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Two novel DNA variants associated with glucose-6-phosphate dehydrogenase deficiency found in Argentine pediatric patients

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ABSTRACT

Objective: The enzyme glucose-6-phosphate dehydrogenase (G6PD) catalyses the first step in the pentose phosphate pathway, producing nicotinamide adenine dinucleotide phosphate (NADPH). NADPH plays a crucial role in preventing oxidative damage to proteins and other molecules in cells, mostly red blood cells. G6PD deficiency has an x-linked pattern of inheritance in which hemizygous males are deficient, while females may or may not be deficient depending on the number of affected alleles. We report two novel DNA variants in the G6PD gene detected in two male probands with chronic nonspherocytic hemolytic anemia (CNSHA), who were referred for hematological evaluation.

Method: Probands and their relatives underwent clinical, biochemical, and molecular assessment.

Results: Two novel DNA variants, c.995C > T and c.1226C > A, were found in this study. At the protein level, they produce the substitution of Ser332Phe and Pro409Gln, respectively. These DNA variants were analyzed in the female relatives of probands for genetic counseling.

Conclusions: The novel DNA variants were classified as class I based on the clinical, biochemical, and molecular evaluations performed.

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme, which is vital for cells. Complete absence of enzyme activity has not been reported in humans, suggesting its incompatibility with life [1]. G6PD catalyses the first step in the pentose phosphate pathway, leading to the production of NADPH. NADPH plays a crucial role in preventing oxidative damage to proteins and other molecules, mostly red blood cells (RBC) [2]. G6PD deficiency is known to affect more than 400 million people around the world. Its distribution is linked to malaria, being more prevalent in Africa, Asia, the Mediterranean Area, and the Middle East. However, due to global migration, currently, it can be found worldwide [3].

The deficiency is associated with mutations in the G6PD gene. G6PD is encoded on the X chromosome, and therefore, the disease has an X-linked inheritance pattern. The affected gene determines the expression of protein variants with different levels of enzymatic activity (OMIM*305900) [4].

The clinical signs in affected males are extremely variable, ranging from chronic nonspherocytic hemolytic anemia (CNSHA), neonatal jaundice (NJ), and hemolytic attacks after fava beans ingestion, infections, or drug exposure in asymptomatic individuals [5].

All known variants can be grouped into 5 classes according to the level of activity in RBC and the clinical manifestations [1]. G6PD variants that result in CNSHA are designated class I, while variants that do not result in CNSHA are designated class II or class III, depending on the severity of the reduction in enzyme activity in RBC. Class IV variants have normal activity and class V is reserved for variants with increased activity.

Here, we report two novel DNA variants in the G6PD gene detected in two male probands with CNSHA referred to our institution for clinical and hematological evaluation.

2. Materials and methods

2.1. Patients

Two boys with CNSHA (proband nos. 1 and 2) were referred at the age of 3.3 and 2.8 years, respectively, for clinical, biochemical, and molecular characterization due to CNSHA.

Both had similar clinical signs and symptoms: neonatal jaundice that required phototherapy, CNSHA, and several episodes of acute hemolytic attacks. Cholecystectomy was performed in both patients at the age of 12.3 years (proband no. 1) and 11.9 years (proband no. 2).

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Table 1
Hematological profiles and G6PD activity.

Markers	Proband no. 1	Proband no. 2
Sex/age (years)	M/3.3	M/2.8
RBC ($\times 10^6$/μL)	2.45	2.22
Hemoglobin (g/dL)	8.6	7.6
Reticulocytes (%)	17.3	25.7
MCV (fL)	108.7	102.2
Total/direct bilirubin (mg/dL)	2.7/0.4	2.5/0.6
Activity (U/gHb)	0.46	1.7
Activity (%)	5.01	7.6
Activity normal control* (U/g Hb)	9.2	9.2
Normal range (U/gHb)	7.1–13.2	7.1–13.2

Enzyme in a stabilized human red cell hemolysate base.
* A normal control sample (namely, normal level) consisted of a lyophilized sample containing G6PD.

One sister of proband no. 1 and both mothers were included in the study.

Written informed consent for molecular and biochemical studies was provided by the parents.

