Study of the Molecular Defects in Glucose Phosphate Isomerase-Deficient Patients Affected by Chronic Hemolytic Anemia

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We have studied four unrelated Italian patients with chronic hemolytic anemia associated with glucose phosphate isomerase (GPI) deficiency. Using intronic primers, we were able to detect the gene alterations on the genomic DNA of the patients. Five different mutations were identified among the eight mutated alleles found: three missense mutations (301A, 584T, 1028G), one nonsense mutation (286T), and a four nucleotides deletion [Del 1473-IVS16(+2)]. All of these were new except for mutation 1028G, which was previously identified in a Japanese variant (GPI Nariita). Two patients were homozygotes (301A/301A and 1028G/1028G), whereas the other two were compound heterozygotes sharing a common mutation (286T/584T and Del1473-IVS16(+2)/584T).

GLOUCOSE PHOSPHATE isomerase (GPI; glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) is an enzyme in the glycolytic pathway that catalyzes the reversible conversion of glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P). GPI has an essential role in carbohydrate metabolism; in fact, its substrates (G6P and F6P) are intermediates of glycolysis, gluconeogenesis, and pentose phosphate pathways. Moreover, GPI has been recently found to be identical to neuroleukin, which is a neurotrophic factor for spinal and sensory neurons, and it is also produced by lectin-stimulated T lymphocytes. This finding suggests that GPI may also have a function outside the carbohydrate metabolism.

GPI deficiency, which was first described by Baughan et al. in 1968, is an autosomal recessive disease and represents the third most common hereditary red blood cell (-C) deficiency results only in chronic nonspherocytic hemolytic anemia associated with mental retardation or neuromuscular symptoms. In rare cases this anemia is accompanied by mental retardation or neuromuscular symptoms. The gene encoding for human GPI (neuroleukin) is located on the long arm of chromosome 19 and the cDNA of 1.9 kb of length coded for 558 amino acids. So far, more than 40 variants of GPI deficiency have been reported, but only 8 of these have been characterized at the molecular level.

In this investigation, we describe the results of molecular study of four Italian patients affected by GPI deficiency. Five different mutations were identified among the eight mutated alleles found; four of these had not been described previously. Moreover, the molecular results have been related to the biochemical properties of the mutant enzymes and to the clinical pattern.

MATERIALS AND METHODS

Patients. Four unrelated patients affected by chronic nonspherocytic hemolytic anemia associated with GPI deficiency were investigated. Two of them (MA and CA) are newly diagnosed patients; they were born in the same area of Southern Italy from nonconsanguineous parents.

Proband MA is a 7-year-old boy born at full term after an uneventful pregnancy and delivery. Exchange transfusion was needed after birth. Thereafter, he had moderate anemia with mild splenomegaly and hyperbilirubinemia; severe hemolytic episodes occurred during infection of the upper respiratory tract, requiring 2 to 3 blood transfusions per year. No neurologic or muscular impairment was detectable.

Proband CA is a 19-year-old woman born after an uncomplicated pregnancy and delivery. Neonatal jaundice was intense, but exchange transfusion was not required. The patient was noted to be markedly anemic at the age of 9 months and then had a history of chronic anemia with jaundice and occasional very severe hemolytic crises triggered by infections (Hb values ranging from 5.3 to 9.0 g/dL). The overall transfusion requirement was 46 packed RBC units. Cholecystectomy was performed at the age of 12. When first seen by us at the age of 18, the patient was anemic and icteric, without liver and spleen enlargement. No signs of mental retardation, muscular weakness, or ataxia were detected.

The clinical history and results of enzyme characterization in patients MM and OC have already been reported. After the original description, patient MM had 10 years of additional follow-up.

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In this period, he displayed a twofold increase in serum ferritin levels (from 380 to 734 ng/mL) in the absence of both blood transfusions and ineffective erythropoiesis, as documented by erythremic studies.

The patients underwent the following hematologic investigations: complete blood count, reticulocyte count, screening for abnormal or unstable hemoglobins, RBC osmotic fragility, autohemolysis, direct antiglobulin test, Ham and sucrase hemolysis test, and assay of the most important erythrocyte enzyme activities of glycolytic and pentose phosphate pathways. Bilirubin, iron status parameters, transferrin receptor (TfR), and erythropoietin (Epo) concentrations were also determined in the serum. The most important clinical and hematologic data at the time of diagnosis are summarized in Table 1.

The diagnosis of GPI deficiency was made by exclusion of the most common causes of hemolytic anemia and by the demonstration of a reduced GPI activity (from 15% to 35% of normal) in all patients, with the other RBCs’ enzyme showing normal or increased activity. The residual GPs from patients CA and MA were purified from RBCs and biochemically characterized.

