Two new variants of G6PD deficiencies in Singapore

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ABSTRACT

Identification of mutations in G6PD gene is performed as an epidemiologic investigation of G6PD deficiency in many countries. In order to understand the hereditary background of G6PD deficiency in a population, screening of mutations is required not only in exonic regions but also for intron and promoter regions. One hundred male neonatal samples diagnosed as with G6PD deficiency by newborn screening in Singapore were used in this study. The multiplex PCR using the multiple tandem forward primers and common reverse primer (MPTP) method was carried out to detect the common 11 mutations in south-east Asia such as Gaohe 95A>G, Orissa 131C>G, Vanua-Lava 383T>C, Mahidol 487G>A, Mediterranean 563C>T, Coimbra 592C>T, Viangchan 871G>A, Chatham 1003G>A, Union 1360C>T, Canton 1376G>T and Kaiping 1388G>A. Samples whose mutations were unidentified by MPTP method were scanned at cording region, intron and promoter region by direct sequencing.Out of 100 samples, 90 samples (90.0%) were identified with one of the above mentioned common mutations. Eight out of 10 samples whose mutations were unidentified by MPTP method carried exonic mutations which had been previously reported such as Murcia 209A>G, Quing Yuan 392G>T, Nankang 517T>C, Chinese5 1024C>T. Two novel mutations were identified in these samples: one had a novel mutation (25C>T); the remaining sample carried a 49 bp deletion in intron 12.

Keywords: G6PD deficiency, new mutation, Singapore, deletion, intron.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common hereditary enzymopathy. It affects more than 400 million people worldwide, and is prevalent in tropical Africa, the Middle East, tropical and subtropical Asia, some areas of the Mediterranean, and Papua New Guinea. Main clinical presentations include neonatal jaundice, severe chronic nonspherocytic hemolytic anemia and acute hemolytic anemia triggered by drugs, infections or fava bean ingestion. However, most individuals may present clinically as asymptomatic and without ever experiencing a hemolytic episode. More than 400 biochemical variants and about 140 different G6PD mutations have been described in previous reports.[1]

Identification of mutations in G6PD gene is performed as an epidemiologic investigation of G6PD deficiency in many countries. However, about the molecular basis of 10.0% of the samples remained unidentified since the screening of mutation by this approach was often limited to the most commonly occurring mutations.[2-4] In order to understand the hereditary background of G6PD deficiency in a population, screening of mutations is required not only in exonic regions but also for intron and promoter regions.

In this study, samples diagnosed with G6PD deficiency through newborn baby screening in Singapore were analyzed by multiplex PCR followed by sequencing of coding regions and splicing sites in introns in order to clarify the mutations in G6PD deficienct subjects.

SUBJECTS AND METHODS

Full term newborns (n=4830) delivered at National Hospital of Singapore from January 1994 to December 1997 were studied. Umbilical cord blood samples from these babies were screened for G6PD deficiency immediately after birth using the modified Bernstein Assay, and were then diagnosed as G6PD deficienct by quantitative enzyme analysis. One hundred male samples were available for extraction of DNA from blood samples and were used for molecular characterization of the variants in this study.

The total genomic DNA was extracted from peripheral blood leukocytes of these affected individuals using standard phenol-chloroform method. The selected DNA samples were then subjected to molecular characterization by the MPTP (multiplex PCR using multiple tandem forward primers and common reverse primer) method.[5,6] A two-step PCR amplification was carried out by primers using PCR conditions previously

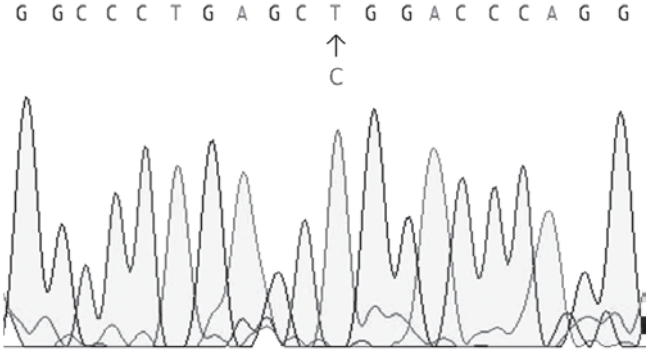

Fig. 1. Direct sequencing around C>T transition

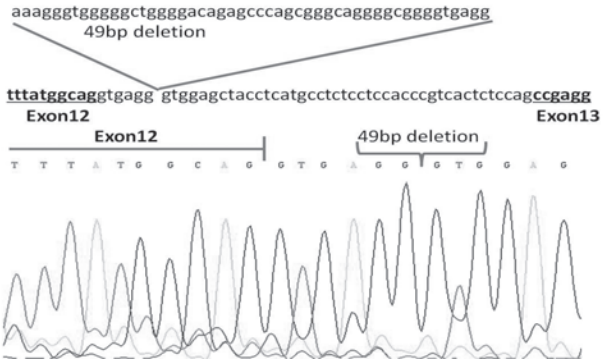

Fig. 2. Site and sequence of 49bp deletion in Intron 12

described in order to detect the 11 known common mutations in exons 2, 3, 5, 6, 9, 11 and 12.[6]

