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Molecular Heterogeneity of Glucose-6-Phosphate Dehydrogenase Deficiency in Vietnam

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In 2003-4 the authors were able to collect 120 samples with glucose-6-phosphate dehydrogenase (G6PD)-deficiency in Vietnamese. These samples were screened by multiplex polymerase chain reaction (PCR) using multiple tandem forward primers and a common reverse primer (MPTP). 79 (65.8%) out of 120 samples could be identified as the Single Nucleotide Polymorphisms (SNPs). Those variants with the SNPs contained G6PD Viangchan (G871A), G6PD Gaohe (A96G), G6PD Kaiping (G1388A), G6PD Canton (G1376T), G6PD Union (C1360T) and G6PD Chatham (G1003A). In 24 specimen from female patients, not only the heterozygote type but also the homozygote types were obtained. Those homozygote types were very high (29.2 %) in the female identified Vietnamese population.

Key words

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, Vietnam, Polymerase chain reaction, Multiplex PCR using multiple tandem forward primers and a common reverse Primer (MPTP)

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X chromosome-linked recessive disease and one of the most common inherited disorders of mankind more than 400 million people are affected worldwide. It is evi-

dence that the deficient alleles have been adapted against malaria infection ^{1,2}. And different mutations are unique to populations in different parts of the world where this disease is common; those are G6PD Mahidol in Thailand ³ and G6PD Kaiping in the Singapore Chinese population⁴. But there is very few information about G6PD deficiency in Vietnam.

Multiplex polymerase chain reaction (PCR) using multiple tandem forward primers and a common reverse primer (MPTP) was recently established as a comprehensive screening method for mutations. MPTP method was used in this study to scan for mutations of the G6PD gene. This method has been applied to identify the targeting site of G6PD mutations in Singapore ⁵, Philippines ⁶, and Malaysia ⁷. A preliminary study about G6PD deficiency in Vietnam has reported ^{8,9}. In this study, with an approval of an ethical review board about human genetic analyses the authors analyzed the molecular bases on G6PD gene in G6PD deficiency patients.

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Materials and Methods

Screening for G6PD enzyme activity was estimated by the fluorescent spot test¹⁰⁾ or the modified formazan method as follows. For preparation of agar plates, the following were dissolved at 55°C in 4 mL buffer consisting of 0.1 mol/L Tris-HCl and 0.01 mol/L MgCl₂, 25 mg of G6PNa₂, 5 mg of NADP, 5 mg of 3(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) and 5 mg of phenazine methosulfate (PMS). Four hundred milligrams of agar was melted in 36 mL of same buffer at 55°C. These two solutions were mixed and poured into a 13 x 6 x 2 cm plate. After hardening, the agar plate (gel thickness, approximately 5 mm) was wrapped with aluminum foil and stored in a refrigerator.

The blood samples from male newborns were taken on at least the 48th hour of life, dropped on filter paper cards (Schleicher and Schuell #903C Dassel, Germany), completely dried at room temperature for at least 4 h and then stored at 2-5°C. A disc (diameter, 1/8 inch) was punched from dried blood sample and placed completely in contact with the agar

surface. This plate was incubated at 37°C for at least 8 h. After the incubation period, the diameter of the formazan blue ring was measured.

For quantitative determination of G6PD activity using patient's erythrocytes as sample material according to its operating manual, the commercial assay kit from Sigma Co. was used. The cut-off level to determine enzyme deficiency was 118 mU/10⁹ erythrocytes.

Whole blood samples collected in EDTA were taken after the informed consent was obtained for all patients. Their age ranged from newborn to 35 years. The patients were unrelated family members. DNA samples were extracted by the phenol chloroform method. For screening mutations of the G6PD gene, MPTP was essentially employed. The sequences of primers, the target of genomic DNA and the length of PCR product for first and second PCR are listed in Tables 1 and 2.