The study was approved by the Ethics Committee of the "Hospital de Pediatría Prof. Dr. Juan P. Garrahan" and complies with ethical guidelines of the Declaration of Helsinki of the World Medical Association, the International Ethical Guidelines for Biomedical Research of the Council for International Organizations of Medical Sciences, and the UNESCO Declaration on Bioethics and Human Rights. The study was conducted according to the guidelines of Good Practice in Clinical Research in Humans of the Argentine Ministry of Health.

2.2. Laboratory and enzyme analysis

Hematological data were analyzed in blood samples collected with EDTA using Sysmex XS800i (Sysmex Corporation, Kobe, Japan).

Brewer test was performed as a screening test for G6PD deficiency [6,7], and G6PD enzymatic activity was measured by an spectrophotometric assay (Trinity Biotech) [8].

2.3. Genomic analysis

DNA was isolated from peripheral blood leukocytes using the salting-out method. The G6PD gene was studied by PCR-sequencing of the 12 coding exons and intronic flanking regions using the primers and the strategy described previously [9]. Exonic and intronic flanking regions were directly sequenced using the Sanger method and the ABI PRISM® 3130 automated capillary sequencer. RefSeq.NG_009015.2 was the reference sequence used for analysis performed using the SeqScape v2.6 (Applied Biosystems, Buenos Aires, Argentina).

2.4. In silico tools

The possible impact of a not previously reported amino acid change in enzyme structure or activity was analyzed using the in silico tools PolyPhen-2 (Polymorphism Phenotyping v2) [10] and SIFT (Sorting Intolerant From Tolerant) [11].

2.5. Protein analysis

Structural analysis was based on the X-ray structure deposited in the Protein Data Bank: 2BHL crystal containing the glucose-6P domain, and 2BH9 crystal containing the $NADP^+$ domain. Mutated protein models were created with MODELLER 9.11, and previous structures were used as templates. Visual molecular dynamics was used for molecular visualization and analysis of the protein [12,13].

3. Results and discussion

The hematological profile and G6PD activity of both patients are described in Table 1.

The sequencing analysis of the exons and intronic flanking regions of the G6PD gene identified two novel DNA variants that have not been previously reported (Fig. 1). Neither of the nucleotide changes, searched in the databases Exome Aggregation Consortium (ExAC), 1000 Genomes (1000G), Single Nucleotide Polymorphism Database (dbSNP), and Human Gene Mutation Database (HGMD), were found.

The NM_001042351.2:c.995C > T DNA change was detected in proband no. 1. This variant was found in exon 9 and determines the Serine332Phenylalanine (Ser332Phe) amino acid change. The NM_001042351.2:c.1226C > A change was detected in proband no. 2. The variant is located in exon 10 and is associated with the Proline409Glutamine (Pro409Gln) amino acid change.

PolyPhen-2 and SIFT predicted that both amino acid changes (Ser332Phe and Pro409Gln) are probably damaging and deleterious.

Both the mother and the sister of proband no. 1 were heterozygous for the DNA variants found and did not show signs or symptoms of the disease phenotype, a fact that is usually expected for X-linked conditions.

To analyze the impact of amino acid change, the results structures were modeled using the previous crystals of G6PD as a template. The model shows that both mutated amino acids are located far from the active site, meaning that the interpretation of the change in protein function is not straightforward. The analysis of Ser332Phe using the X-ray structure shows that the main effect is a breakage of the hydrogen bond between the side chains of serine 332 and aspartic acid 251 (Fig.