The unidentified samples by MPTP method were further subjected to direct sequencing. Direct sequencing was carried out by a DNA sequencer (ABI PRISM 310; PE Biosystems, Connecticut, USA).

Samples with no mutations in coding region detectable by above methods were subjected to direct sequencing of intron regions, extending about 100 bp from 5' or 3' splice sites. Direct sequencing of a promoter region was also performed on the same sample. The following primers are used to amplify the first PCR and direct sequencing of promoter region including the core promoter region of G6PD gene.[7] The 912 bp promoter region was amplified by forward and reverse primers, (5'-gggggaggaatcaagaagagac and 5'-gtaaaatccttgtggggcatgg) on first PCR. Then direct sequencing for the 574bp promoter region was carried out using forward and reverse primers, (5'-gggggaggaatcaagaagagac and 5'-ttacctgcgcttcgtcgtc).

Table-1: Primers for 1st PCR

Primer	Sequence	Exon	PCR Product
2-a	5'-cccagaggaactctcaagaaa	2	287bp
2-b	5'-aacaattagttggaaaagctga		
3-a	5'-agtagtgatcctgagtagtg	3-4	571bp
3-b	5'-tatgattggcggagaaaacg		
5-a	5'-acacggactcaaagagag	5	341bp
5-b	5'-tggtgggagcactgcctg		
6-a	5'-caaaaccaatgaggaagcca	6-7	987 bp
6.7-2	5'-ataaaaccgtggagtgcttgg		
9-a	5'-tcttgcagcttgtcactagga	8-9	1207bp
9-b	5'-cttgtatctgttgccgtagg		
10-13a	5'-cacaccctgagagagctggt	10	583 bp
G6	5'-cacggagctcgtcgctgag		
11-a	5'-actccacatggtggcag	11-13	619 bp
12-a	5'-aggaatgtgcagctgaggtcaa		

RESULTS

The first pair of PCR primers used to amplify each coding region are shown in Table-1. The primers for direct sequencing are summarized in Table-2. The first pair of PCR primers used to amplify each intron are shown in Table-3. The primers used for direct sequencing of intron region are shown in Table-4. About 90.0% of the G6PD deficient neonates in Singapore could be identified as carrying the common 10 mutations by MPTP- method (Table-5).

The distribution of mutations were Canton 1376G>T 24 (24.0%), Viangchan 871G>A 18(18.0%), Kaiping 1388G>A 11 (11.0%), Vanua-Lava 383T>C 11(11.0%), Mediterranean 563C>T 6 (6.0%), Gaohe 95A>G 6 (6.0%), Mahidol 487G>A 5 (5.0%), Orissa 131C>G 4 (4.0%), Coimbra 592C>T 3(3.0%) and G6PD Chatham 1003G>A 2 (2.0%), respectively. Union 1360 C>T which is common in Philippines was absent in this study.[8] However, ten samples did not carry these common mutations.

By direct sequencing the unidentified 8 samples were detected to carry four known mutations such as Quing Yuan 392G>T 4 (4.0%), Chinese 5 1024C>T 2 (2.0%), Murcia 209A>G 1 (1.0%) and Nankang 517T>C 1 (1.0%). One sample was detected to carry an unreported mutation in a coding region of exon 2, 25C>T (Table-6). This sequence of this novel mutation is shown in Fig. 1. This 25C>T mutation results in an amino acid substitution from arginine to tryptophan.