The PCR reactions were performed as follows: For first PCR except exons 11 and 12, 10 µl of solution containing 1 µl of 10 x Buffer, 0.5 µl of 2.5mM dNTPs, 0.2 µl of 0.2 µM primer mixture, 1 µl of 30 µg DNA and

Table 1. List of the primers for first PCR

Exon	Primer ^{*1}	Sequence	Product
2	2-a	5'- cccagaggaactctcaagaaa	288 bp
	2-b	5'- aacaattagttggaaaagctga	
3-4	3-a	5'- agtagtgatcctgagtagtg	538 bp
	4-b	5'- ccccgacacgctcatagagt	
5	5-a	5'- acacacggactcaaagagag	341 bp
	5-b	5'- tggaggagcactgcctg	
6	6-a	5'- caaaaccaatgaggaagcca	652 bp
	9-b	5'- cttgtatctgttgccgtagg	
9	9-a	5'- tcttcagctgtgcacatagg	1207 bp
	9-b	5'- cttgtatctgttgccgtagg	
11-12	11-a	5'- actccacatggtggcag	618 bp
	12-a	5'- aggaatgtgcagctagag	

*1 a: forward primer, b: reverse primer

Table 2. List of the primers for MPTP

Exon	Specific primers for wild type	Product(bp)	Specific primers for mutant type ^{*1}	Product(bp)	G6PD variant
2	2-C ^{*2} 5'-catgggtgagtatct				
	2-95 5'-cagtcgggatacacac	64	2-95M 5'-cagtcgggatacacG	98	Gaohe (96A>G)
	2-R ^{*3} 5'-ctccatgccctcagc	98			
3	3-C 5'-accccaccatctg	61			
	3-131 5'-gacctggccaaga	83	3-131M 5'-gacctggGcaaga	83	Orissa (131C>G)
	3-R 5'-gcctgtccctggc				
	5-C 5'-tgcagcctctac	103			
5	5-376/383 5'-catgaatgccctc	73	5-383M 5'-catgaatgccCc	73	Vanua Lava(383T>C)
	5-R 5'-ctcgtagtcggtc				
	6-C 5'-gggaggaggacctg	119			
	6-487/493 5'-agcagaggctgga	158	6-487M 5'-tcccagcagaAg	162	Mahidol (487G>A)
6	6-563 5'-acatctctccctg	82	6-563M 5'-ccaaccacatctTc	88	Mediterranean (563C>T)
	6-592 5'-atctaccgcatcg	52	6-592M 5'-accagatctctacTg	58	Coimbra (563C>T)
	6-R 5'-accatgagggtctgc				
	9-C 5'-gagaggggcagg	146			
9	9-871 5'-tctcaggtcaagg	229	9-871M 5'-ctctcaggtcaagAt	229	Viangchan (871G>A)
	9-1003 5'-gtccacaccacc	94	9-1003M 5'-ggtcaccaccAc	95	Chatham (1003G>A)
	9-R 5'-gttctgccttgcg				
	11-C 5'-tcctcacagaac	128			
11	11-1360 5'-cacttcgtgcgc	55	11-1360M 5'-atgcacttcgtgTg	58	Union(1360C>T)
	11-R 5'-catagcccacaggt				
	12-C 5'-ctatgtccctcag	73			
	12-1376 5'-acgagctccgtg	55	12-1376M 5'-gacgagctccTtg	57	Canton (1376C>T)
12	12-1388 5'-aggcctggcgt	45	12-1388M 5'-ggcctggcAtat	4	Kaiping (1388G>A)
	12R 5'-ccagctcaatctgg				

^{*1} Capital letter in the sequence of primers shows the nucleotide change

^{*2} Primer 'C' was used for the control of amplification.

^{*3} Primer 'R' was used for the common reverse primer in each primer set.

