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Complete molecular characterisation of glucose-6-phosphate dehydrogenase (G6PD) deficiency in a group of Malaysian Chinese neonates

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Abstract

We performed DNA analysis on cord blood samples of 128 Chinese male neonates diagnosed as G6PD deficiency in Hospital Universiti Kebangsaan Malaysia by a combination PCR-restriction enzyme digest technique, Single Stranded Conformation Polymorphism analysis and DNA sequencing. We found 10 different G6PD-deficient mutations exist. The two commonest alleles were G6PD Canton 1376 G>T (42.3%) and Kaiping 1388 G>A (39.4%) followed by G6PD Gaohe 592 G>A (7.0%), Chinese-5 1024 C>T, Nankang 517 T>C (1.5%), Mahidol 487 G>A (1.6%), Chatham 1003 G>T (0.8%), Union 1360 C>T (0.8%), Viangchan 871 G>A (0.8%) and Quing Yang 392 G>T (0.8%). Sixty eight percent (88/125) neonates in this study had neonatal jaundice and 29.7% developed hyperbilirubinemia >250 μmol/l. The incidence of hyperbilirubinemia >250 μmol/l was higher in G6PD Kaiping (43.8%) than G6PD Canton (22%) ($p < 0.05$). There was no significant difference in the incidence of neonatal jaundice, mean serum bilirubin, mean age for peak serum bilirubin, percentage of babies requiring phototherapy and mean duration of phototherapy between the two major variants. None of the 88 neonates required exchange transfusion. In conclusion we have completely characterized the molecular defects of a group of Chinese G6PD deficiency in Malaysia. The mutation distribution reflects the original genetic pool and limited ethnic admixture with indigenous Malays.

Key words: Glucose-6-phosphate dehydrogenase, G6PD, molecular variants, Chinese

INTRODUCTION

Glucose-6-phosphate dehydrogenase deficiency is the commonest enzymopathy in humans, estimated to affect 400 million individuals worldwide. G6PD-deficient individuals are usually asymptomatic but acute haemolysis may occur with oxidative stress induced by ingestion of drugs, certain types of food, exposure to certain chemical substances or when there is accompanying infection or hypoxia. Rarely, it may cause chronic non-spherocytic haemolytic anemia. One of the most important complications of G6PD deficiency is severe neonatal hyperbilirubinemia and the risk of developing kernicterus, a problem especially seen in G6PD-deficient individuals in the Mediterranean and Asia.[1,2,3,4] To date at least 130 mutations have been identified to cause G6PD deficiency in various populations in the world.[5] Advances in molecular techniques have allowed the molecular characterisation of G6PD gene in any population to be carried out with ease. G6PD deficiency is common in Malaysia with an overall incidence of 3.1% among males and is more prevalent in ethnic Malays and Chinese and less common among the Indians.[6,7] It is an important cause of severe neonatal jaundice and kernicterus. All newborn babies are screened for G6PD deficiency as part of a national programme to prevent complications of severe hyperbilirubinemia and kernicterus. Earlier, we reported the existence of 4 mutations in a small group of Malaysian Chinese male G6PD-deficient individuals and subsequently reported G6PD mutations in Malays.[8,9,10] We present here the results of a complete characterization of the molecular defects of Chinese G6PD deficiency in 128 Malaysian Chinese male neonates.

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MATERIALS AND METHODS

Subjects

One-hundred-and-twenty-eight male Chinese neonates born in Hospital Universiti Kebangsaan Malaysia (HUKM) who were diagnosed as G6PD-deficient by routine screening test between June 1999 to June 2002 were studied. Cord blood samples collected in EDTA from neonates in the labour room and sent for routine screening for G6PD deficiency in the Haematology laboratory were used for determination of G6PD red cell activity and DNA extraction. As routinely practiced in HUKM, results of all G6PD screening tests were sent back to the ward within 24 hours of receiving the specimen and mothers of babies diagnosed as G6PD deficiency were explained of the problems related to the condition by the attending paediatrician and were advised to have the babies kept in the hospital for a minimum of five days to be observed for clinical jaundice. Daily monitoring was carried out clinically and by serial estimation of serum bilirubin. Neonates with serum bilirubin level exceeding 180 µmol/l within the first 48 hours of birth and/or exceeding 250 µmol/l were subjected to phototherapy.[11] Those infants with bilirubin level > 340 µmol/l would require exchange transfusion. Neonates were discharged after the fifth day when they showed both clinical and biochemical improvement with a downward trend of serum bilirubin to a level of less than 230 µmol/l. Babies born to mixed parentage, those born prematurely (36 weeks gestation) and those with clinical evidence of infections and or haematoma as a result of birth injury were excluded from the study. Written informed consent were obtained. This study was approved by the Institutional Review Board.

