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

By Hitoshi Kanno, Hisaichi Fuji, Akira Hiro, Yoji Ishida, Shochi Ohga, Yasuhiro Fukumoto, 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 glucose 6-phosphate (G6P) 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 GPIcDNA showed homozygous missense mutations 1028CAG -+ CGG (343Gln - Arg), 14ACC -+ ATC (5Thr - Ile), 671ACG -+ 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 169/160, Asp341, and Lys518, 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.

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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. In humans, the GPI gene locus is located on chromosome 19, and the gene spans more than 40 kb, including 18 exons and 17 introns. Human GPI has been purified from several tissues such as muscle, erythrocytes, and the placenta, 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. Neureoleukin (NLK), a protein that acts as both a neurotrophic factor and a lymphokine, has been isolated from mouse salivary glands; subsequently, the primary structure of NLK was found to be identical to GPI by comparison of the cDNA sequences. 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. Hereditary hemolytic anemia associated with GPI deficiency was first reported in 1968. 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. Frequency of heterozygosity was estimated as 2.3 in 1,000 subjects in the Japanese population. In the most severe case, GPI deficiency caused hydrops fetalis. Enzymatic characterization has shown that most GPI variants were unstable, but enzyme kinetics were not necessarily impaired. 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, 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 hemolysis 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).

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A 54-year-old Japanese man (GP 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. Laboratory data at admission were as follows: Hb level, 11.2 g/100 mL; reticulocyte level, 10.6%; serum LDH, 326 IU; total bilirubin, 2.5 mg/100 mL; direct bilirubin, 7.0% of normal mean value. GP Iwate showed a decreased level of RBC enzyme activity in GP1 activity in GP1 Iwate was 4.8 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). GP1 activity of her mother was 27.6 IU/g Hb, 51.0% of the normal mean value, suggesting heterozygosity for the mutant GP1 gene. The RBC enzyme analysis showed that GP1 activity in GP1 Narita had a homozygous mutation at no. 1028 CAG to CTA. In turn, the levels of fructose-6-phosphate (F6P; 2.4 mmol/L RBC; range, 4.9 to 10.1 nmol/mL RBC [mean ± SD]). In turn, the levels of fructose-1,6-diphosphate (FDP; 9.8 mmol/L RBC; range, 1.0 to 5.5 nmol/mL RBC [mean ± SD]), glyceraldehyde-3-phosphate (GAP; 8.7 mmol/L RBC; range, 0 to 5.1 nmol/mL RBC [mean ± SD]), and nicotinamide adenine dinucleotide (NAD; 93.9 mmol/L RBC; range, 50.0 to 68.2 mmol/mL RBC [mean ± SD]) were increased relative to normal mean values. The ATP level was within normal limits (1,192 nmol/mL RBC).

**RESULTS**

RBC enzyme analysis showed that GP1 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). GP1 activity of her mother was 27.6 IU/g Hb, 51.0% of the normal mean value, suggesting heterozygosity for the mutant GP1 gene. The RBC enzyme activities and glycolytic intermediates were measured by protocols standardized previously by the International Committee for Standardization in Haematology or methods described by Minakami et al.31

Cellulose-treated RBCs were homogenized in 4 vol of 4 mol/L guanidinium isothiocyanate solution containing 25 mmol/L sodium acetate (pH 6.0) and 0.12 mol/L β-mercaptoethanol, and reticulocyte RNA was purified by density gradient ultracentrifugation on a 5.7 mol/L cesium chloride cushion.32 Genomic DNA was purified from white blood cells by the standard proteinase K-sodium dodecyl sulfate method.33

The oligonucleotides used in this study are listed in Table 1. Two micrograms of human reticulocyte total RNA was reverse transcribed in a 20 μL mixture of 50 mmol/L Tris/HCl (pH 8.3), 40 mmol/L KCl, 8 mmol/L MgCl2, 0.5 mmol/L dNTP with 10 pmol of GP1 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. Aliquots of 1/10 of the cDNA mixture were then subjected to PCR amplification in a 50 μL mixture of 1× LAPCR buffer (included in the kit), 0.4 mmol/L dNTP with 10 pmol each of primers GP1-2 and GP1-7, and 2.5 U of LTAq DNA polymerase. The reaction mixture was subjected to 30 cycles of amplification at 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 210 seconds in GeneAmp PCR System 9600 (Perkin Elmer Cetus). The amplified cDNA spanned 1,736 bp covering the entire coding sequence and parts of the 5′- and 3′-untranslated regions were subsequently cloned into the pCR II vector by TA cloning kit (InVitrogen, San Diego, CA). Clones were sequenced by the dideoxy chain termination method with fluorescent deoxyxynucleotide (Dye Terminator Cycle Sequencing Kit; Perkin Elmer) and the primers GP1-2, -3, -4, -5, -6, -7, -8, -9, -11, and -12.