In the remaining sample, a 49bp deletion in intron12 was observed by direct sequencing. The deletion site and sequencing results are shown in Fig. 2. Indeed, this PCR fragment was 49 bp shorter than the normal control PCR product when analyzed by gel electrophoresis as shown in Fig. 3. This deletion occurred in 7th to 56th nucleotide from 5'-splicing site in intron 12 which consists of 99 nucleotide. This resulted in a shift of six

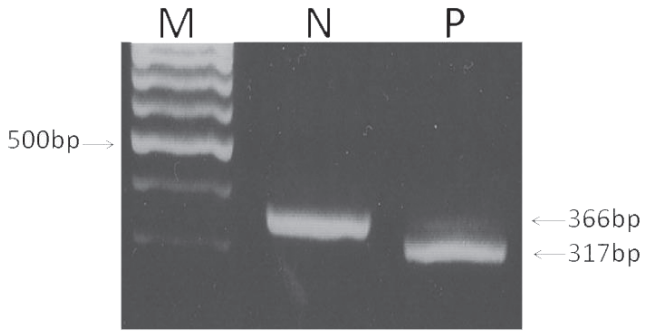

Fig. 3 Electrophoretic patterns of PCR products with or without 49 bp deletion.

bases in the splicing site at 3'-end of exon 12 and was interestingly sandwiched between two similar GTGAGG-residues. There was no mutation in promoter region of this sample.

DISCUSSION

Most individuals with G6PD deficiency may present as asymptomatic clinically and may not have experienced a hemolytic episode without a trigger event. The severity of this disease is associated with the type of molecular variants. As the World Health Organization recommended all newborn to be screened for G6PD deficiency,[9] the mass screening of G6PD deficiency for newborn babies has been performed in south-east Asian countries.[10,11]

Table-2: Primers for sequencing of cording region

Primer	Primer sequence	Exon
2-a	5'-cccagaggaactctcaagaaa	2
2-b	5'-aacaattagttggaaaagctga	
3-a	5'-agtagtgatcctgagtagtg	3-4
3-b	5'-tatgattggcggagaaaacg	
5-a	5'- acacggactcaaagagag	5
5-b	5'-tggtgggagcactgcctg	
E6-seq	5'-aggtgttgagccagaggg	6
E6-RV	5'-aactgaccttgggcctctgt	
E7-seq	5'-aggacgaattcctccagaac	7
6.7-2	5'-ataaaa tgcttgg	
E8-1	5'-ggagctaaggcgagctctgg	8
E8-3	5'-tgcctcgtcacagatgggcc	
E9-FN	5'-tgaccagggaaggccattg	9
E9-RN	5'-agctctctcagggtgtgga	
10.13-a	5'-cacaccctgagagagctggt	10
G6	5'-cacggagctcgtcgctgag	
11-a	5'-actccacatggtggcag	11-12
B12	5'-catgaggtagctccaccctca	
B13-seq	5'-ttatggcaggtgaggaaagg	13
12-a	5'-aggaatgtgcagctgaggtcaa	

Table-3: 1st PCR primers for amplification of intron region

Primer	Primer sequence	Amplified Intron	Products (bp)
Int1F	5'-taacccatcaaccactcccc	Intron 1 & 2	589
Int1R	5'-aggcaggagaatcgcttgaa		
Int2F	5'-gcgtttatgtcttctgggtc	Intron 2-7	2533
E6.7-2	5'-ataaaaccgtggagtgcttgg		
E6seq	5'-aaggtgttgagccagagggt	Intron 7-12	2771
G3	5'-aggaatgtgcagctgaggtcaa		
Int13F2	5'-agacgagctgatgaagagagtg	Intron 13	707
Int13R2	5'-aagcaaatgacaaggggacagg		

Out of hundred male neonatal samples diagnosed as a G6PD deficient by newborn screening in Singapore, 98 samples were identified as ones carrying known common mutations in G6PD gene coding region by MPTP method (90 samples) and direct sequencing (8 samples).

By further direct sequencing analysis, a novel mutation was detected as a C>T transition in exon 2. The co-authors had previously identified a new G6PD deficiency variant in Thailand as G6PD Songklanagarind 196T>A,[4] but this is different from the C>T transition identified in this study. Indeed, the latter mutation resulted in an amino acid substitution from arginine to tryptophan and has not been reported in the databases of G6PD variants[12] and SNP database.[13] The mutation site is not located in the binding sites of substrate or coenzyme, while these amino acids have wide differences in biochemical properties from each other. Arginine carries the long side chain with multiple H-bonds and is a basic amino acid. On the other hand, Tryptophan has aromatic ring system and is hydrophobic. These amino acids substitution shows negative score in BLOSUM62 substitution matrix, showing heterogeneity between these amino acid residues.[14]

The splicing process is catalyzed by the spliceosome, complex of small nuclear ribonucleoprotein (snRNP) particles associated with additional large number of proteins.[15,16] These proteins assemble to the consensus sequences in the intron of pre-mRNA. In following splicing process, cleaved 5'-end of intron assembles to branch site in intron.[17,18]

The 49 bp deletion in intron12 may cause a decrease in G6PD activity associated with splicing errors of pre-mRNA. This deletion occurred between the 7th to 56th nucleotide from 5' splicing site in the intron 12 and may possibly include the consensus sequence of 5' splicing site.[19]