G6PD activity assay

Red cell G6PD activity assays were performed in all 128 neonates. Based on the principle of measurement of rate of absorbance of reduced NADP+ in red cell haemolysate, the assay was carried out at 25^0C according to manufacturer's instruction, using the G6PD Kit by RANDOX Laboratory LTD (United Kingdom). Measurment of absorbance was performed on the Hitachi Autoanalyser 717, Boehringer Mannheim (USA). All G6PD activity assays were performed within 24 hours of sample collection.

DNA extraction

Total genomic DNA was isolated from peripheral cord blood leucocyte of G6PD-deficient neonates according to manufacturer's instruction using High Pure Template DNA Preparation Kit, Roche Diagnostics Corporation (USA).

DNA amplification and restriction enzyme analysis

In mutation analysis we adopted the strategy of firstly screening all the G6PD-deficient samples for 5 known mutations (nt 1376 G>T, nt 1388 G>A, nt 1024 C>T and nt 95 A>G) previously reported to occur in Malaysian Chinese G6PD deficiency and nt 487 G>A, a common allele in Malays. Samples negative for these mutations were then subjected to Single Stranded Conformation Polymorphism (SSCP) analysis, followed by DNA sequence analysis of those exons that showed electrophoretic mobility shift on SSCP gel. Mutations at nt 1376 G>T, nt 1388 G>A, and nt 1024 C>T were detected using PCR-based techniques involving amplification of target gene with oligonucleotide primers designed to create restriction sites followed by restriction enzyme digests of amplified products with Xho I, Nde I and Mbo II.[12] For the detection of mutation 95 A>G, primers used were designed to create artificial restriction enzyme site for Mlu I.[12] The PCR reaction was optimized for each reaction by using the following concentrations: $MgCl_2$ 1.5 mmol/l, DNTP 200 µmol/l, each primer 0.1 µmol/l and Taq polymerase 1.5U. The PCR was performed on the DNA Thermal Cycler 480 (Perkin Elmer) using annealing temperatures calculated according to Tm of primer pairs and run at 30 cycles with a further 10 minutes extension at 72^0 C. Following digestion with the appropriate restriction enzymes amplified products were electrophoresed on 3.5% Nu-sieve agarose gel.

SSCP and DNA Sequencing

Samples negative for the 5 known mutations were subjected to Polymerase Chain Reaction-Single Stranded Conformation Polymorphism (PCR-SSCP) analysis using primer sequences for G6PD exons amplification as previously described.[13] Amplifications were carried out using fluorescent-tagged primers and amplified fragments were electrophoresed on MDE gel (Mutation Enhancement Gel Solution with 5% polyacrylamide, Biowhittaker Application, USA) at 35^0C using the ABI PRISM 377 DNA Sequencer. The amplified product were analysed using the software Genescan ABI PRISM 3.1. DNA sequencing was performed on exons

showing mobility shift and in samples that showed absence of mobility shift by SSCP in any of the G6PD gene exons DNA sequencing were performed on all the 12 exons of the G6PD gene using primers similar to that used for SSCP.[13] All PCR amplifications were carried out using the DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Purification of the PCR product for the preparation of the sequence template were carried out using CONCERT™ RAPID Gel Extraction System (Life Technologies USA). Cycle sequencing was performed according to the manufacturer's instruction using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit version 2.0 (Applied Biosystems, USA). Sequenced products were electrophoresed on 4.5% polyacrylamide (Long Ranger) using the automated DNA sequencer (ABI Prism 377 DNA Sequencer, Applied Biosystems USA) and the analysis of the sequence was carried out using the ABI PRISM Sequence Navigator software. Six mutations discovered by SSCP and DNA sequencing were subsequently detected in the remaining samples by PCR-enzyme restriction methods, established in the laboratory using similar PCR condition as above and the annealing temperatures set at Tm of primers, using primer sequences as spreviously described.[11,12,14] All mutations found by using PCR-restriction enzyme method were confirmed by DNA sequencing.