Reference subjects. Thirty normal individuals coming from various Italian regions were used as the reference population.

Hematologic assays and enzymes studies. Routine hematologic investigations were performed according to Dacie and Lewis. The assay of glycolytic and pentose phosphate pathway enzyme activities were performed as reported elsewhere, following the methods of Beutler. Purification and biochemical characterization of RBC GPI were performed as previously described.

Serum iron, total iron binding capacity, and transferrin saturation (TS) were determined by standard methods. Serum ferritin was measured by an enzyme immuno assay procedure (IMX System; Abbott Laboratories, Abbott Park, IL). Serum Epo (s-Epo) and TfR (s-TfR) levels were determined by enzyme-linked immunoassays (for s-Epo, Epo-ELISA [Medac Diagnostika, Hamburg, Germany]; for s-TfR, Clinigen [Amgen Diagnostics, Thousand Oaks, CA]).

DNA extraction. Blood samples were collected from the four patients and a group of normal individuals. Leukocytes were isolated and genomic DNA was extracted using standard manual methods.

Single-strand conformation polymorphism (SSCP) analysis. The 18 exons of the GPI gene of the four patients were screened by SSCP analysis. Each exon was amplified by polymerase chain reaction (PCR) using 200 ng of genomic DNA, in a 10 μL mix containing: 33.5 mmol/L Tris/HCl, pH 8.8, 1.65 mmol/L MgCl₂, [α-32P]dATP (1 μCi), 0.2 mmol/L dNTPs, 25 ng of each primer, and 0.6 U of Taq DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). The samples were heated at 98°C for 5 minutes to denature the genomic DNA and then processed for 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds in a Thermacycler (Perkin-Elmer Corp, Norwalk, CT). Ten microliters of SSCP loading buffer[22] was added to the PCR and 5 μL of the sample was loaded, after denaturation at 98°C for 3 minutes, on a 5% polyacrylamide gel containing 5% glycerol and 0.5× TBE. Electrophoresis was performed at 25 V/cm for 6 hours at room temperature.

Sequence analysis. SSCP analysis was followed by direct sequencing of the exons in which band shifts were observed. Direct sequence was performed on genomic DNA of the remaining patients until mutations were identified. Sequence was performed using a Cycle Sequencing Kit (Strategene, La Jolla, CA) using as a template double-strand DNA. The oligonucleotides and the methods used for SSCP and sequence analysis have been reported by Xu et al. Confirmation of the new mutations by endonuclease restriction enzymes and population study. The mutations were confirmed by sequence of both strands or by use of restriction endonuclease enzymes. When a mutation did not create or destroy a restriction endonuclease site, a mismatched oligonucleotide primer that creates an informative restriction site was prepared to confirm the mutation. The genomic DNA of 30 normal individuals were investigated for the presence of the missense mutations. PCR was applied to amplify a portion of the GPI gene and restriction endonuclease enzymes were used to examine the presence of the mutations. The oligonucleotides and endonuclease restriction enzymes (New England Biolabs, Beverly, MA) used are reported in Table 2. A population study was also performed for the nucleotide substitution 473 T → G that creates a restriction endonuclease site for the enzyme AcI. The oligonucleotides 5’-ttgagecgaggctctgct-3’ and 5’-gaaagaacagctgattg-3’ were used to amplify the specific fragment.

RNA extraction and cDNA study. Total RNA was extracted from leukocytes using Trizol Reagent (total RNA isolation reagent; Gibco BRL, Gaithersburg, MD). First-strand cDNA was made from the total RNA, using oligo (dT) and reverse transcriptase, as described. DNA extending from cDNA at 1360 to cDNA at 1592 was amplified by 30 cycles under the following conditions: at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; 50 ng of sense oligonucleotide 5’-AAAGTCCAGAGGACCTTTG-3’ and antisense oligonucleotide 5’-CCATCAAGCTGACCCCTAT-3’ was used. The same oligonucleotides were used to perform the sequence of the PCR product.

