Molecular Analysis of Glucose Phosphate Isomerase Deficiency Associated With Hereditary Hemolytic Anemia

By Hitoshi Kanno, Hisaichi Fujii, Akira Hirono, Yoji Ishida, Shoichi Ohga, Yasuhiko Fukimoto, Kenji Matsuzawa, Satoru Ogawa, and Shiro Miwa

We report here two new cases of glucose phosphate isomerase (GPI) deficiency associated with hemolytic anemia and present the results of molecular analysis of the five Japanese GPI variants. A Japanese girl (GPI Fukuoka) had an episode of prolonged neonatal jaundice and at 3 years of age was admitted due to acute hemolytic crisis occurring with upper respiratory tract infection. Red blood cell (RBC) GPI activity was decreased to 11.8% of normal and the reduced glutathione (GSH) level of RBCs was slightly decreased. A 54-year-old Japanese man (GPI Iwate) was hospitalized due to chronic active hepatitis, and compensated hemolysis was noted. RBC GPI activity of the proband was decreased to 18.8%, and the GSH content was about half of the normal mean value. Sequencing of the reticulocyte GPlcDNA showed homozygous missense mutations 1028CAG → CGG (343Gln → Arg), 14ACC → ATC (5Thr → Ile), 671ACC → ATG (224Thr → Met), and 1615GAC → AAC (539Asp → Asn) in GPI Narita, GPI Matsumoto, GPI Iwate, and GPI Fukuoka, respectively. We also identified GPI Kinki as a compound heterozygote of 1124ACA → AGA (375Thr → Arg)/1615GAC → AAC (539Asp → Asn). Our findings, together with the previous results of other investigators, showed that the GPI gene mutations so far identified were heterogeneous, although most GPI variants had common biochemical characteristics such as heat instability and normal kinetics. Several amino acid substitutions were identified in the proximity of the catalytically important amino acid residues such as Ser/Asp 159/160, Asp341, and Lyr518, which have been identified in the structural analysis of the pig GPI. The molecular characterization of human GPI variants, therefore, may provide new insights into the genotype-phenotype correlation of GPI deficiency as well as the structure-function relationship of this enzyme.

© 1996 by The American Society of Hematology.

GlucoSE PHOSPHATE isomerase (D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9; GPI) catalyzes interconversion of glucose-6-phosphate and fructose-6-phosphate in the Embden-Meyerhof glycolytic pathway. GPI is an essential enzyme for carbohydrate metabolism in all tissues, and null expression of the murine GPI gene has been found to be embryonic lethal.1,2 In humans, the GPI gene locus is located on chromosome 19,3 and the gene spans more than 40 kb, including 18 exons and 17 introns.4,6 Human GPI has been purified from several tissues such as muscle, erythrocytes, and the placenta.7,10 and no tissue-specific isoforms have been reported. However, in several species and tissues, including human placenta, various GPI isoforms presumably modified at the posttranslational level have been identified.9,10 Neureoleukin (NLK), a protein that acts as both a neurotrophic factor and a lymphokine, has been isolated from mouse salivary glands;14 subsequently, the primary structure of NLK was found to be identical to GPI by comparison of the cDNA sequences.15,16 The cDNA sequence encodes 558 amino acid residues. The enzyme consists of two identical subunits with the molecular weight of approximately 63,000, whereas NLK is active as a monomer. The crystal structure of porcine GPI has been elucidated at 2.6-Å resolution.17

Hereditary hemolytic anemia associated with GPI deficiency was first reported in 1968.18 Since then, more than 40 families have been reported, making this the third most common red blood cell (RBC) enzymopathy after glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiency.19-22 Frequency of heterozygosity was estimated as 2.3 in 1,000 subjects in the Japanese population.23 In the most severe case, GPI deficiency caused hydrops fetalis.24 Enzymatic characterization has shown that most GPI variants were unstable, but enzyme kinetics were not necessarily impaired.25 These observations make it quite difficult to distinguish each variant by biochemical analysis. Thus, to investigate the genetic background of GPI deficiency, analysis at the DNA level might be essential. Eight GPI-deficient families have been reported to date by our group,26 and two newly discovered Japanese families with GPI deficiency, GPI Fukuoka and GPI Iwate, are presented in this report. We also describe here the molecular abnormalities identified in the five GPI variants, including the new cases.