Data Analysis

Data on incidence of jaundice, age of onset of jaundice, peak serum bilirubin value, age in days when the serum bilirubin peaked, birth weight, mode of treatment and duration of phototherapy were obtained from the patient's record and analysed. Comparisons of clinical and biochemical data were carried out for the two major mutations (G6PD Canton nt 1376 G>T, and G6PD Kaiping 1388 G>C) using the SPSS (Statistical Package for Social Scientist) version 10.0 (Chicago, USA). Kolmogorov-Smirnov test was used to determine normality of distribution. For comparisons of continous variables with normal distribution (G6PD activity, serum bilirubin, age for peak bilirubin and duration of phototherapy) student's test was used. The Fisher's Exact test was used for comparison of discrete variables (incidence of jaundice and onset of jaundice). P value of less than 0.05 was considered significant.

RESULTS

Prevalence of G6PD deficiency

A total of 15277 newborn babies (7728 males, 7542 females) were screened for G6PD deficiency using the semiquantitative fluorescent spot test during the period of study. The overall prevalence of G6PD deficiency among males was 4.7% (371 out of 7737) and among females was 1.4% (108 of 7542). Out of 7737 male babies 4740 were Malays, 2124 were Chinese and 365 Indian. The prevalence of male G6PD deficiency were 4.6%, 6.0% and 1.3% in the Malays, Chinese and Indians respectively. The prevalence of female G6PD deficiency were 1.4%, 1.65 and 0.49% in Malays, Chinese and Indians respectively.

G6PD Mutations

The overall results of mutation analysis of 128 Chinese G6PD-deficienct neonates are as follows : G6PD Canton 1376 G>T (46.1%) and Kaiping 1388 >A (37.5%), Gaohe 592 G>A (7.0%), Chinese-5 1024 C>T (3.1%), Nankang 517 T >C (1.5%), Mahidol 487 G>A (1.6%), Chatham 1003 G >T (0.8%), Union 1360 C>T (0.8%), Viangchan 871 G>A (0.8%) and Quing Yang 392 G>T (0.8%). SSCP analysis showed amplicon mobility shifts and were useful in locating the site for the following mutations: G6PD Union 1360 C>T and Chatham 1003 G>A. Figures 1 - 4 show the electrophoretic agarose gel analysis for mutations Chinese 5 nt 1024, Gaohe nt 95, Union nt 1360 and Quing Yang nt 392. Figure 5 shows the DNA sequence electropherogram for G6PD Nankang mutation T>C nt 517.

G6PD Activity

G6PD activity assay showed that 120/128 (93.7%) had severe enzyme deficiency (<10% mean normal G6PD activity). There was no significant difference in the mean G6PD actvitity between the two major variants (p>0.05, students t test). The results of mutation analysis and G6PD activity assay for G6PD-deficient neonates are shown in Table 1. All 4 cases of G6PD Chinese 5 1024 C>T had moderate G6PD deficiency.

Neonatal jaundice

Sixty eight percent (86 of 126) of G6PD-deficient neonates developed neonatal jaundice. There was no significant difference in incidence of neonatal jaundice (p>0.05, chi square test) and in the mean serum bilirubin (p>0.05 student's t

