Diverse Point Mutations Result in Glucose-6-Phosphate Dehydrogenase (G6PD) Polymorphism in Taiwan

By Tang K. Tang, Ching-Shan Huang, May-Jen Huang, Ka-Bik Tam, Chiao-Hwa Yeh, and Chieh-Ju C. Tang

Glucose-6-PHOSPHATE dehydrogenase (G6PD; EC 1.1.1.49) deficiency is the most common human enzymopathy, affecting more than 200 million people worldwide. Although greater than 400 variants have been described based on clinical and biochemical criteria, little is known about the molecular basis of these G6PD deficiencies. Recently, the gene that encodes human G6PD has been cloned and sequenced, which enables us to examine directly the heterogeneity of G6PD at the DNA level. During the past 10 years, we have examined the G6PD activity in 21,271 newborn Chinese infants (11,400 males and 9,871 females) and identified 314 (2.8%) males and 246 (2.5%) females having low G6PD activity. The G6PD gene from 10 randomly selected affected individuals and their relatives was polymerase chain reaction (PCR) amplified, subcloned, and sequenced. Our results indicate that at least four types of mutation are responsible for the G6PD polymorphism in Taiwan. The first type of mutation (487 G → A) was found in an affected Chinese with a G to A change at nucleotide 487, which results in a (163)Gly to Ser substitution. The second type of mutation (493 A → G) is a novel mutation that has not been reported in any other ethnic group and was identified in two affected Chinese. This mutation causes an A to G change at nucleotide position 493, producing an (165)Asn to Asp substitution. Interestingly, the 487 G → A and 493 A → G mutations create Alu I and Ava II recognition sites, respectively, which enabled us to detect these two mutations by PCR/restriction enzyme (RE) digestion method. The third mutation (1376 G → T) was found in four affected Chinese. This mutation causes a G to T change at nucleotide position 1376 that results in an (459)Arg to Leu substitution. The 1376 G → T mutation seems to be the dominant allele that causes G6PD deficiency in Taiwan. Finally, two affected Chinese were identified having the fourth mutation (1388 G → A). This mutation causes a G to A change at nucleotide 1388 that produces an (463)Arg to His substitution. Our studies provide the direct proof of the genetic heterogeneity of G6PD deficiency in the Chinese populations of Taiwan and the PCR/RE digestion method is suitable for simultaneous detection of the 487 G → A and 493 A → G mutations.

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MATERIALS AND METHODS

Subjects for enzyme assay. Blood samples were obtained from the subjects who were admitted to the Cathay General Hospital (Taipei, Taiwan). The screening for G6PD activity in the blood samples from these subjects was performed with an automated enzyme-linked method as described.14

Polymerase chain reaction (PCR) amplification of genomic DNA. Total genomic DNA was isolated from whole blood cells as previously described.19 PCR amplification of the entire coding sequence of G6PD gene was performed using eight sets of primers (Table 1). The synthetic oligonucleotides were assembled on a DNA synthesizer (Applied Biosystems, Inc, Foster City, CA) and purified as described by Tang et al.14 The amplification reaction mixture (100 μL) contained 1 μg DNA in 10 mmol/L Tris- HCl pH 8.3, 1.5 mmol/L MgCl2, 200 μmol/L each dNTP, 100 ng each primer, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT).

Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) is an X-linked enzyme. It plays an important role in the generation of NADPH, which is required for reductive biosynthesis and for detoxification of hydrogen peroxide and other compounds in mature red blood cells. G6PD deficiency predisposes to a number of diseases, including drug- or food-induced acute hemolytic anemia, severe chronic nonspherocytic hemolytic anemia, and neonatal jaundice. Until now, more than 400 G6PD variants have been described by extensive biochemical studies that include measuring the G6PD activity, the electrophoretic mobility of the enzyme, its pH optimum curve, the Km values for its substrates, the enzyme thermostability, and the use of substrate analogues.15

Fortunately, before many G6PD variants had been described, a standardized procedure for biochemical analysis of G6PD deficiency were established by the World Health Organization (WHO).2 However, even with these standardized methods, it is still difficult to ensure that the G6PD variants that were considered identical may prove to be different. For example, the G6PD A− variant, which was previously regarded as homogeneous mutant, is the result of at least three different mutations.16 Conversely, different variants such as G6PD Dallas, Birmingham, and Sassari all turn out to be identical to G6PD Mediterranean.17−19 Other similar cases have also been reported.2

Recently, the gene encoding human G6PD was cloned and the coding sequence derived from cDNA clones has been determined.20 This finding allows the direct examination of mutations that cause the heterogeneity of G6PD variants. It is clear that, instead of painstaking biochemical characterization, the determination of the nucleotide sequences of different G6PD variants will be performed to determine differences between variants. Indeed, using this approach, the identification of mutations of the G6PD gene has been successfully established.21,22 We report here the identification of four G6PD mutations by direct cloning and sequencing of the G6PD gene isolated from affected Chinese with G6PD deficiency in Taiwan. The molecular defects of these G6PD mutations will be discussed.