To confirm nucleotide changes detected in the cDNA, 0.5 μg of genomic DNA was amplified by PCR in a 50 μL mixture of 10 mmol/L Tris/HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.001% gelatin, 0.2 mmol/L dNTP with 25 pmol of each primer listed in Table 2, and 1.25 U of AmpliTaq DNA polymerase. The reaction mixture was subjected to 30 cycles of amplification at 94°C for 20 seconds, 60°C for 10 seconds, and 72°C for 10 seconds in a GeneAmp PCR System 9600. The amplified DNA was digested with the restriction endonucleases listed in Table 2 to verify the nucleotide changes found in the cDNA.

**MATERIALS AND METHODS**

Restriction endonuclease were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). Avian myeloblastosis virus reverse transcriptase (AMVRT) was obtained from Seikagaku Kogyo (Tokyo, Japan). Taq DNA polymerase (AmpliTaq) was obtained from Perkin Elmer Cetus (Norwalk, CT). For the long polymerase chain reaction (PCR) experiment, a commercial kit (LAPCR kit version 2) from Takara Shuzo was used. NuSieve GTG agarose and SeaKem HGT agarose were obtained from FMC Bioproducts (Rockland, ME). DNA was sequenced using a DNA sequence analysis system (Model 373A; Applied Biosystems, Foster City, CA). The computer software DNASTAR (Hitachi Software Engineering, Yokohama, Japan) was used to analyze nucleotide and amino acid sequences.

RBC enzyme activities and glycolytic intermediates were measured by protocols standardized by the International Committee for Standardization in Haematology or methods described by Minakami et al.31

### Table 1. Oligonucleotides Used in This Study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI-1</td>
<td>5'-GTGCCAGGAACTGACAGT-3'</td>
</tr>
<tr>
<td>GPI-2</td>
<td>5'-GGGGAAGACTACAGAGGACG-3'</td>
</tr>
<tr>
<td>GPI-3</td>
<td>5'-ATCGAGGAGGGCCGACG-3'</td>
</tr>
<tr>
<td>GPI-4</td>
<td>5'-CGTACCTTCCGCCCCAGG-3'</td>
</tr>
<tr>
<td>GPI-5</td>
<td>5'-AGTGGTTTTCCAGGCCTGCG-3'</td>
</tr>
<tr>
<td>GPI-6</td>
<td>5'-CAGGGAACAACTGACAGT-3'</td>
</tr>
<tr>
<td>GPI-7</td>
<td>5'-TCAGTGTACCTCTGAGCTG-3'</td>
</tr>
<tr>
<td>GPI-8</td>
<td>5'-CGTCTAGGCTAGTCTGAGT-3'</td>
</tr>
<tr>
<td>GPI-9</td>
<td>5'-GTATCTGTCATGATACGCTG-3'</td>
</tr>
<tr>
<td>GPI-10</td>
<td>5'-CTGAAGGTTGGTCAGGCTG-3'</td>
</tr>
<tr>
<td>GPI-11</td>
<td>5'-CGGAGCTTCATCAACATTGGCAT-3'</td>
</tr>
<tr>
<td>GPI-12</td>
<td>5'-AACATGAGGGGTCGCCAGG-3'</td>
</tr>
<tr>
<td>GPI-13</td>
<td>5'-GACCTTATACCTACAGGAGG-3'</td>
</tr>
<tr>
<td>GPI-14</td>
<td>5'-TTGGCCCGCTTGAGAAACA-3'</td>
</tr>
<tr>
<td>GPI-15</td>
<td>5'-GACGCAAAAGGCCTGAGGT-3'</td>
</tr>
<tr>
<td>GPI-16</td>
<td>5'-TCTATTGTTCGCAAGAATCTG-3'</td>
</tr>
<tr>
<td>GPI-17</td>
<td>5'-GTGAGATATCTCTCTTGAGAGG-3'</td>
</tr>
<tr>
<td>GPI-18</td>
<td>5'-TGGAGCTGGAAAGCAGCTG-3'</td>
</tr>
</tbody>
</table>

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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.\textsuperscript{27} The substituted Gln is adjacent to the active site of the pig GPI, \textsuperscript{341}Asp, suggesting that this mutation would affect the catalytic activity of GPI.

A homozygous missense mutation, 14 ACC to ATC (14T, 5 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 Migration rate of this variant towards the cathode on the starch gel electrophoresis described previously.\textsuperscript{23} The substrate of the human GPI \textsuperscript{341}Asp, suggesting that this mutation would affect the catalytic activity of GPI.