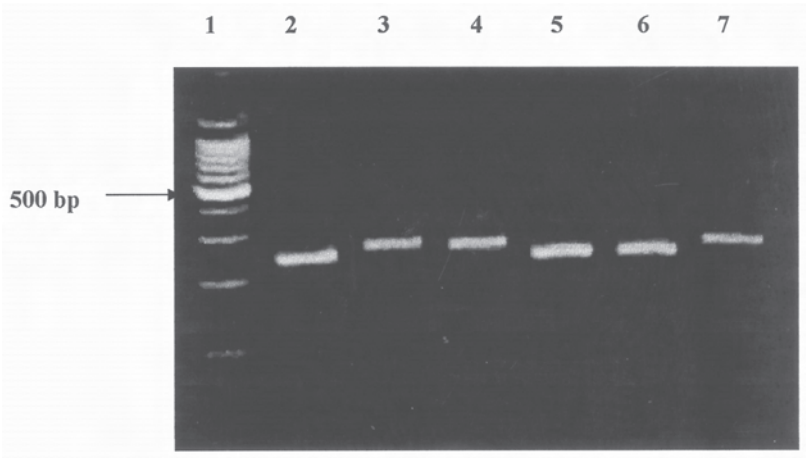

Fig 1: Detection of G6PD Gaohe nt 95 A>G by PCR/Xho I enzyme digest. Lane 1 is 100 bp marker. Lanes 2 and 3 are positive control showing a 223 bp and negative control showing 260 bp DNA fragments respectively. Lanes 4 & 7 represent individuals with normal allele (260 bp DNA fragment) and lanes 5 & 6 represent individuals with mutant alleles showing 223 bp DNA fragment

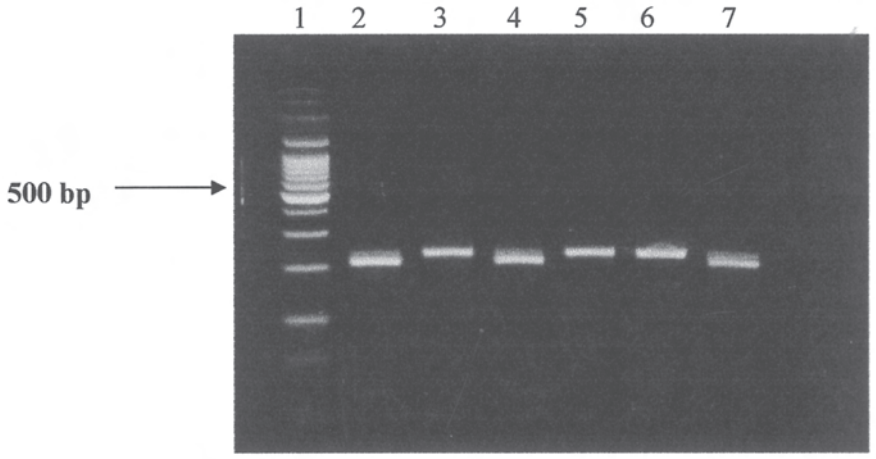

Fig 2: Detection of G6PD Kaiping nt 1388 G>A by PCR/Nde 1 enzyme digest. Lane 1 is the 100bp DNA marker. Lane 2 is the positive control showing 205 bp DNA fragment and lane 3 is the negative control showing the 227 bp DNA fragment. Lanes 4 & 7 represent individuals with mutant alleles (205 bp DNA fragment) and lanes 5 & 6 represent individuals with normal allele (227 bp DNA fragment).

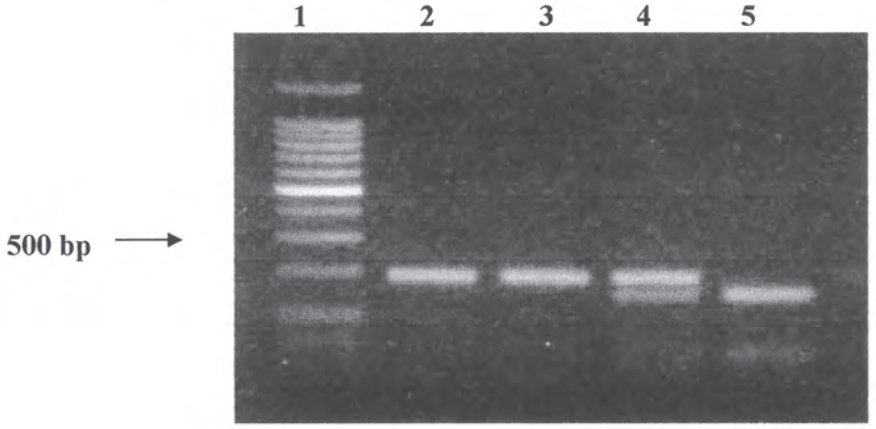

Fig 3 : Detection of G6PD Chinese 5 nt 1024 C>T by PCR/Mbo II digest. Lane 1 is 100bp marker. Lane 2 is negative undigested control, lane 3 normal control showing a 187 bp DNA fragment. Lane 4 represents a female heterozygous showing both the 187 and 150 bp DNA fragments and lane 5 represent individuals with mutant alleles showing a 150 bp DNA fragment