From the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; the Department of Laboratory Medicine, The Cathay General Hospital, Taipei, Taiwan; and the Department of Zoology, National Taiwan University, The Republic of China.

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Address reprint requests to Tang K. Tang, PhD, Institute of Biomedical Sciences, Academia Sinica, 128 Academy Rd, Sec. 2, Taipei, Taiwan 115, The Republic of China.

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were purified cycler (Cetus) as follows: denaturation for 35 cycles at 94°C for 1.5 minutes, annealing at 58°C for 1.5 minutes, and elongation at 72°C for 3 minutes.

BioProducts, Rockland, ME). The purified DNA was phosphorylated and blunt end-ligated into a vector (Promega-Biotech, Madison, WI).

The PCR-amplified products were electrophoresed with P1 and P2, which were used for amplification of exon 2 and exons 3/4, respectively, as previously been reported.7,9

Norwalk, CT). The reactions were performed on a DNA thermal cycler (Cetus) as follows: denaturation for 35 cycles at 94°C for 1.5 minutes, annealing at 58°C for 1.5 minutes, and elongation at 72°C for 3 minutes.

Restriction-enzyme (RE) analysis of PCR products. The genomic DNAs from normal and affected individuals were PCR amplified with P1 (5'-GCCTCAGTATGCGACGCTCTGAT-3') and P2 (5'-CTCTGACGAGGCTCCTCCCGAAGGC-3') primers. The amplified 130-bp fragment, encompassing both 487 G and 493 A mutations, was extracted with phenol/chloroform, precipitated with ethanol, and digested with appropriate RE. Because these two mutations are only 6 bp apart, a primer set (Pl/P2) was designed to amplify a 119-bp (119)Alu I and 11-bp (11)Alu I sites, respectively, this finding enable us to directly examine these two mutations without sequencing the whole gene, but, instead, by simply cleaving the PCR products with appropriate RE.

RESULTS

G6PD activity assay. The normal mean value of G6PD activity at 30°C was first established for the Chinese population in our laboratory. It was 8.10 ± 2.04 IU/g hemoglobin (Hb) for newborn babies, 6.46 ± 1.24 IU/g Hb for children, and 5.24 ± 1.01 IU/g Hb for adults. Using an automated enzyme-linked method (see Materials and Methods), we examined 21,271 (11,400 males and 9,871 females) Chinese newborn infants. Our results showed that 314 (2.8%) males and 246 (2.5%) females had low G6PD activity. Among these, 10 randomly selected subjects and some of their relatives, who were considered to be G6PD deficient in this screening, were further analyzed at the DNA level.

Determination of the G6PD mutations. The G6PD gene consists of 13 exons and 12 introns spanning approximate 20 kb of genomic DNA.10 The coding region is located in exons 2 to 13. The entire coding regions of G6PD gene from normal and G6PD-deficient individuals were PCR amplified using eight sets of primers (Table 1). Each amplified fragment was subcloned and sequenced.

At least four different types of G6PD mutations were identified in 9 of 10 subjects examined (Fig 1 and Table 2). The first type of mutation (487 G → A) was found in a Taiwanese with a G to A change at nucleotide 487 that results in a (163)Gly to Ser substitution. The second type of mutation (493 A → G) different from the wild-type sequence G6PD B was originally found in a male Hakkanese. This novel mutation, which has not been reported in any other ethnic group, has an A to G change at nucleotide 493, resulting in the substitution of (463)Arg to His substitution (Fig 1 and Table 2). Interestingly, the 487 G → A and 493 A → G mutations create Alu I and Ava II recognition sites, respectively, which are useful for later distinction between the normal and mutant alleles (Table 2). The 493 A → G mutation was also identified in a Taiwanese (Fig 2A, lane 5) by the PCR/Ava II method described in the next section. The third type of mutation (1376 G → T) was identified in a Taiwanese, which showed a G to T change at nucleotide 1376 resulting in an (459)Arg to Leu substitution (Fig 1). The same mutation was also identified in her affected daughter and three other unrelated individuals with G6PD deficiency (Table 2). Finally, the fourth type of mutation (1388 G → A), found in both a Hakkanese and a Taiwanese (Table 2), was a G to A change at nucleotide 1388 that results in an (463)Arg to His substitution (Fig 1). A summary of G6PD mutations in these affected Chinese of Taiwan is listed in Table 2.

