since the first report of the disease [14] about fifty cases have been reported throughout the world [1,15]. Deficiency of the enzymatic activity occurs in individuals homozygous or compound heterozygous for GPI gene mutations and affects mostly erythrocytes causing hereditary nonspherocytic hemolytic anemia (HNSHA). Diagnosis is based on determination of the GPI activity in the red blood cells by enzyme quantitative assay. The major clinical features include variable degrees of jaundice, slight-to-moderate

* Corresponding author at: Research Centre for Anthropology and Health (CIAS), Department of Life Sciences, University of Coimbra, Rua Arco da Traição, 3000-456 Coimbra, Portugal.
E-mail address: lmanco@antrop.uc.pt (L. Manco).

http://dx.doi.org/10.1016/j.bcmd.2016.06.002
1079-9796/

splenomegaly, an increased incidence of gallstones, and mild to severe anemia that is normochromic in most of the cases [1]. In severe cases GPI deficiency was associated with *hydrops fetalis* and neonatal death [16]. Few patients present with neuromuscular dysfunctions defined by muscle weakness and mental retardation [17].

The gene encoding GPI is located on chromosome 19q13.1 [18], contains 18 exons [19], and the cDNA of 1.9 kb translates a protein of 558 amino acids. The molecular characterization of GPI deficient variants shows that the gene defects are mostly missense mutations leading to protein instability or negatively influence the enzyme catalytic activity [1,15]. Until now 34 GPI pathogenic variants have been documented [1,20,21] (http://www.biobase-international.com), including 28 missense, three nonsense, two splicing and one recently described frameshift mutation (submitted).

A Portuguese GPI deficient patient was previously reported with hemolytic anemia associated with hyperbilirubinemia and splenomegaly, showing severe neurological impairment [22], however, at that time, molecular analysis could not be performed. In this work we present the clinical features and genotypic analysis of two additional unrelated Portuguese patients with GPI deficiency.

2. Methods

2.1. Patients

Patient 1 is a 31-year-old female diagnosed at the age of 10 with GPI deficiency. This woman has a palpable spleen (2 cm bellow the costal margin), a mild chronic hemolytic anemia (Hb 10–11.5 g/dL), macrocytosis (MCV 105 fL), reticulocytosis (180–200 $\times 10^9$/L reticulocytes), unconjugated hyperbilirubinemia (total bilirubin 34 μmol/L; indirect 32 μmol/L) and with iron overload (ferritin 650 ng/mL, transferrin saturation 66%) with no evidence of cardiac or hepatic hemosiderosis. Red blood cell GPI activity was 8 IU/g Hb (control 38 IU/g Hb). PK activity was 7.1 IU/g Hb (control 6.0 IU/g Hb) and G6PD activity was 6.8 IU/g Hb (control 6.2 IU/g Hb). She presents infrequent more severe hemolytic episodes associated with intercurrent infections.

Patient 2 is a 54-year-old male, presenting a mild hemolytic anemia (Hb 12.1 g/dL; reticulocytes 156 $\times 10^9$/L), macrocytosis (MCV 100 fL), hyperferritinemia (450 ng/mL) and unconjugated hyperbilirubinemia (total bilirubin 62 μmol/L; indirect 59.1 μmol/L). GPI activity was 5 IU/g Hb (control 49 IU/g Hb). PK activity was 10 IU/g Hb (control 5.3 IU/g Hb) and G6PD activity was 11.2 IU/g Hb (control 9.4 IU/g Hb). His medical history included a total splenectomy after splenic injury at age of 18-year-old.

2.2. Hematological and biochemical analysis

Routine hematological studies were conducted with standard methods [23]. Red cell enzyme activities, expressed as IU/g Hb, were measured in hemolysates according to the methods recommended by the International Committee for Standardization in Hematology [24].