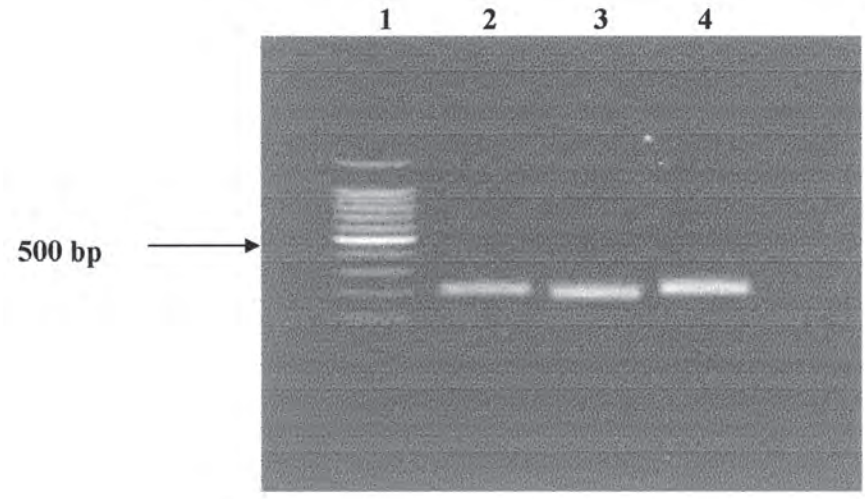

Fig 4 : Detection of G6PD Quing Yang 392 C>T by PCR/Bst E II digest. Lane 1 is 100 bp marker. Lane 2 represents an individual with mutant allele showing a 202 bp DNA fragment. Lane 3 represents an individual with normal allele showing a 182 bp DNA fragment. Lane 4 contains undigested amplified DNA fragment.