RESULTS

Table 3 reports the molecular and biochemical data in the four unrelated GPI-deficient patients together with the respective variant names. Although parents’ consanguinity was not known to be present in the families investigated, two patients were found to be homozygous. A common mutation was detected in the two remaining compound heterozygous patients. Therefore, only five different mutations were detected among the eight mutated genes identified: a nonsense mutation (286C → T), a deletion of four nucleotides [1473-IVS16(+2)] and three missense mutations (584C → T, 301G → A, and 1028A → G). Figure 1 shows the position of the new mutations in the cDNA in comparison with the
The enzyme characterization in the two electrophoretic patterns. Moreover, one difference from the published normal individuals, thus excluding the possibility of poly-

The small and capital letters used to report the oligonucleotides refer to intron primers and exon primers, respectively. The bold type in the oligonucleotide represents a mismatched nucleotide. Quotation marks indicate that an artificial restriction site has been introduced by using a mismatched oligonucleotide.

Table 2. Oligonucleotides and Endonuclease Restriction Enzymes Used to Confirm the GPI Mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sense Oligonucleotide</th>
<th>Antisense Oligonucleotide</th>
<th>Size of PCR Fragment</th>
<th>Enzyme</th>
<th>Size of Normal Fragments</th>
<th>Size of Mutated Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>301G → A</td>
<td>5’-catgccccagtggggcat-3’</td>
<td>5’-GCAGAAAGACTTCATCTTTGTC-3’</td>
<td>166</td>
<td>Nla III</td>
<td>4 + 16 + 166</td>
<td>4 + 16 + 68 + 98</td>
</tr>
<tr>
<td>584C → T</td>
<td>5’-ATGGAACTCAGACCGGACA-3’</td>
<td>5’-caaaagggaccaatggcagc-3’</td>
<td>167</td>
<td>“Nfi I”</td>
<td>42 + 126</td>
<td>19 + 23 + 126</td>
</tr>
<tr>
<td>1028A → G</td>
<td>5’-cagagggcgctgggtg-3’</td>
<td>5’-ggttcgagacctcagcga-3’</td>
<td>325</td>
<td>Kpn I</td>
<td>63 + 262</td>
<td>35 + 63 + 227</td>
</tr>
</tbody>
</table>

The mutations identified in the homozygous state (301A and 1028G) result in a marked enzyme instability without strongly affecting the substrate affinity (slightly modified in patient OC only). The enzyme characterization in the two compound heterozygotes showed that heat stability was strikingly reduced in patient CA and almost normal in patient MA, without alteration of the affinity for the substrates and electrophoretic pattern.

**DISCUSSION**

Using SSCP and direct DNA sequence analysis, we have identified five different mutations [286T, Del 1473-IVS16(+2), 584T, 301A, and 1028G] in a group of four unrelated Italian GPI-deficient patients. All were new but one, 1028G, which had already been described in a Japanese family. Although consanguinity was not reported, two patients turned out to be homozygous. Moreover, mutation 286T and deletion 1473-IVS16(+2) were found in two compound heterozygotes in association with mutation 584T, suggesting that these subjects had a common ancestor.

The 286T mutation is the first nonsense mutation detected in GPI deficiency and is the most drastic among the mutations so far reported. Because the GPI cDNA coding region is 1,677-bp long, corresponding to 558 amino acids, this mutation causes the lack of more than 80% of the normal protein structure. Therefore, the gene product is inactive and is not likely to survive the intracellular proteolysis. The deletion 1473-IVS16(+2) causes the disruption of the splice site and the activation of a cryptic splice site, as confirmed by the computer analysis using the Staden method. Despite the drastic alteration, we cannot exclude that the enzyme is expressed. If so, it is likely to be unstable and catalytically inactive, as suggested by the lack of lysine 517, which has been postulated as a possible catalytic site in pig protein. The abnormal gene product could interact with the product of 584T allele, giving rise to the formation of unstable dimers.

Mutation 286T and deletion 1473-IVS16(+2) undoubtedly account for the enzyme deficiency. On the contrary, the cause-and-effect relationship between a single amino acid substitution and the enzyme defect is less certain. Therefore, the three nucleotide substitutions that induce an amino acid change (101Val → Met, 195Thr → Ile, and 343Gln → Arg) can only be considered the putative disease-producing mutations. However, by comparing the amino acids sequence among several species (T. Brucei, Yeast, Mouse, Pig, and Human), we found the three missense mutations to involve highly conserved amino acids. This suggests that these amino acid residues are important in maintaining the GPI activity.

