Molecular Analysis of Eight Biochemically Unique Glucose-6-Phosphate Dehydrogenase Variants Found in Japan

By Akira Hirono, Hisaichi Fujii, Toshikuni Takano, Yasuko Chiba, Yoichi Azuno, and Shiro Miwa

We analyzed the molecular mutations of eight known Japanese glucose-6-phosphate dehydrogenase (G6PD) variants with unique biochemical properties. Three of them were caused by novel missense mutations: G6PD Musashino by 185 C→T, G6PD Asahikawa by 695 G→A, and G6PD Kamiube by 1387 C→T. Predicted amino acid substitutions causing asymptomatic variants G6PD Musashino (62 Pro→Phe) and G6PD Kamiube (463 Arg→Cys) were located in regions near the amino or carboxyl end of the polypeptide chain, whereas an amino acid change 232 Cys→Tyr causing a class 1 variant G6PD Asahikawa was located in the region where amino acid alterations in some class 1 variants were clustered. The other five variants had known missense mutations, namely, G6PD Fukushima, 1246 G→A, G6PD Morioka, 1339 G→A, and G6PD Iwate, G6PD Niigata and G6PD Yamaguchi, 1160 G→A, which cause variants, G6PD Tokyo, G6PD Santiago de Cuba, and G6PD Beverly Hills, respectively.

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RESULTS AND DISCUSSION

In the World Health Organization (WHO) proposal of standard procedures for characterizing glucose-6-phosphate dehydrogenase (G6PD) variants in 1967, more than 400 G6PD variants have been identified by their biochemical properties. Efforts to characterize the diverse variant enzymes have proven fruitful in understanding some basic features of G6PD deficiency such as ethnic heterogeneity or relationships between functional abnormalities and clinical manifestations. On the other hand, the DNA mutations causing G6PD deficiencies have been analyzed since 1987, and nearly 100 molecular variants have so far been reported. It is well established that some of the biochemical variants bear an identical mutation, or that distinct mutations can cause a phenotypically identical variant. Indeed, of 52 unique biochemical variants, which have been studied at the DNA level, only 28 are molecularly unique.

The tertiary structure of G6PD from Leuconostoc mesenteroides has recently been determined. A model of the tertiary structure of the human enzyme has been subsequently deduced, and it has shed some light on the pathogenesis of some mutations. Although biochemical characterization has lost its significance as a means of identifying variants, it still provides useful information about the functional abnormalities of variant enzymes. It is, therefore, important to reanalyze fully characterized biochemical variants using current molecular techniques and to evaluate the biochemical properties to elucidate structure-function relationships. We report here a molecular study of eight biochemically distinct variants in blood samples from Japanese individuals.

MATERIALS AND METHODS

G6PD variants. We examined the G6PD variants, G6PD Musashino, G6PD Asahikawa, G6PD Iwate, G6PD Niigata, G6PD Fukushima, G6PD Morioka, and G6PD Kamiube in this study. Case histories of the patients and the biochemical properties of the variant enzymes have been reported elsewhere. The biochemical properties of the variants are summarized in Table 1. G6PD Musashino and G6PD Kamiube are asymptomatic variants found in a G6PD deficiency screening of healthy Japanese males. The other six variants belong to class 1. Chronic nonpherocytic hemolytic anemia was the only clinical manifestation of the patients, except for one with G6PD Niigata who was complicated by severe hemochromatosis of unknown etiology and died at age 25.

Preparation of genomic DNA. Fresh blood samples were obtained from the patients with G6PD Iwate, G6PD Morioka, and G6PD Yamaguchi after obtaining their informed consent. Genomic DNA was isolated from the buffy coat and purified by standard means. Because fresh blood samples were no longer available for G6PD Musashino, G6PD Asahikawa, G6PD Fukushima, and G6PD Kamiube, genomic DNA of these variants was isolated from stored cellulose-treated blood as described. Genomic DNA of the patient with G6PD Niigata was isolated from a bone marrow smear that was the only available material from the patient. A glass slide with the bone marrow smear was soaked in running water for 1 hour to remove dust and contaminating DNA materials. The slide was dried, incubated with 600 μL of lysis solution (0.1 mg/mL proteinase K and 0.5% sodium dodecyl sulfate [SDS]) at 55°C for 1 hour, then the smeared cells were scraped into the solution with a spatula and gently mixed by pipetting. The cells were completely lysed by further incubation at 55°C for 3 hours. Genomic DNA was then purified by the standard method.