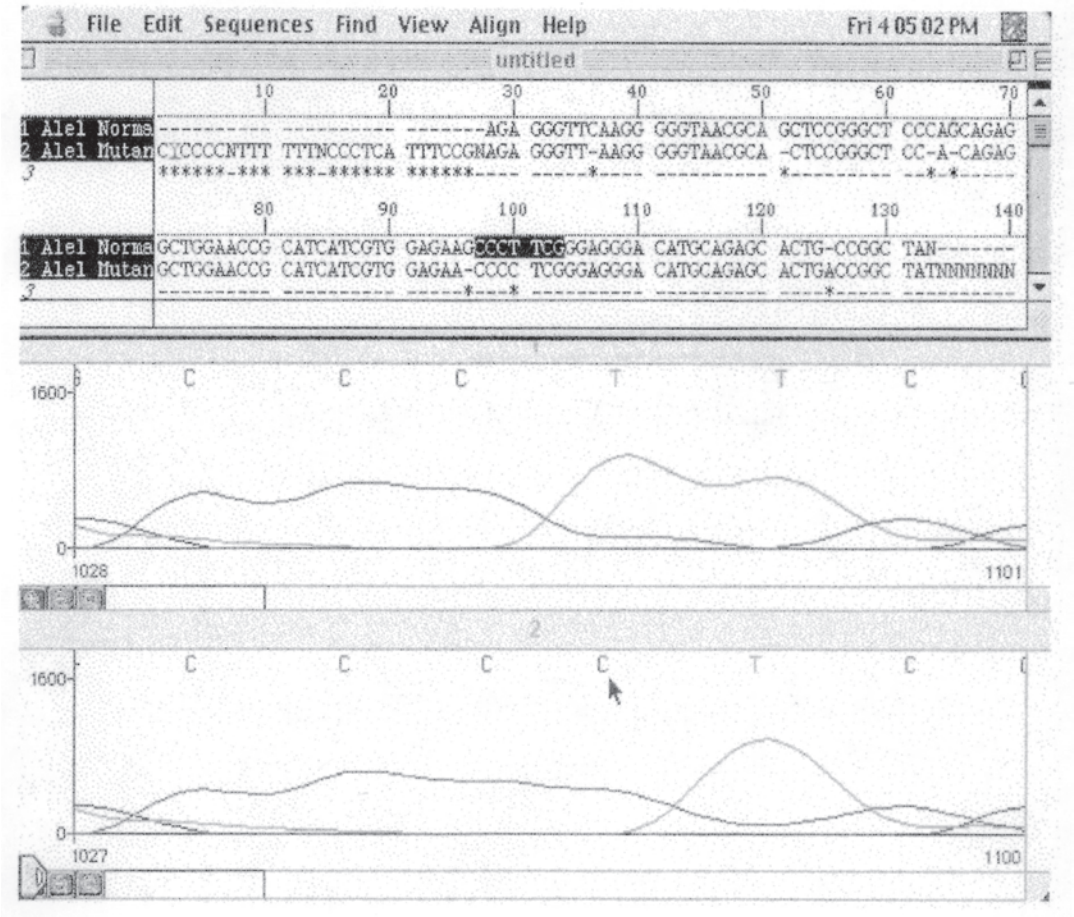

Fig 5 **:** DNA analysis sequence electropherogram showing nt 517 T > C mutation (G6PD Nankang) in exon VIII of G6PD gene

TABLE 1: G6PD variants and red cell G6PD activity in Chinese G6PD-deficient neonates

G6PD variant	G6PD Activity IU/gm Hb +/- SD	No of Cases Severe G6PD deficiency*	No of cases Moderate G6PD deficiency *
Canton 1376 G>T (59)	0.46 +/- 0.46	58 (98.3%)	1(1.7%)
Kaiping1388 G>A (48)	0.67+/-0.5	46 (95.8%)	2 (9.8%)
Gaohe 95 A>G (9)	0.50+/- 0.34	9 (100%)	0
Chinese-5 1024 C>T (4)	1.6 +/-0.26	0	4 (100%)
Nankang 517 T>C (2)	0.79; 0.83	2 (100%)	0
Mahidol 487 G>A (2)	0.33;0.79	2 (100%)	0
Chatham 1003 G>A (1)	0.96	1	0
Union 1360 C>T (1)	1.09	1	0
Viangchan 871 G>A (1)	1.3	1	0
Quing Yang 392G>T (1)	2.07	0	1
Total n=128	0.616 ±0.52	120 (93.7%)	8 (6.3%)

Comparison of mean G6PD activity between the major variants did not show any significant difference: Canton vs Kaiping p=0.362, Canton vs Gaohe p=0.940 dan Kaiping vs Gaohe p=0.557. All cases of G6PD Chinese 5 had moderate G6PD deficiency. *Severe deficiency : G6PD activity < 10 % normal mean, moderate deficiency: G6PD activity 10-60% normal mean.

test) between the two major variants. Thirty eight of the 126 (29.7%) babies had serum hyperbilirubinemia > 250 µmol/l and the proportion was significantly higher in the G6PD Kaiping group than the Canton group (p=0.014) (Table 2). Fifty one babies were examined for onset of neonatal jaundice, 40% developed jaundice on second day of life and 60% after 48 hours of birth . None developed jaundice within the first 24 hours of life (Table 3). The overall mean age for peak serum bilirubin was 4.89 day of life +/-2.7 days. The mean peak serum bilirubin value for the Canton variant appear to be higher than Kaiping but comparison by student's t test analysis showed no significant difference between these two major variants (p>0.05). Seventy seven percent of babies with neonatal jaundice required treatment with phototherapy and the mean duration of phototherapy was 3.67+/-2.2 days and there was no signifant difference between the two major variants (p>0.05, student's t test) (Tables 2 & 3).