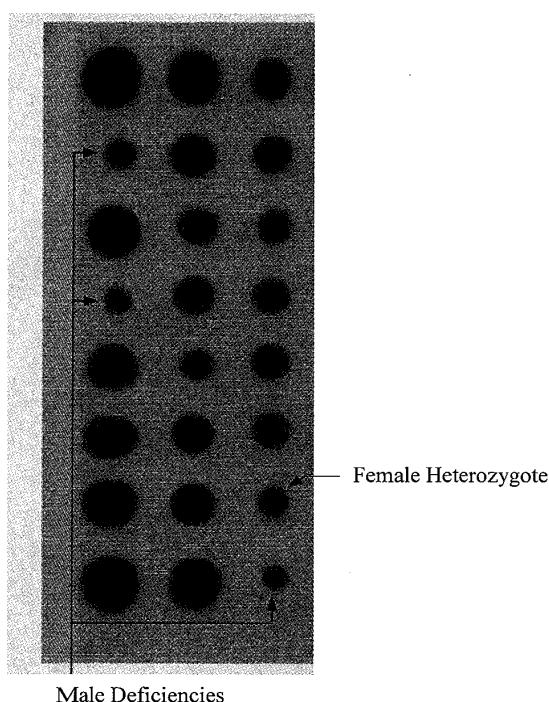


Figure 1. Ranges of Formazan ring results

0.5 μ l of 5U AmpliTaq Gold. For exons 11 and 12, PCR solution was changed into 1 μ l of 10 x Buffer, 1 μ l of Q solution and 0.5 μ l of 5U Quiagen Hot Start Taq. PCR was heated at 95 $^{\circ}$ C for 10 min and 35 cycles of denaturation at 95 $^{\circ}$ C, annealing at 58 $^{\circ}$ C and elonga-

tion at 72 $^{\circ}$ C for 30 sec per step and followed final elongation at 72 $^{\circ}$ C for 10 min.

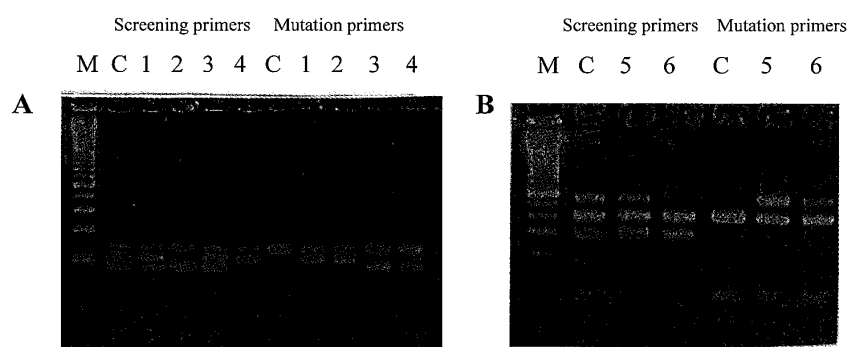
For MPTP, 10 μ l of solution containing 1 μ l of 10 x Buffer, 1 μ l of 25mM MgCl₂, 1 μ l of 2.5mM dNTPs, 0.5 μ l of 0.2 μ M primer mixture, 1 μ l of 10-fold diluted first PCR product and 0.5 μ l of 5U AmpliTaq Gold. PCR was heated at 95 $^{\circ}$ C for 10 min and 30 cycles of denaturation at 95 $^{\circ}$ C, annealing at 52 $^{\circ}$ C and elongation at 72 $^{\circ}$ C for 30 sec per step.

Each PCR product was electrophoresed on a 4 % agarose gel (Metaphor/Seakem HGT 3:1) containing 1.0 μ g/ml ethidium bromide and photographed under long-ultraviolet light.

Results

We chose three sampling areas from the long geographical feature of Vietnam. Hanoi and Hor Binb of Hanoi suburbs were selected as northern areas, Khanh Hoa and Hue as central area, and Ho Chi Minh City as southern area. Those collected areas included in malaria pollution area.

As the specimens of Formazan ring method we were able to collect 250 in Hoa Binh. 45



4% agarose gel electrophoresis of exon 12 and 9 products with a screening-primer set or a mutation-primer set for MPTP method.

M: Marker DNA (50bp ladder)

C: Healthy control

1: Patient 1 (G6PD Canton, heterozygote)

2: Patient 2 (G6PD Canton, homozygote)

3: Patient 3 (G6PD Kaiping, heterozygote)

4: Patient 4 (G6PD Kaiping, homozygote)

5: Patient 5 (G6PD Viangchan, heterozygote)

6: Patient 6 (G6PD Viangchan, homozygote)

Figure 2. Typical electrophoretic patterns in MPTP method in female variants

specimens (18.0 %) were equal to or less than 7 mm in the diameter of Formazan ring. They revealed 'positive screening', probably G6PD deficiencies¹¹⁾. Those results in one plate were shown in Figure 1. This high value in Hoa Binh well accorded with data by the fluorescent spot test examined previously. Furthermore, 174 specimens (2.9 %) out of 5,899 newborn babies in Hanoi and 27 specimens (5.5%) out of 491 newborn babies in Tu Du Hospital in Ho Chi Minh City were 'positive screening'. These values were approximately equal to a result from newborn babies more than 20,000 in Filipino male¹²⁾.