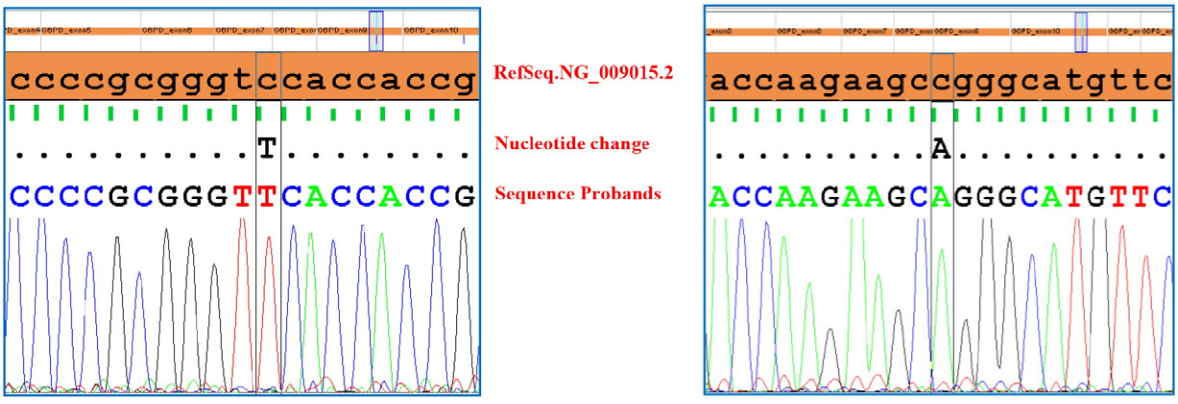

Fig. 1. Electropherogram of the probands. On the right, a part of the sequence of exon 9 showing the c.995C > T DNA variant (Ser332Phe). On the left, the segment of exon 10 showing the c.1226C > A DNA variant (Pro409Gln). The reference sequence NG_009015.2 can be seen in both electropherograms.

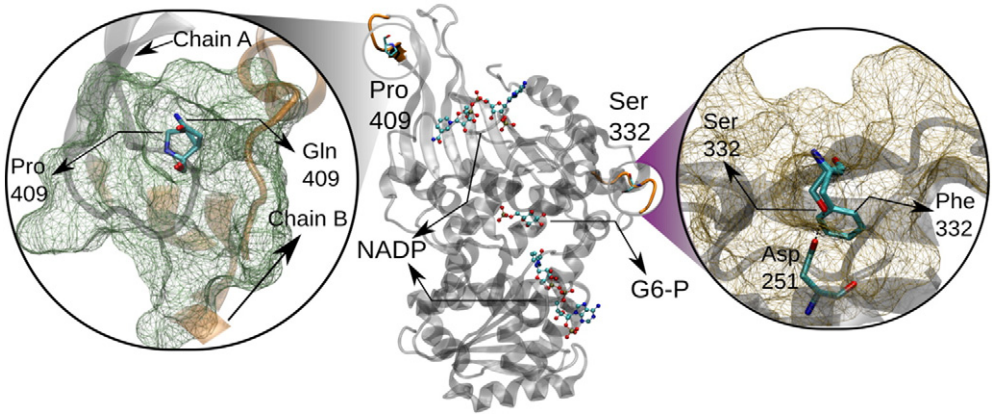

Fig. 2. Structure of glucose 6-phospathe dehydrogenase. In the middle, the general structure of G6PD with the binding sites of $NADP^+$ and glucose 6-phosphate, showing their respective ligands. In the right panel, a zoom of the region with both wild type and Pro409Gln change superimposed. In the left panel, a zoom of the region with both wild type and Ser332Phe superimposed.

2). We hypothesize that the amino acid replacement could generate a conformational change in the loop at the access of the active site, which may affect the enzyme activity disturbing the substrate uptake.

Additionally, the Pro409Gln change affects the domain which is relevant for dimerization and is located on an intersubunit contact surface. The affected proline is at the end of a β-sheet and, because it is a rigid amino acid, it usually provokes the rupture of secondary structure elements. Consequently, the change of proline for another amino acid could alter the structure and β-sheet, affecting dimer formation. The homodimeric or tetrameric structure is essential for protein stability and enzymatic activity [4] and therefore, the Pro409Gln change could affect normal dimerization, decreasing the activity of the enzyme (Fig. 2).

A previously described different mutation (HGMD: CM044625) at the same position 409 (Pro409Arg) leads to changes in G6PD kinetics and determines an unstable variant in comparison to the wild type [14]. This finding allows us to hypothesize that proline plays an important role in enzyme stability and function.

Although functional assays would be important to demonstrate the pathogenicity of these new variants, unfortunately, it was not possible to perform them at our laboratory.

4. Conclusions

This study describes two novel G6PD DNA variants associated with G6PD deficiency. These variants could be classified as Class I based on biochemical and molecular analyses, in silico tools predictions, and the clinical features of the probands.

The importance of identifying a mutation responsible for G6PD deficiency in a family relies on the possibility to investigate the presence of the same mutation in the females who are probably carriers in order to diagnose the disorder and provide genetic counseling. Proper genetic counseling enables the correct management of male newborns and the avoidance of exogenous agents that might trigger hemolytic attack.

We decided to name the Ser332Phe variant "G6PD Quilmes" and the Pro409Gln variant "G6PD Merlo" according to the place of birth of the probands.

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