DISCUSSION

The overall prevalence of male G6PD deficiency in this study is 4.7% with a racial breakdown of 4.6%, 6.0% and 1.3% in the Malays, Chinese and the Indians respectively, a finding comparable to previous reports.[6,7,15] We reported earlier the existence of 4 common Chinese mutations in 38 Chinese male neonates with G6PD deficiency in the UKM Neonatal Unit, Hospital Kuala Lumpur.[9] In this study by using a combination of techniques that includes PCR-enzyme restriction analysis, SSCP analysis and DNA sequencing we have succeeded in characterizing completely the Chinese G6PD mutations in a group of Malaysian neonates. Following this we have also established easy PCR-based/ restriction methods for the detection of almost all the G6PD mutations and applied the technique to determine the prevalence of the variants in the studied population. It is known that, although many G6PD mutations exist in each population, only a few are polymorphic and occur at high frequencies. The molecular defects in all the 128 Chinese G6PD-deficient neonates were characterized and we found the existence of 10 different mutations causing Chinese G6PD deficiency. We did not find any novel mutation. As shown earlier, the two commonest alleles causing Chinese G6PD deficiency in at least 84% of the cases were G6PD Canton nt 1376 G>T (46.1%) and G6PD Kaiping nt 1388 G>C (37.5 %). The finding of these two common alleles in the Chinese is in concordance with many other reported studies in other Chinese populations.[17,18,19,20] In addition to these two common alleles, 4 other mutations

TABLE 2: G6PD activity and serum bilirubin in G6PD-deficient Chinese neonates

G6PD Variant (%)	Mutation	Neonatal Jaundice		
		No Cases (%)	Min serum bilirubin μmol/l	No Cases serum bilirubin > 250 (μmol/l)
Canton	1376G>T (n= 59)	42 (71.2 %)	215.9 ± 77.0	13 (22%)
Kaiping	1388G>A (n=48)	37 (77.0%)	248. ± 48.6	21 (43.8%)
Gaohe	95 A>G (n=9)	2 (22.2%)	222.0	0
Nankang	517 T>C n=2	1 (50%)	162	0
Chinese-5	1024 C>T (n=4)	2 (50%)	253.0; 253	2 (50%)
Mahidol	487 G>A (n=2)	2 (100%)	294; 246	1
Chatham	1003 G>A(n=1)	1	295	1
Union	1360 C>T(n=1)	0	-	-
Viangchan	871 G>A (n=1)	0	-	-
Quing Yang	392 G>T (n=1)	0	-	-
Total	128 (100%)	87 (68.0%)	232.4 ±65.5	38(29.7%)

Canton vs Kaiping: incidence of neonatal jaundice (p=0.321, Chi square); mean serum bilirubin (p=0.123, Student t test). Incidence of serum hiperbilirubinemia >250 μmol/l for G6PD Kaiping was significantly higher than G6PD Canton (p=0.014); Gaohe vs Kaiping p=.003 dan Gaohe vs Canton p=0.010 (Fisher exact test).

that occur at much lower frequencies are found and they are: G6PD Gaohe 592 G >A (7.0%), Chinese-5 1024 C> T (3.1%), Nankang 517 T >C (1.5%), and Quing Yang 392 G> T (0.8%). These 4 mutations have also been shown to be Chinese-specific alleles in studies in mainland China and Taiwan where they were found to exist in a number of Chinese ethnic minorities[20,21,22,23] The existence of all these Chinese-specific mutations reflect the original genetic pool of Malaysian Chinese who are direct descendents of immigrants from the provinces of Guangdong, Guangxi and Fujian in south mainland China. Two variants which are known to be common in Southeast Asian populations i.e G6PD Mahidol G487A (1.6%) and Viangchan G871A (0.8%), occur at very low frequencies in our Chinese G6PD-deficient neonates.[24,25,26,27] Our own study have shown that these two mutations are highly polymorphic in the Malays.[10] These findings suggest absence of genetic crossovers from the Malays to the Chinese although these two ethnic groups have been around each other for at least 2 centuries. An interesting observation is the existence of G6PD Union 1360 C>T (0.8%), a mutation that occur widespread in unrelated populations and G6PD Chatham 1003 G>T (0.8%) a variant found in a patient in Damascus and two Chinese Indonesians.[28,29] The presence of these 4 non-Chinese mutations at low frequencies in our Chinese neonates may suggest that these mutations have arisen independently and may not be the result from population movement.