Mutation analysis. Synthetic oligonucleotide primers used for the polymerase chain reaction (PCR) and direct sequencing were as described. PCR-based single strand conformation polymorphism (PCR-SSCP) analysis was performed by our modified method using a mini gel system and ethidium bromide staining. Amplified exons were directly sequenced by an automated DNA sequencer using a dyeoxy fluorescent dye termination protocol (Dye Terminating Cycle Sequencing Kit; Perkin Elmer, Norwalk, CT). The mutation was verified either by detecting the altered restriction site or by sequencing both strands. The position of each mutation was expressed using the cDNA numbers.

RESULTS AND DISCUSSION

The results of molecular studies are summarized in Table 2. DNA samples from G6PD Musashino, G6PD Asahikawa,
Table 1. Biochemical Characteristics of G6PD Variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Class (%)</th>
<th>Electro- Activity (%)</th>
<th>Km G6P (μmol/L)</th>
<th>Km NADP* (μmol/L)</th>
<th>Km NADPH (μmol/L)</th>
<th>2-Deoxy G6P (%)*</th>
<th>Gal-6P (%)*</th>
<th>Deamino NADP⁺ (%)¹</th>
<th>pH Optimum</th>
<th>Heat Stability</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Musashino</td>
<td>3</td>
<td>17.5</td>
<td>90</td>
<td>32</td>
<td>2.3</td>
<td>8.3</td>
<td>5.1</td>
<td>16.3</td>
<td>80</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Asahikawa</td>
<td>1</td>
<td>3.8</td>
<td>98</td>
<td>30</td>
<td>18</td>
<td>2.1</td>
<td>7.1</td>
<td>11.9</td>
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<td>Biphasic</td>
<td>Labile</td>
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<tr>
<td>Iwate</td>
<td>1</td>
<td>2.3</td>
<td>75</td>
<td>37</td>
<td>40</td>
<td>3.0</td>
<td>4.3</td>
<td>4.3</td>
<td>51</td>
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<td>Labile</td>
</tr>
<tr>
<td>Niigata</td>
<td>1</td>
<td>1.8</td>
<td>38</td>
<td>47</td>
<td>7.3</td>
<td>2.3</td>
<td>32</td>
<td>540</td>
<td>37</td>
<td>Acidic</td>
<td>Labile</td>
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<tr>
<td>Yamaguchi</td>
<td>1</td>
<td>3.5</td>
<td>57</td>
<td>37</td>
<td>15</td>
<td>7.6</td>
<td>4.1</td>
<td>19.0</td>
<td>94</td>
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<td>Labile</td>
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<tr>
<td>Beverly Hills</td>
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<td>&lt;0.1</td>
<td>93</td>
<td>41</td>
<td>15</td>
<td>ND</td>
<td>&lt;4</td>
<td>ND</td>
<td>61</td>
<td>Biphasic</td>
<td>Labile</td>
</tr>
<tr>
<td>Fukushima</td>
<td>1</td>
<td>2.8</td>
<td>86</td>
<td>31</td>
<td>50</td>
<td>4.4</td>
<td>3.0</td>
<td>11.0</td>
<td>37</td>
<td>Normal</td>
<td>Labile</td>
</tr>
<tr>
<td>Tokyo</td>
<td>1</td>
<td>4.4</td>
<td>90</td>
<td>65</td>
<td>5.5</td>
<td>ND</td>
<td>2.5</td>
<td>55</td>
<td>61</td>
<td>Normal</td>
<td>Labile</td>
</tr>
<tr>
<td>Morioka</td>
<td>1</td>
<td>4.5</td>
<td>100</td>
<td>30</td>
<td>3.4</td>
<td>19.4</td>
<td>6.1</td>
<td>5.3</td>
<td>81</td>
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<td>Labile</td>
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<tr>
<td>Santiago de Cuba</td>
<td>1</td>
<td>15</td>
<td>80</td>
<td>50</td>
<td>43</td>
<td>ND</td>
<td>&lt;4</td>
<td>ND</td>
<td>ND</td>
<td>Labile</td>
<td>Labile</td>
</tr>
<tr>
<td>Kamiube</td>
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<td>24.5</td>
<td>100</td>
<td>57</td>
<td>3.0</td>
<td>8.0</td>
<td>3.7</td>
<td>11.0</td>
<td>52</td>
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<td>Normal</td>
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<tr>
<td>B (control)</td>
<td>4</td>
<td>100</td>
<td>100</td>
<td>31-71</td>
<td>2.6-6.6</td>
<td>4.9-12.9</td>
<td>1.6-6.4</td>
<td>4.3-12.7</td>
<td>51-69</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Abbreviations: Gal-6P, galactose-6-phosphate; ND, not determined.