Identification of 487 G → A and 493 A → G mutations by PCR/RE digestion method. Because the mutations occurring at nucleotide positions 487 and 493 create Alu I and Ava II sites, respectively, this finding enable us to directly examine these two mutations without sequencing the whole gene, but, instead, by simply cleaving the PCR products with appropriate RE. Because these two mutations are only 6 bp apart, a primer set (P1/P2) was designed to amplify a 130-bp DNA fragment that encompassed both mutations (see Materials and Methods). Simultaneous examination of the 487 G → A and 493 A → G mutations can be simply achieved by digestion of the same PCR product with appropriate RE.

Figure 2A shows the detection of 493 A → G mutation by PCR/Ava II digestion method. Because an internal Ava II site is present in the 130-bp PCR-amplified product, two fragments (119 and 11 bp) were generated in normal allele. However, in patients with 493 A → G mutation, three fragments (86, 33, and 11 bp) were produced. In Fig 2A (lane 5), a major (86 bp) and a weak (33 bp) band were identified in an affected male Taiwanese, which indicated the presence of 493 A → G mutation. The 11-bp fragment is too small to be detected in this gel. The same mutation was also found in an affected female Hakkanese (Fig 2A, lane
6), whose son had previously been identified as having a point mutation at position 493 (Fig 1). Two major bands (119 bp and 86 bp) seen on the gel indicated the heterozygous status of this female patient. The PCR-amplified fragments from two normal (lanes 2 and 3) and one male patient (lane 4; Taiwanese) did not show the presence of 493 A → G mutation.

Using a similar approach, the affected male Taiwanese with a previously described 487 G → A mutation (Fig 1) was analyzed by PCR/Alu I digestion. Because two internal Alu I sites are present in the normal allele, four fragments (17, 20, 45, and 48 bp) instead of three (17, 45, and 68 bp) were produced from the mutant allele (487 G → A). However, the separation of 45- and 48-bp fragments is indistinguishable, and because of their small size, 17- and 20-bp fragments were undetectable in the agarose gel. Therefore, only one major band (45 bp + 48 bp) was actually seen in the mutant allele (Fig 2B, lanes 3 and 4). However, in a normal individual, two major fragments (45 bp and 68 bp) were detected (Fig 2B, lane 2). Our results indicate that the PCR/RE digestion method is suitable for the detection of 487 G → A and 493 A → G mutations.

**DISCUSSION**

The prevalence of G6PD deficiency varies greatly throughout the world. In some Asiatic populations, G6PD deficiency is known to exist with a relatively high frequency. In Taiwan, the current Chinese population may be divided into four groups (Taiwanese, Mainland Chinese, Hakkanese, and aborigines) based on their origins and migration times.

Taiwanese is the largest Chinese population, descending from emigrants who left Mainland China during the 17th to 19th centuries. Most of these emigrants were from Fuchien province located on the southeast coast of China. The second largest population in Taiwan is Mainland Chinese, who had resided originally in many provinces throughout Mainland China and migrated to Taiwan during the period.

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**Table 2. G6PD Mutations identified in Affected Chinese of Taiwan**

<table>
<thead>
<tr>
<th>No. of Cases</th>
<th>Subjects (ethnic group)</th>
<th>Nucleotide Substitution</th>
<th>Amino Acid Substitution</th>
<th>Exon No.</th>
<th>Restriction Site Created</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Taiwanese</td>
<td>487 GGC-AGC</td>
<td>163 Gly-Ser</td>
<td>6</td>
<td>Alu I</td>
</tr>
<tr>
<td>1</td>
<td>Taiwanese</td>
<td>493 AAC-GAC</td>
<td>165 Asn-Asp</td>
<td>6</td>
<td>Ava II</td>
</tr>
<tr>
<td>1</td>
<td>Hakkanese</td>
<td>1376 CGT-CTT</td>
<td>459 Arg-Leu</td>
<td>12</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>Taiwanese</td>
<td>1388 CGT-CAT</td>
<td>463 Arg-His</td>
<td>12</td>
<td>No</td>
</tr>
</tbody>
</table>

Among the 10 subjects examined, one subject did not show any of the known mutations described above.
between 1948 and 1950. The third population is Hakkanese (Taiwan-Hakka), originally from Chung Yuan, who emigrated from the Kwangtung and Fuchien provinces on the southern coast of China, and who came to Taiwan primarily during the 16th and 17th centuries. The native Taiwan aborigines are a much smaller group, containing at least nine distinct tribes whose ancestors are believed to have arrived in Taiwan from Mainland Asia several thousand years ago.