2.3. Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard methods and polymerase chain reaction (PCR) to amplify GPI exons and adjacent intronic regions was performed with primers and conditions reported in Beutler et al. [25]. PCR products were purified with a ExoSap IT (Valencia, CA, USA) following the manufacturer's instructions and sequenced using the ABI Prism BigDye® Terminator V 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3130 genetic analyzer (Applied Biosystems). Screening of 50 unrelated normal individuals for the new missense mutation was performed by RFLP with the restriction endonuclease DdeI.

2.4. Structural modelling

To better understand the structural implications of a novel missense mutation identified in the human GPI protein, we have used Modeller 9v15 [26] to build a three-dimensional model of the mutant protein. The model was generated based on the crystallographic structure of the human GPI with pdb code: 1NUH [27]. During the homology modelling process, Modeller starts by creating a single point mutation in the original structure and ends by optimizing and refining the mutant side-chain by conjugated gradient and simple Molecular Dynamics simulation [28]. In the end, PROCHECK [29] was used to perform a full stereochemical validation of the generated model.

The probable phenotypic effect of amino acid change for the new missense mutation was also evaluated by using *in silico* tools commonly used for missense variant interpretation, including: the PolyPhen-2 (Polymorphism Phenotyping v. 2) software [30] (http://genetics.bwh.harvard.edu/pph2/), MutationAssessor [31] (http://mutationassessor.org), PROVEAN [32] (http://provean.jcvi.org/index.php), SIFT [33] (http://sift.jcvi.org), MutationTaster [34] (http://www.mutationtaster.org) and MutPred v.1.2 [35] (http://mutpred.mutdb.org), using the coordinates ENST00000356487, ENSP00000348877 and UniProtKB - P06744.

3. Results

The hematological, biochemical and molecular findings of the two unrelated Portuguese patients with GPI deficiency are summarized in Table 1. The two patients suffer from a mild hemolytic anemia (with Hb levels ranging from 10 to 12.7 g/mL) associated with macrocytosis, reticulocytosis, slight splenomegaly, hyperbilirubinemia and hyperferritinemia. The diagnosis of GPI deficiency was made after exclusion of the most common causes of hemolytic anemia and by the demonstration of a reduced GPI enzyme activity (about 80% in patient 1 and 90% in patient 2).

Sequencing of genomic DNA in patient 1 revealed homozygosity for a new missense mutation in exon 3, transversion c.260G > C, that changes a codon GGT to GCT, leading to the amino acid substitution p.Gly87Ala. Sequence analysis showed both mother and father heterozygous for the variant c.260G > C. Patient 2 genomic DNA sequencing revealed compound heterozygosity for two different mutations: the same missense mutation c.260G > C (p.Gly87Ala), along with a single nucleotide deletion in exon 14, c.1238delA, resulting in the incorporation of incorrect amino acids into the protein after residue 413 and predicting premature termination of translation at codon 436 (p.Gln413Arg fs*24).

The novel missense mutation was confirmed by RFLP since the c.260G > C change creates a restriction site for DdeI. Sequencing of the

Table 1
Hematological and biochemical data in the two Portuguese patients with GPI deficiency.

Parameters	Patient 1	Patient 2	Reference values
Age	31 years	54 years	–
Sex	Female (F)	Male (M)	–
RBC ($\times 10^{12}$/L)	2.88	3.67	M: 4.5–5.5 F: 3.8–4.8
Hb (g/dL)	10–11.5	12.2	M:13–17.5 F: 12–16
MCV (fL)	105	102	80–100
MCH (pg)	34.7	33.3	27–32
MCHC (g/dL)	32.8	32.7	32–35
RDW (CV %)	11	10.8	11.6–14
WBC ($\times 10^9$/L)	3.6	14.5	4–10
Platelets ($\times 10^9$/L)	313	530	150–400
Reticulocytes ($\times 10^9$/L)	183 (5.7%)	156 (4%)	50–100
Serum ferritin (ng/mL)	450	650	9–120
Unconjugated bilirubin (μmol/L)	32	59.1	<12.8
G6PD (IU/g Hb)	6.8	11.2	6.5–13
PK (IU/g Hb)	9.1	10	9–14
GPI (IU/g Hb)	8	5	45–75