¹ % of G6P.
² % of NADP⁺.

G6PD Fukushima, G6PD Morioka, and G6PD Kamiube showed clear mobility shifts in various exons. Direct sequencing analyses showed the nucleotide substitution responsible for each single-strand conformation change. However, the exon fragments from G6PD Iwate, G6PD Niigata, and G6PD Yamaguchi showed no mobility shift. We sequenced the whole coding region of these variants and found a single nucleotide substitution in each of them.

Molecular analysis showed that G6PD Iwate, G6PD Niigata, and G6PD Yamaguchi all bore the same missense mutation 1160 G→A originally identified in G6PD Beverly Hills.¹⁹ These are the second, the third, and the fourth Japanese unrelated cases with the 1160 G→A mutation. The identical mutation has also been found in two Caucasian patients with G6PD Genova²⁰ and G6PD Worcester²¹, respectively.⁵ The mutation destroys the cleavage site for Hha I. The mother of the patient with G6PD Niigata was confirmed heterozygous by the restriction analysis. Although these four variants share the same distinctive properties including slow electrophoretic mobility, increased Km nicotinamide adenine dinucleotide phosphate (NADP⁺) and marked heat instability, they vary in substrate specificity and pH optima. Because the biochemical characterization of G6PD Iwate, G6PD Niigata, and G6PD Yamaguchi were performed in the same laboratory using the same reagents and instruments, such variation is likely to be due to different degrees of denaturation of enzyme proteins. The extremely high utility rate of galactose-6-phosphate by G6PD Niigata is unique, and no other variants, except for G6PD Kobe²²,²³ that uses the substrate analogue 130 times faster than G6P, have this feature. The high utility of galactose-6-phosphate by G6PD Kobe appeared only after the enzyme was partially purified from hemolysate or a crude lymphoblastoid cell lysate from the patient (A. Hirono, unpublished observations, September 1983). This finding, as well as the marked heterogeneity of G6PD Niigata, G6PD Iwate, and G6PD Yamaguchi in galactose-6-phosphate utilization, suggests that the high activities of G6PD Kobe and G6PD Niigata towards the substrate analogue are not the direct outcome of a structural alteration caused by the mutations. Posttranslational factors are likely to participate in exposing their unique properties.

G6PD Fukushima had the missense mutation 1246 G→A, which was originally identified in G6PD Tokyo.²⁴ This mutation creates a new Sty I restriction site. The biochemical heterogeneity of G6PD Fukushima¹² and G6PD Tokyo²⁵ is minimal, namely the utility of deamino NADP⁺ is decreased in the former, and their having the same mutation is reasonable. The 1246 G→A mutation is the most common class I mutation among Japanese, and it has been found in four unrelated families in addition to the present patient.¹⁷ The same mutation was also identified in a Scottish²⁶ and an Italian²⁷ family. The origin of the 1246 G→A mutation in