CASE REPORTS

A 3-year-old girl (GPI Fukuoka) with an episode of prolonged neonatal jaundice was admitted to the Kyushu University Hospital due to acute hemolytic crisis occurring with upper respiratory tract infection. Laboratory data at admission were as follows: hemoglobin (Hb) level, 6.9 g/100 mL; reticulocyte level, 12.1%; RBC morphology, polychromasia; serum lactate dehydrogenase (LDH), 1,056 IU; total bilirubin, 3.5 mg/100 mL; direct bilirubin, 0.7 mg/100 mL; indirect bilirubin, 2.8 mg/mL. There was no consanguinity in her family. In the chronic phase, the Hb value recovered to 11.2 g/100 mL with persistent reticulocytosis (9.8%) and hyperbilirubinemia (total bilirubin, 4.0 mg/100 mL; direct and indirect bilirubin, 0.4 and 3.6 mg/100 mL, respectively).

From the Okinaka Memorial Institute For Medical Research, Tokyo, Japan; the Tokyo Women’s Medical College, Tokyo, Japan; the Iwate Medical University, Iwate, Japan; the Kyushu University School of Medicine, Fukuoka, Japan; the Narita Red Cross Hospital, Chiba, Japan; the Maruko Central Hospital, Nagano, Japan; and the Osaka Medical Center and Research Institute For Maternal and Child Health, Osaka, Japan.

Submitted March 13, 1996; accepted May 13, 1996.

Supported in part by a Scientific Research Grant from the Ministry of Education, Science, and Culture, and by a Research Grant for Specific Diseases from the Ministry of Health and Welfare, Japan.


Address reprint requests to Hitoshi Kanno, MD, PhD, Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato-ku, Tokyo 105, Japan.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8806-0035$00/0

Blood, Vol 88, No 8 (September 15), 1996: pp 2321-2325
A 54-year-old Japanese man (GP1 Iwate) was hospitalized to receive interferon therapy for chronic active hepatitis as a result of hepatitis C virus infection. Routine laboratory tests showed chronic hepatitis and hemolysis of unknown etiology. Laboratory data at admission were as follows: Hb level, 11.2 g/100 mL; reticulocyte level, 10.6%; direct bilirubin, 2.5 mg/100 mL; indirect bilirubin, 0.8 mg/100 mL. Study of the family history showed that the grandparents of the proband were first cousins. 

Clinical history, hematologic data, and enzymatic characteristics of GPI Narita,24,27 GPI Matsumoto,27,28 and GPI Kinki29 have been reported previously.

MATERIALS AND METHODS

Restriction endonucleases were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). Avian myeloblastosis virus reverse transcriptase (AMVRT) was obtained from FMC Bioproducts (Rockland, ME). DNA was sequenced using a DNA sequencer type 373A (Applied Biosystems, Foster City, CA). The computer software DNASIS (Hitachi Software Engineering, Yokohama, Japan) was used to analyze nucleotide and protein sequences. 

<table>
<thead>
<tr>
<th>Oligonucleotides Used in This Study</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI-1</td>
<td>5'-GTTGACAGCGAGGACAGGCGA-3'</td>
</tr>
<tr>
<td>GPI-2</td>
<td>5'-GGGGAGTCCACAGGGAGGC-3'</td>
</tr>
<tr>
<td>GPI-3</td>
<td>5'-ATCGAGGAGAGGGCCAGGAA-3'</td>
</tr>
<tr>
<td>GPI-4</td>
<td>5'-CTGGACTCTTCCGCGAGCCG-3'</td>
</tr>
<tr>
<td>GPI-5</td>
<td>5'-AGGGAAAAAACAGGCTTGCC-3'</td>
</tr>
<tr>
<td>GPI-6</td>
<td>5'-CCAGGGAACAAGAGCTTGCCG-3'</td>
</tr>
<tr>
<td>GPI-7</td>
<td>5'-TCAGTGTACCTTCTGAGC-3'</td>
</tr>
<tr>
<td>GPI-8</td>
<td>5'-GGTGCAATCTACCTAGTGTCG-3'</td>
</tr>
<tr>
<td>GPI-9</td>
<td>5'-GTTACTGCTGATCAGAGCTC-3'</td>
</tr>
<tr>
<td>GPI-10</td>
<td>5'-CTGAAAGTGTGAGCAAGC-3'</td>
</tr>
<tr>
<td>GPI-11</td>
<td>5'-CGAGGTCGTACCAAATGTGG-3'</td>
</tr>
<tr>
<td>GPI-12</td>
<td>5'-ACCATGAGGGGTCCCAGGTC-3'</td>
</tr>
<tr>
<td>GPI-13</td>
<td>5'-GGACCTTCTACTACCAAGGAA-3'</td>
</tr>
<tr>
<td>GPI-14</td>
<td>5'-TGCCGCGGCTGAGGAAAACC-3'</td>
</tr>
<tr>
<td>GPI-15</td>
<td>5'-GACGCAAAACGCGTACATAG-3'</td>
</tr>
<tr>
<td>GPI-16</td>
<td>5'-CTTAGCTACCAAGAACTTGG-3'</td>
</tr>
<tr>
<td>GPI-17</td>
<td>5'-GTGAGATATTCCTTCAGAGGAG-3'</td>
</tr>
<tr>
<td>GPI-18</td>
<td>5'-TGGAAGTGGGAAAGACGCTG-3'</td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotides Used in This Study