The association of jaundice and the risk of kernicterus in G6PD-deficient Malaysian babies have been well established. [30,31,32] In this group of G6PD-deficient neonates, 68% developed neonatal jaundice and at least one third of the neonates are at risk of severe hyperbilriubinemia with peak serum bilirubin level reaching 250 μmol/l or more despite phototherapy. However, none progressed to the level that required exchange transfusion. An interesting observation is the finding of a significantly higher proportion of babies in the G6PD Kaiping group (43.2%) with serum hyperbilriubinemia >250 μmol/l compared to the Canton group (22%) ($p<0.05$). This finding should be interpreted with caution as other studies have shown that G6PD Canton has been associated with increased rate of exchange transfusion.[19,33] In this study the mean

TABLE 3: G6PD variants and neonatal jaundice in Chinese G6PD-deficient neonates

G6PD variant	No of neonates with neonatal jaundice (n=51)		Mean age (days) peak bilirubin ±SD (n=45)	No of cases with phototherapy (%) (n=62)	Duration of phototherapy (mean+/-SD) (n=62)
	Onset of jaundice 24-48 jam	>48 jam			
Canton n=42	13/26	13	5.09 ± 3.3	23/29 (79.3%)	3.65± 1.99
Kaiping n=37	5	14/19 (73%)	4.56 ± 1.85	20/26 (76.9%)	3.47± 2.09
Gaohe n= 2	1	1	8 ; 3	1/2	6.0
Nankang n=1	1	0	2	1/1	2
Chinese-5 n=2	1	1	5	1/1	1
Mahidol n=2		1	17*	1/2	nil
Chatham n=1	-	1	8	1/1	10
Viangchan n=0	-	-	-	-	-
Union n=0	-	-			
Quing Yang n=0	-	-	-	-	-
Total 87	20	31	4.89± 2.7	48 (77.4%)	3.67 ± 2.2

The two variants were compared for age for peak bilirubin (p=0.188 Student's t test) (outlier *), mean duration of phototherapy (p=0.892, student's t test). Mean birth weight (kg) for the Chinese neonates showed no significant difference between the variants (p=0.567 ANOVA)

age for bilirubin to reach its peak is the fourth day of life. However, it has been observed that although there is no significant difference in the mean age for peak serum bilirubin between these two variants, the mean age for G6PD Canton appears to be higher and the variation wider than G6PD Kaiping. Infants are usually discharged on the fifth day of life and it is possible that some of these infants may have their bilirubin reaching its peak at home. These could account for the readmissions of G6PD-deficient neonates with jaundice to the neonatal NICU post-discharged (unpublished data). These findings, although strengthens the notion that G6PD-deficient infants should not be discharged before the fourth day of life, as the danger lies in the fact that we may miss the day when serum bilirubin peaked i.e when infant is most susceptible to developing kernicterus, also suggests that there is a group of infants who get readmitted for severe neonatal jaundice. Further studies are required to establish the relationship between type of variant and type of cases that are readmitted and explore the usefulness of variant analysis as clinical indicators. The current protocol of managing G6PD-deficient neonates that requires babies to be discharged on the 5th day may be missing neonates whose serum bilirubin may reach its peak late. Modification of the current protocol may be required for optimal management. All neonates developed jaundice after the first day of life and 60% developed jaundice after 48 hours of life. These findings are consistent with the notion that G6PD deficiency causes late-onset jaundice. The duration of phototherapy ranged from 1.2 days to 5.9 days with a mean of 3.7 days. None developed jaundice on the first day of life, a feature that is usually associated with the rare class I G6PD variant that causes chronic non-nonspherocytic haemolytic anemia.[28] There was no signifant difference in the mean peak serum bilirubin between the two major variants. The peak serum bilirubin value may not be representative of true values since treatment with phototherapy of the hyperbilirubinemic neonates would have prevented the levels from reaching their natural peak. We find that majority (93.7%) of neonates have severe enzyme deficiency (red cell G6PD activity < 10% normal mean) and this finding is consistent with the findings from our previous study.[34] Another observation is the finding of variant G6PD Chinese-5 showing red cell G6PD activity of

moderate deficiency, consistent with other studies.[9,19] We find that the severity of enzyme deficiency does not correlate with the severity of serum hyperbilirubinemia and hence it cannot be used as a predictor of severe jaundice.

In conclusion, we have completely characterised the molecular defects of G6PD deficiency in a group of Malaysian Chinese neonates. The two commonest alleles are G6PD Canton and G6PD Kaiping, accounting for at least 84% of the cases of G6PD deficiency in the Chinese in Malaysia. The pattern of mutation distribution reflects the original gene pool of Malaysian Chinese with limited evidence of genetic admixture with the indigenous Malays.

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