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.\textsuperscript{1,2} The molecular characterization of GPI variants showed that the gene defects were mostly point mutations that led to enzymatic instability. Homozygous 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 expression has been observed in homozygous PK variants' RBCs.\textsuperscript{30} 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,\textsuperscript{40} because the L(liver)-type PFK that was physiologically expressed in RBCs could compensate for the M-type PFK deficiency.\textsuperscript{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.\textsuperscript{25} although elevated FDP levels were identified in the RBCs of GPI Narita and GPI Matsumoto.\textsuperscript{26,28}

The elevation of F6P, FDP, and GA3P levels might reflect acceleration of the pentose phosphate shunt that is activated by blockade of G6P isomerization.\textsuperscript{25}

Clinical profiles, hematologic data, RBC GPI activity, and

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**Table 2. Strategy for Identification of the GPI Mutations in Genomic DNA Sequences**

<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>14ACC → ATC</td>
<td>1</td>
<td>GPI-7/10</td>
<td>Smal</td>
<td>106 + 39</td>
<td>145</td>
</tr>
<tr>
<td>671ACG → ATG</td>
<td>7</td>
<td>GPI-13/14</td>
<td>BsmAI</td>
<td>35 + 18 + 16</td>
<td>53 + 16</td>
</tr>
<tr>
<td>1028CAG → CGG</td>
<td>12</td>
<td>GPI-9/15</td>
<td>KpnI</td>
<td>79</td>
<td>57 + 22</td>
</tr>
<tr>
<td>1124ACA → AGA</td>
<td>13</td>
<td>GPI-16/17</td>
<td>MnlI</td>
<td>198 + 40</td>
<td>106 + 93 + 40</td>
</tr>
<tr>
<td>1815GAC → AAC</td>
<td>18</td>
<td>GPI-2/18</td>
<td>HpaI</td>
<td>80 + 90</td>
<td>170</td>
</tr>
</tbody>
</table>

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**Table 3. Summary of the GPI Variants Analyzed in This Study**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Hb (g/dL)</th>
<th>Reticulocytes (%)</th>
<th>GP (%) of mean</th>
<th>Mutation</th>
<th>Amino Acid Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 GPI Narita</td>
<td>M</td>
<td>8.8</td>
<td>23.6</td>
<td>37.6</td>
<td>1028G/1028G</td>
<td>343Gln → Arg/343Gln → Arg</td>
</tr>
<tr>
<td>2 GPI Matsumoto</td>
<td>F</td>
<td>8.6</td>
<td>8.5</td>
<td>40.6</td>
<td>14T/14T</td>
<td>5Thr → Ile/5Thr → Ile</td>
</tr>
<tr>
<td>3 GPI Iwate</td>
<td>M</td>
<td>11.2</td>
<td>10.8</td>
<td>18.8</td>
<td>671T/671T</td>
<td>224Thr → Met/224Thr → Met</td>
</tr>
<tr>
<td>4 GPI Fukuoka</td>
<td>F</td>
<td>10.7</td>
<td>9.8</td>
<td>11.8</td>
<td>1615A/1615A</td>
<td>539Asp → Asn/539Asp → Asn</td>
</tr>
<tr>
<td>5 GPI Kinki</td>
<td>F</td>
<td>10.7</td>
<td>10.2</td>
<td>5.2</td>
<td>1124G/1124A</td>
<td>375Thr → Arg/539Asp → Asn</td>
</tr>
</tbody>
</table>
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.16 Previously, we reported the homozygous missense mutations of GPI Narita and GPI Matsumoto,17 and the same mutation as that of GPI Narita was reported in an Italian patient, GPI Moscone.18

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.32 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.

Table 4. Comparison of the Amino Acid Residues Substituted in the GPI Variants

<table>
<thead>
<tr>
<th></th>
<th>5</th>
<th>224</th>
<th>343</th>
<th>375</th>
<th>539</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Thr</td>
<td>Thr</td>
<td>Gin</td>
<td>Thr</td>
<td>Asp</td>
</tr>
<tr>
<td>Pig</td>
<td>Thr</td>
<td>Thr222</td>
<td>Gin341</td>
<td>–</td>
<td>Asp534</td>
</tr>
<tr>
<td>Mouse</td>
<td>Thr</td>
<td>Thr224</td>
<td>Gin343</td>
<td>Thr375</td>
<td>Asp539</td>
</tr>
<tr>
<td>Yeast</td>
<td>Thr</td>
<td>Thr232</td>
<td>Gin352</td>
<td>Thr384</td>
<td>Asp540</td>
</tr>
</tbody>
</table>

Abbreviation: – no amino acid sequence homology.

Previous enzymatic characterization of the GPI variants indicated that most GPI variants were heat-labile but had normal kinetics, 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.

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

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