After the formazan ring method or the fluorescent spot test, 120 Vietnamese could be recalled as 'positive screening' and obtained their fresh blood. There were 75 male and 45 female patients whose age ranged from newborn to 35 years. Those were unrelated family members.

By MPTP method, actually, 79 (65.8 %) out of 120 patients could be identified, and the other 41 (34.2%) samples were uncharacterized. The major mutation was a G to A substitution at nt.871 in the G6PD genome (G871A). This agreed with males and females and was called as G6PD Viangchan shown in Table 3-A. Other mutations were G6PD Canton (G1376T), G6PD Kaiping (G1388A), G6PD Gaohe (A96G), G6PD Union (C1360T) and G6PD Chatham (G1003A). A silent mutation at nt.1311 excluded from their mutations. This variant was a silent mutation and observed about around 10% in Japan, The typical electrophoretic migration patterns with their specific primers about female specimen were shown in Figure 2 and their distribution results were summarized in Table 3-B.

In particular, seven cases out of 24 identified cases were the homozygote-type; 4 cases of G6PD Viangchan, 2 cases of G6PD Canton and 1 case of G6PD Kaiping. In the remaining 17 cases, each mutation was heterozygote-type.

Five different variants such as G6PD Mediterranean (C563T), G6PD Vanua Lava (T383C), G6PD Coimbra (C592T), G6PD Orissa (C131G) and G6PD Mahidol (G487A) could not be observed in this study.

Discussion

The rate of appearance of G6PD deficiency was 3 % in Hanoi and 18 % in Hoa Binh by a mass-screening test based on its activity⁹⁾. As for the incidence the local difference was clear, but the distribution of each variant seemed to make a little difference.

The genetic revelation rates of the specimen were high with 79.2 - 89.5 % in Hanoi, Hue and Khanh Hoa but low with 39.2 - 45.8 % in Hoa Binh and Ho Chi Minh city, respectively. It was unknown the reason why the samples from Hoa Binh was extremely low revelation rate. There may be a limit in a method to scan only exons in G6PD genome. Two possibilities can be considered about this reason. It is whether an absolutely new variant is in 7 exons screened in this study or in else where such as some introns, remaining 6 exons and promoter sites. As most of the genetic scans in whole exons are almost complete, the importance in latter cases is pointed out recently.

G6PD Viangchan was superior in all areas. Indeed G6PD Viangchan was first described in Laos¹³⁾, G6PD Canton, G6PD Gaohe and G6PD Kaiping in the Chinese¹⁴⁾.

The distribution of G6PD deficiency in Vietnam and the neighboring countries was shown in Table 4. Six kinds of variants identified in this study were recognized commonly in the neighboring countries. Its distribution type resembles the Philippines, but seven kinds such as G6PD Mediterranean (C563T), G6PD Vanua Lava (T383C), G6PD Coimbra (C592T), G6PD Orissa (C131G) and G6PD Mahidol (G487A) are not recognized. It was unexpected that a lot of recognized Mahidol in a neighboring country, Thailand, was not de-

Table 3. Distribution of six major G6PD gene mutations in Vietnamese populations analyzed by MPTP method.