...of AMVRT. The reaction mixture was incubated at 42°C for 120 minutes, and then AMVRT was inactivated by heating at 95°C for 10 minutes. Aliquots of 1/10 of the cDNA mixture were then subjected to PCR amplification in a 50 µL mixture of 1 x LAPCR buffer (included in the kit), 0.4 mM/L dNTP with 10 pmol each of primers GPI-1 primer, 100 U of ribonuclease inhibitor (Takara Shuzo), and 20 U of AMVRT. The reaction mixture was incubated at 42°C for 120 minutes, and then AMVRT was inactivated by heating at 95°C for 10 minutes. 

RESULTS

RBC enzyme analysis showed that GPI activity in GPI Fukuoka was 6.4 IU/g Hb (11.8% of normal mean value) and the reduced glutathione (GSH) level of the proband's RBCs was slightly decreased to 47.8 mg/100 mL RBC (74.9% of normal mean value). GPI activity of her mother was 27.6 IU/g Hb, 51.0% of the normal mean value, suggesting heterozygosity for the mutant GPI gene. The RBC GPI activity in GPI Iwate was decreased to 10.2 IU/g Hb (18.8% of normal mean value), and GSH content was 50.9% of normal mean value. GPI Iwate showed a decreased level of fructose-6-phosphate (F6P; 9.8 mM/L RBC; range, 4.9 to 10.1 mM/L RBC [mean ± SD]). In turn, the levels of fructose-1,6-diphosphate (FDP; 9.8 mM/L RBC; range, 1.9 to 5.5 mM/L RBC [mean ± SD]), glyceraldehyde-3-phosphate (GAP; 8.7 mM/L RBC; range, 0 to 5.1 mM/L RBC [mean ± SD]), and nicotinamide adenine dinucleotide (NAD; 93.9 mM/L RBC; range, 50.0 to 68.2 mM/L RBC [mean ± SD]) were increased relative to normal mean values. The ATP level was within normal limits (1.192 mM/L RBC). 

The human reticulocyte GPIcDNA sequences of the reticuloocyte-rich control subject and from all of the GPI variants examined showed a sequence discrepancy at nucleotide no. 603 CCC instead of CCG, which was deposited in GenBank (accession no. K03515). Because previous studies5,8,38 have not described this variation, we verified our results by genomic sequencing further 10 normal Japanese subjects and found that all had 603C. These results suggested that it might be a common DNA polymorphism in the Japanese population or a sequencing error. Xu and Beutler26 previously reported two DNA polymorphisms in this gene; however, none of these has been detected in Japanese GPI variants. 

GPI Narita had a homozygous mutation at no. 1028 CAG...
to CGG (1028G, 343 Gln to Arg). This mutation replaced a neutral with a basic amino acid compatible with the faster migration rate of this variant towards the cathode on the starch gel electrophoresis described previously. The substituted Gln is adjacent to the active site of the pig GPI, but not in yeast GPI.'6.37 (Table 3). The Thr residue is phylogenetically conserved among mouse, human GPI, and pig, and human GPI, but not in yeast GPI.'4.16.37 (Table 3).

The missense mutation 671ACG → ATG (671T, 224 Thr to Ile), was identified in GPI Matsumoto; this amino acid substitution changed the secondary structure of the amino-terminal region the GPI monomer. This variant migrated as normal on electrophoresis and had the normal Michaelis constant for F6P, but was markedly heat labile. The Thr residue is phylogenetically conserved among mouse, pig, and human GPI, but not in yeast GPI.'4.16.37 (Table 3).

The heterozygous missense mutation, 14 ACC to ATC (14T, 5 Thr to Ile), was identified in GPI Matsumoto; this amino acid substitution caused an alteration in hydrophobicity. The Thr residue is phylogenetically conserved among mouse, pig, and human GPI, but not in yeast GPI.'4.16.37 (Table 3).

Thus, it was possible for us to study the hemolytic anemia associated with GPI deficiency at the molecular level. To date, 15 GPI variants have been analyzed at the molecular level, and 13 missense mutations, 1 nonsense mutation, and 1 splice site mutation have been reported.