Table 3. Some Molecular and Biochemical Data of the GPI-Deficient Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Variant Name</th>
<th>Mutation</th>
<th>Amino Acid Substitution</th>
<th>Activity (IU/g Hb)</th>
<th>Km (μM)</th>
<th>Hb (g/dL)</th>
<th>Thermostability*</th>
<th>Electroph. Mobility†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>Bari</td>
<td>286C → T/584C → T</td>
<td>96Arg → End/196Thr → Ile</td>
<td>14.9</td>
<td>182</td>
<td>49</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CA</td>
<td>Mola</td>
<td>Del 1473-IVS16(+2)/584C → T</td>
<td>Splice site/195Thr → Ile</td>
<td>12.9</td>
<td>80</td>
<td>2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MM</td>
<td>Sarasina</td>
<td>301G → A/301G → A</td>
<td>101Val → Met/101Val → Met</td>
<td>7.2</td>
<td>150</td>
<td>20</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>OC</td>
<td>Morcone</td>
<td>1028A → G/1028A → G</td>
<td>343Gln → Arg/343Gln → Arg</td>
<td>7.2</td>
<td>72.5</td>
<td>9</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reference values</td>
<td></td>
<td></td>
<td></td>
<td>48 ± 8.3</td>
<td>130.6 ± 32.5</td>
<td>66 ± 12</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Percentage of activity after 60 minutes of incubation at 48°C.
† Percentage of normal mobility.
‡ Reference values are expressed as the mean ± 1 SD.
Moreover, the observation that none of these mutations was detected in the normal population and that 1028G has already been reported in association with chronic hemolytic anemia further supports the correlation between these mutations and the enzyme defect.

Mutation 584T causes the change from the polar amino acid threonine to a hydrophobic amino acid isoleucine. In patient MA (286T/584T), the allele 584T is responsible for the residual enzyme activity (30% of normal), with the other being unable to express an active protein. The mutant enzyme displays a normal electrophoretic pattern and substrate affinity and almost normal thermostability. This last finding is rather unusual, because a common characteristic of mutant GPIs is a marked heat lability, which has been related to the loss of enzyme activity and to the occurrence of hemolytic crises during infections. We would therefore expect homozygotes for GPI 584T to express about 60% of normal activity and to be normal hematologically, as are heterozygotes for severe mutations.

The mutation 301A was found at the homozygous state in patient MM. The parents were born in the same small village but they were not aware of the consanguinity. The amino acid substitution 101Val → Met results in marked decrease in the enzyme stability without affecting the kinetic properties and the electrophoretic mobility of the protein. The enzyme instability is then likely to be the main cause of the defective activity. The clinical pattern of this mutation was characterized by severe hemolytic episodes during childhood and by an effective response to splenectomy, followed by full compensation of hemolysis.

Mutation 1028G found in GPI Morcone has already been reported associated with chronic hemolytic anemia in a Japanese variant (GPI Narita). The fact that this mutation has been found in two ethnic groups could mean either that the origin of the mutation is very old or that the same mutation arose in more than one individual. A study of the polymorphisms linked with the GPI gene could be useful to determine if this mutation has a single origin. Recently, two polymorphic sites have been identified by Xu and Beutler in the GPI gene, 489 A → G and 1356 G → C. Knowing the state of the polymorphisms in GPI Narita may help to draw further conclusions. Although the two variants have the same mutation, their biochemical properties seem to be different concerning the Km for F6P and electrophoretic mobility. Also, the finding of a normal electrophoretic pattern in GPI Morcone despite the amino acid substitution (Gln → Arg) is unexpected. These inconsistencies are not surprising, because the methods of biochemical characterization are not very accurate; moreover, the difference in the electrophoretic methods used and the instability of the mutant enzyme may represent additional causes of discrepancy between biochemical and genetic results. A similar situation has also been reported for other defective enzymes of the RBC, such as glucose-6-phosphate dehydrogenase and pyruvate kinase. From a clinical point of view, homozygosity for mutation 1028G results in from moderate to severe hemolytic anemia, with occasional transfusion requirement until splenectomy is performed.

The observation that the nucleotide change 473 T → G is present in the normal Italian population and in the patients similarly reported by Fujii et al suggests that this substitution may represent a cloning artifact from the published sequence, although a rare polymorphism cannot be excluded.

Information on iron status and erythropoietic activity in GPI deficiency is scanty, although it is known that, in PK deficiency, the other most common glycolytic enzymopathy, iron overload, frequently occurs as a consequence of various factors, including hyperhemolysis, ineffective erythropoiesis, and splenectomy. In the present study, the Epo levels were in the normal range and the expansion of the erythropoiesis, as measured by circulating TIR, slightly increased in all of the examined patients. Iron stores were elevated in all but one, mainly due to transfusion treatment. However, the observation that patient MM, 10 years after splenectomy, had doubled his iron stores in the absence of ineffective erythropoiesis and blood transfusions suggests the necessity of monitoring iron status in this disease. This is particularly needed in cases of splenectomy, which is known to increase iron stores in patients with hemolysis.

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