Among these four groups, the Hakkanese has been reported as having the highest prevalence (4.52%) of G6PD deficiency. The prevalences for other populations in Taiwan are 1.57% (Mainland Chinese), 0.33% (Taiwanese), 3.5% (aborigines, Ami tribe), and 0.3% (aborigines, all other tribe), respectively.

We reported here a relatively high prevalence (2.6%) of G6PD deficiency among 21,271 newborn Chinese infants. Nucleotide sequence analysis of the DNAs isolated from 10 of these patients and their relatives indicates that at least four types of mutation are responsible for the G6PD polymorphism in the Chinese of Taiwan (Fig 1 and Table 2).

The first type of mutation (487 G → A) was identified in a Taiwanese. The single base change results in a (163)glycine to serine substitution. The same mutation was also found in G6PD Mahidol variant, which has been reported to be one of the most common deficient variants in South East Asia. The second type of mutation (493 A → G) is a novel mutation that has not been reported in any other ethnic groups and was identified in a Hakkanese and a Taiwanese. The 493 A → G mutation results in an (165)Asn to Asp amino acid substitution in the G6PD protein. The biochemical features of this mutation have not been characterized.

Interestingly, both 487 G → A (163 Gly → Ser) and 493 A → G (165 Asn → Asp) mutations are located in exon 6, which is close to the putative G6P-binding domain and the single amino acid change that occurred at these two positions is identical with the G6PD from Drosophila, rat, and human (Table 3). In addition, both 487 G → A and 493 A → G mutations create Alu I and Ava II sites, respectively. In this report, we designed a specific primer set (P1/P2) that may allow to simultaneously detect both mutations in the same PCR-amplified product by the PCR/RE digestion method (Fig 2).

The third type of mutation (1376 G → T) was identified in three Taiwanese and one Hakkanese. This single base mutation leads to one amino acid change from (459)Arg to Leu that is conserved in both human and rat (Table 3). The same mutation was also found in G6PD Canton variant and three other Chinese G6PD variants (Taiwan-Hakka, Gifu-like, and Agrigento-like) in Guangdong, China. Finally, a Taiwanese and a Hakkanese were identified as having 1388 G → A mutation. This mutation, causing a single amino acid substitution from (463)Arg to His, is highly conserved in human, rat, and Drosophila (Table 3). The same mutation was also identified in five other Chinese variants from Southern China. Interestingly, both mutations (1376 G → T and 1388 G → A) are located in exon 12, which is close to the putatively NADP-binding domain and exon 10.

Table 3. Comparison of the Partial Amino Acid Sequences of Human, Rat, and Drosophila G6PD That Encompassed Mutated Regions

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
<th>Drosophila</th>
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<tr>
<td></td>
<td>163</td>
<td>165</td>
<td>459</td>
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<tr>
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<tr>
<td>Rat</td>
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<tr>
<td>Drosophila</td>
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In this report, 10 unrelated Chinese with low G6PD activity were examined. At least four types of mutation have been identified in the Chinese population of Taiwan. The Taiwanese and Hakkanese who have been screened with G6PD deficiency contain 493 A → G, 1376 G → T, and 1388 G → A mutations (Table 2). Interestingly, none of the three mutations described here are specific for the G6PD deficiency in either Taiwanese or Hakkanese. This could be partially due to the intermarriage between Taiwanese and Hakkanese after they moved to Taiwan. In addition, among 10 subjects examined, only one Taiwanese was found to contain 487 G → A mutation. At the present time, it is difficult to tell whether this mutation is specific for Taiwanese or not. Further analysis of more patients may answer this question.

It is interesting to note that the 1376 G → T mutation seems to be the most common allele that causes the G6PD deficiency in Taiwan, because 4 of 10 subjects examined had this mutation, whereas the mutations occurring at nucleotide 493, 1376, and 1388, respectively, are three major alleles that make up 80% (8 of 10) of the disease gene. These results were further confirmed by our later studies in which the total sample population was increased to 94 affected Chinese (Chang et al, manuscript in preparation). In addition, among 10 subjects examined, one individual did not show any of these four known mutations, which suggests that some unidentified mutation(s) could lead to the G6PD deficiency in the Chinese of Taiwan.

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