Because both the glycolysis and the pentose-phosphate shunt are indispensable for RBC metabolism, complete deficiency of the enzymes involved in those metabolic pathways may result in embryonic lethal effect, as previously shown in murine GPI system. The molecular characterization of GPI variants showed that the gene defects were mostly point mutations that led to enzymatic instability. Heterozygous state of null mutation of the enzymes governed by a single locus gene may be lethal. In contrast, in hemolytic anemia associated with deficiencies of other glycolytic enzymes such as PK and phosphofructokinase (PFK), null expression of genes for major isoforms in RBCs had been shown to be compensated by other isozyme genes. In severe PK deficiency, either persistence or reactivation of the fetal (M2)-type PK gene expression has been observed in homozygous PK variants’ RBCs.36,37 For another example, the M(muscle)-type PFK deficiency, the type VII glycogen storage disease, caused mild hemolytic anemia despite drastic homozygous mutations of the M-type gene,40 because the L(liver)-type PFK that was physiologically expressed in RBCs could compensate for the M-type PFK deficiency.41

Study of the glycolytic intermediates of GPI Iwate showed decreased F6P and elevated FDP levels in the proband’s RBCs, whereas the G6P level was within the normal range. Levels of F6P and FDP, metabolites distal to the step catalyzed by GPI, were reported to be decreased in most cases of GPI deficiency,25 although elevated FDP levels were identified in the RBCs of GPI Narita and GPI Matsumoto.26,28 The elevation of F6P, FDP, and GA3P levels might reflect acceleration of the pentose phosphate shunt that is activated by blockage of G6P isomerization.25

Clinical profiles, hematologic data, RBC GPI activity, and

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers</th>
<th>PCR Products (bp)</th>
<th>Restriction Enzyme</th>
<th>Normal Allele (%)</th>
<th>Mutant Allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14ACG</td>
<td>ATC</td>
<td>1</td>
<td>GP-7/10</td>
<td>Sma I</td>
<td>106 + 39</td>
</tr>
<tr>
<td>671ACG</td>
<td>ATG</td>
<td>7</td>
<td>GP-13/14</td>
<td>Bsr Al</td>
<td>35 + 18 + 16</td>
</tr>
<tr>
<td>1028CG</td>
<td>CGG</td>
<td>12</td>
<td>GP-9/15</td>
<td>Kpn I</td>
<td>79</td>
</tr>
<tr>
<td>1124ACA</td>
<td>AGA</td>
<td>13</td>
<td>GP-16/17</td>
<td>Mnl I</td>
<td>198 + 40</td>
</tr>
<tr>
<td>1615GAC</td>
<td>AAC</td>
<td>18</td>
<td>GP-2/18</td>
<td>Hpa I</td>
<td>80 + 90</td>
</tr>
</tbody>
</table>

Table 2. Strategy for Identification of the GPI Mutations in Genomic DNA Sequences
results of the molecular characterization of the variant GPI genes are summarized in Table 3. We identified 5 distinct missense mutations in this study, among which 671T(T224M) has been identified in an African-American variant.56 Previously, we reported the homozygous missense mutations of GPI Narita and GPI Matsumoto,32 and the same mutation as that of GPI Narita was reported in an Italian patient, GPI Moscone.43

Single amino acid substitutions such as Q343R and D539N were identified near the putative active sites that have been delineated by structural studies of pig GPI.39 These residues are phylogenetically well conserved, as shown in Table 4. 343Gln corresponds to the 341Gln of pig GPI, adjacent to the reported active site residue 342 Asp. Thus, it is most likely that GPI Narita, a variant GPI with Q343R, may cause alterations in the kinetic characteristics of GPI, although enzymatic analysis did not show any changes in kinetics. To evaluate the altered enzymatic characteristics caused by each mutation, improvements in the purification and characterization of GPI variants are required.

Previous enzymatic characterization of the GPI variants indicated that most GPI variants were heat-labile but had normal kinetics,29 suggesting the homogenous genetic background of this disease compared with other erythroenzymopathies such as PK and G6PD deficiency. However, recent results of GPI gene analysis disclosed fairly heterogeneous genetic abnormalities in this disease. Further studies are necessary to clarify the molecular pathology of GPI deficiency and may also provide important information regarding the genotype-phenotype relationship of GPI-deficient hemolytic anemia.

ACKNOWLEDGMENT

We thank Y. Okamura, A. Sakuma, and J. Oka for their technical assistance.

REFERENCES


Molecular analysis of glucose phosphate isomerase deficiency associated with hereditary hemolytic anemia

H Kanno, H Fujii, A Hirono, Y Ishida, S Ohga, Y Fukumoto, K Matsuzawa, S Ogawa and S Miwa