Table 2. Molecular Abnormalities of G6PD Variants

<table>
<thead>
<tr>
<th>Biochemical Variant Name</th>
<th>Exon</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Molecular Variant Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Musashino</td>
<td>4</td>
<td>185 C → T</td>
<td>62 Pro → Phe</td>
<td>Musashino</td>
</tr>
<tr>
<td>Asahikawa</td>
<td>7</td>
<td>695 G → A</td>
<td>232 Cys → Tyr</td>
<td>Asahikawa</td>
</tr>
<tr>
<td>Iwate</td>
<td>10</td>
<td>1160 G → A</td>
<td>387 Arg → His</td>
<td>Beverly Hills</td>
</tr>
<tr>
<td>Niigata</td>
<td>10</td>
<td>1160 G → A</td>
<td>387 Arg → His</td>
<td>Beverly Hills</td>
</tr>
<tr>
<td>Yamaguchi</td>
<td>10</td>
<td>1160 G → A</td>
<td>387 Arg → His</td>
<td>Beverly Hills</td>
</tr>
<tr>
<td>Fukushima</td>
<td>10</td>
<td>1246 G → A</td>
<td>416 Glu → Lys</td>
<td>Tokyo</td>
</tr>
<tr>
<td>Morioka</td>
<td>11</td>
<td>1339 G → A</td>
<td>447 Gly → Arg</td>
<td>Santiago de Cuba</td>
</tr>
<tr>
<td>Kamiube</td>
<td>12</td>
<td>1387 C → T</td>
<td>463 Arg → Cys</td>
<td>Kamiube</td>
</tr>
</tbody>
</table>
the Italian family is different from that in the Japanese family because the former was associated with a polymorphic C→T mutation at nucleotide 1311 that was absent in the Japanese patients. Because there are few useful haplotype markers for the G6PD gene among Japanese, the origins of the identical mutations found in several unrelated families, such as the 1246 G→A or the 1160 G→A mutation, are difficult to evaluate at present.

The 1339 G→A mutation identified in G6PD Morioka has been identified in G6PD Santiago de Cuba. This mutation creates a Pst I cleavage site. The heterogeneous biochemical properties are again evident in the two identical molecular variants. Although the deduced amino acid substitution 447 Gly→Arg predicts a slow electrophoretic mobility, which was confirmed in G6PD Santiago de Cuba, G6PD Morioka showed a normal electrophoretic mobility.

Molecular analysis of G6PD Asahikawa showed that this variant was molecularly, as well as biochemically unique. The 695 G→A mutation found in G6PD Asahikawa is located in a region just downstream from the G6P binding site where several class 1 variants are clustered. Because this mutation does not alter any commercially available restriction enzyme sites, we examined the mutation in the proband’s family members by SSCP analysis. While the mother was confirmed to be heterozygous, neither of the maternal grandparents carried the mutant allele, suggesting that the mutation of G6PD Asahikawa had arisen spontaneously in one of the proband’s maternal grandparents. G6PD Asahikawa is distinctive in its unusual pH curve: an extremely high peak at pH 4.4 in addition to a low normal peak at 8.4. G6PD Clinic is the only other known variant with an unusual acidic pH optimum. The locations of the amino acid changes of these variants, 232 Cys→Tyr (βG) in G6PD Asahikawa and 405 Met→Ile (βM) in G6PD Clinic, are considerably apart both in the amino acid sequence and in the three-dimensional structure. The extraordinary properties of these variants remain to be further elucidated from the viewpoint of the structure-function relationships.

Two asymptomatic class 3 variants, G6PD Musashino and G6PD Kamiube, had unique missense mutations in exon 4 and 12, respectively. The 185 C→T mutation in G6PD Musashino creates a new Tag I site, while the 1387 C→T mutation in G6PD Kamiube does not affect any restriction sites. The heterozygosity of the probands’ mothers was confirmed by either restriction or SSCP analysis. The missense mutation of G6PD Musashino predicts an amino acid substitution of 62 Pro→Phe. The tertiary structure model of the human G6PD suggests that the mutated residue of G6PD Musashino is located in a junction region between a helix (αa) and a sheet (βB) in the coenzyme domain, which seems to be distant from either the substrate or the coenzyme binding site. The missense mutation of G6PD Kamiube predicts an amino acid substitution of 463 Arg→Cys. This position is also involved in the mutation that causes the class 2 variant G6PD Kaiping (1388 G→A, 463 Arg→His). Because CG to TG and CG to CA mutations in the CpG dinucleotide occur at high frequency and some of the arginine residues are coded by codons CGX, identical arginine residues are often involved in different missense mutations and are changed to other amino acids. In human G6PD variants, the yielded residues are mainly cysteine and histidine. Five Arg→Cys/His substitutions have been reported: residue 81, Konan /Lagosanto /, residue 285, Osaka /Montalbano /, residue 387, Guadalajara /Beverly Hills /; residue 454, Union /Andalusia /; and residue 463, Kamiube/Kaiping. Except for the class 1 variants G6PD Guadalajara and G6PD Beverly Hills, the Arg→Cys variant seems to elicit less severe clinical expression than the Arg→His variant having the mutation in the same codon, although the significance of this phenomenon remains to be evaluated.

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