Table-4: 2nd PCR and sequencing primers for intron region

Primer	Primer sequence	sequencing region
Int1F	5'-taacccatcaaccactcccc	intron 1 (3' end)
Int1R	5'-aggcaggagaatcgcttgaa	intron 2 (5' end)
Int2F	5'-gcgtttatgtcttctgggtc	intron 2 (3' end)
3-4b	5'-tatgattggcggagaaaacgc	intron 3 (5' & 3' end)
		intron 4 (5' end)
Int4F	5'-tcctgaaatctggcct ctgt	intron 4 (3' end)
B2a	5'-tggtgggagcactgcctg	
Int5F	5'-agcgcctcaacagccacat	intron 5 (5' end)
Int5R	5'-ctctggctgggttgggaaa	
E6seq	5'-aaggtgttgagccagagggt	intron 5 (3' end)
E7R2	5'-ttggctgtgtaggggtcca	intron 6 (5' & 3' end)
		intron 7 (5' end)
Int7,8F	5'-caaggggatcaggaagtg	intron 7 (3' end)
Int7,8R	5'-gtgacttctccggggttga	intron 8 (5' end)
E9FN	5'-tgaccagggaaggccattg	intron 8 (3' end)
E9RN	5'-agcaccagctctctcag	intron 9 (5' end)
Int9F	5'-tggagaatgagaggtgggat	intron 9 (3' end)
Int9R	5'-ctgctggtggaagatgtcg	
Int10,11F	5'-agatgatgaccaagaagccg	intron 10 (5' & 3' end)
G3	5'-aggaatgtgcagctgaggtcaa	intron 11 (5' & 3' end)
		intron 12 (5' & 3' end)

In addition, intron12 which spans 99 nucleotides was shortened to 50 nucleotides due to this deletion. The shorter intron has a possibility of inducing constraints on the splicing machinery[20]

Table-5: Eleven common G6PD mutations by MPTP method

Variant	Mutation	Total	Race		
			Chinese	Malay	Others
Gaohe	95 A>G	6	5	1	0
Orissa	131 C>G	4	0	3	1
Vanua-Lava	383 T>C	11	1	10	0
Mahidol	487 G>A	5	1	3	1
Mediterranean	563 C>T	6	1	3	2
Coimbra	592 C>T	3	0	2	1
Viangchan	871 G>A	18	3	14	1
Chatham	1003 G>A	2	0	2	0
Union	1360 C>T	0	0	0	0
Canton	1376 G>T	24	18	3	3
Kaiping	1388 G>A	11	9	2	0
unknown	-	10	7	2	1
		100	45	45	10

Table-6: Mutations in coding region detected by direct sequencing

Variant	Mutation	Total	Race		
			Chinese	Malay	Others
New mutation	25 C>T	1	0	1	0
Murcia	209 A>G	1	0	1	0
Quing Yuan	392 G>T	4	3	0	1
NanKang	517 T>C	1	1	0	0
Chinese 5	1024 C>T	2	2	0	0
unknown	-	1	1	0	0
		10	7	2	1

Although most of G6PD deficiency cases are caused by a point mutation in the coding region, a few reports have been presented showing deletion of several bp in coding regions such as G6PD Sunderland with 3 bp deletion in exon 2,[21] G6PD Nara with 24 bp deletion in exon 9,[22] G6PD StonyBrook with 6 bp deletion in exon7,[23] G6PD Tsukui with 3 bp deletion in exon 6,[24] G6PD Amsterdam with 3 bp deletion in exon 4[25] and G6PD Urayasu with 3bp deletion in exon 5.[24] Only a report presented G6PD Varnsdorf with deletion of the 3'-end in intron 10.[23]

Mutations in coding region show typical patterns among different ethnic groups as previously reported.[2-4] Similarly in this study, G6PD Canton and G6PD Kaiping were mainly detected in Chinese while G6PD Viangchan and G6PD Vanua-Lava were mainly in Malay as summarized in Table-5. G6PD Quing Yuan and Chinese 5 was also detected frequently among the Chinese in this study (Table-6), these mutations should be added to the list of common mutations in Chinese. On the other hand, G6PD Union common in Philippines could not be detected among the samples in this study. Mutations in the promoter region of the G6PD gene was not found despite attempts by the authors in screening this region.

The detection of two new mutations and 4 rare mutations found by direct sequencing can contribute towards design of improved MPTP method by inclusion of new design of the primers in the PCR procedures. In future these findings may contribute towards the improvement in the rate of identification of G6PD deficiency variants in south-east Asia.

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