A) Patients from various areas

Variant	Sampling Area			Hanoi			Hue			Hoa Binh			Ho Chi Minh City			Khanh Hoa		
	Total	Female	Male	Total	Female	Male	Total	Female	Male	Total	Female	Male	Total	Female	Male	Total	Female	Male
Gaohe A96G	7	0	7	0	0	0	0	0	0	0	0	0	1	0	1	6	0	6
Viangchan G871A	41	14	27	4	3	1	18	5	13	2	1	1	5	2	3	12	3	9
Chatham G1003A	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Union C1360T	5	4	1	0	0	0	1	1	0	2	1	1	1	1	0	1	1	0
Canton G1376T	17	5	12	6	2	4	0	0	0	4	2	2	2	1	1	5	0	5
Kaiping G1388A	8	1	7	1	0	1	0	0	0	3	1	2	2	0	2	2	0	2
Uncharacterized	41	21	20	3	0	3	5	3	2	17	8	9	13	7	6	3	3	0
Total samples	120	45	75	15	5	10	24	9	15	28	13	15	24	11	13	29	7	22

B) Female patients

Variant	Sampling Area			Hanoi			Hue			Hoa Binh			Ho Chi Minh City			Khanh Hoa		
	Total	Homo	Hetero	Total	Homo	Hetero	Total	Homo	Hetero	Total	Homo	Hetero	Total	Homo	Hetero	Total	Homo	Hetero
Gaohe A96G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Viangchan G871A	14	4	10	3	1	2	5	3	2	1	0	1	2	0	2	3	0	3
Chatham G1003A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Union C1360T	4	0	4	0	0	0	1	0	1	1	0	1	1	0	1	1	0	1
Canton G1376T	5	2	3	2	2	0	0	0	0	2	0	2	1	0	1	0	0	0
Kaiping G1388A	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
Total samples	24	7	17	5	3	2	6	3	3	5	1	4	4	0	4	4	0	3

tected in Vietnam. We consider that the peculiarity of a personnel exchange reflects in a difference of these distribution patterns.

Many races live in Vietnam and Kinh group is the majority. When 'positive screening' was recalculated about only Muong group in Hoa Binh, its rate rose to 32 % from 18 %. And the variants in Hue were observed in Katu group, It was seemed that the distribution of variants was different in every island of Southeast Maluku in Indonesia¹⁵⁾ was equivalent to a different distribution of variants every Vietnamese ethnic group. Since the G6PD genome is recessive in X chromosome, in the heterozygote female case it is usually expressed, but rarely its genetic expression disappears due to an inactivation of X chromosome. Although many female patients were actually obtained during mass screening test, and their

genotypes were identified as heterozygote- and homozygote-types, most differences were accepted with neither according to perform those quantitative enzyme assays. It was cleared there was genetic heterogeneity of the G6PD gene in Vietnam as seen in the other populations. The authors would like to study on a lot of uncharacterized sample selected in this study and clarify the inconvenience between phenotypic and genotypic ability. In Vietnam, December 2003, International Atomic-Energy Agency (IAEA) organized an international conference. IAEA decided to support and develop the national newborn screening Program, RAS/6/032, in Southeast Asian countries. Its concept was anyone could undergo the examinations of congenital metabolic diseases such as congenital hypothyroidism (CH), G6PD deficiency, phen-

Table 4. Distribution of G6PD deficiency in Vietnam and the neighboring countries

Variant		Vietnam	Thailand	Malaysia	Phillipines	Singapore	Indonesia
Gaohe	A96G	7				5	
Orissa	C131G			2		3	
Songklanagarind ^{*1}	T196A		1				
Vanua Lava	T383C			13	20	14	7
Chinese 4	G392T					1	
Mahidol	G487A		3	8	4	5	
Mediterranean	C563T			18		8	
Coimbra	G592C			5		3	1
Taiwan-Hakka	A493G		1			1	
Viangchan	G871A	41	4	27	63	22	
Chatham	G1003A	1		1	18	2	2
Chinese 5	C1024T						
Union	C1360T	5	3		40		
Canton	G1376T	17	3	2	8	29	3
Kaiping	G1388A	8	2	6		15	4
Uncharacterized		41	3	13	47	15	6
Ratio of identified samples		79/120	16/20	87/100	153/200	107/122	17/23
%		65.8	80	87	76.5	87.7	73.8

^{*1} New variant reported by Laosombat *et al*¹⁶⁾

ylketonuria and congenital adrenal hyperplasia without the test fee. Indeed, the screening of G6PD deficiency among all newborn babies in Vietnam has started since 2004. The authors expect the progress in this project to explore the incidence of G6PD deficiency.

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