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Coexistence of five G6PD variants indicates ethnic complexity of Phuket islanders, Southern Thailand

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Abstract Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy in humans. The prevalence of G6PD deficiency and its molecular basis were studied in Phuket islanders, Southern Thailand. A total of 345 volunteers (123 males and 222 females) were recruited in this study. Infection with *Plasmodium falciparum* or *Plasmodium vivax* was not detected in any of these subjects by polymerase chain reaction (PCR)-based diagnosis. G6PD-deficient individuals were identified with the WST-8/1-methoxy PMS method. The molecular basis of G6PD deficiency was investigated by PCR-direct sequencing procedures or PCR-restriction enzyme fragment length polymorphism assays. The numbers of individuals showing severe and mild G6PD deficiency were 14 and 21, respectively. A high prevalence of G6PD deficiency was observed in subjects with Moken (15.4%) or Thai (15.5%) ethnic background. G6PD Mahidol (487G > A) ($n=14$), G6PD Viangchan (871G > A) ($n=11$), G6PD Gaohe (95A > G) ($n=2$), G6PD Kaiping (1388G > A) ($n=1$), and G6PD Kerala-Kalyan (949G > A) ($n=1$) were identified. The

results suggest that several groups of people of the Asian Continent, such as Burmese, Laotian or Cambodian, Thai and Chinese, participated in the establishment of the ethnic identity of the current ethnic groups of Phuket Island.

Keywords G6PD deficiency · Moken · Urak Lawoi · Phuket islander · Thai ethnicity

Introduction

Humans have encountered various infectious and non-infectious diseases during their long evolutionary history. Malaria caused by *Plasmodium* infection has threatened humans since the establishment of slash-and-burn agriculture (Volkman et al. 2001) and kills over a million people annually; some 3.2 billion living in more than 100 countries or territories are at risk (WHO and UNICEF 2005). Any genetic trait protective against malaria must have been favorable to humans and those who carry such a genetic variant trait have an advantage in survival and, consequently, reproductive success. Although such variants themselves may lead to health problems, they are maintained in a population over many generations under strong selective constraints by malaria as a balanced polymorphism.

Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked enzyme and G6PD deficiency, estimated to be carried by more than 400 million individuals worldwide (Beutler 1996), is the most common enzymopathy in humans (WHO 1989). The main clinical manifestations of G6PD deficiency are neonatal jaundice and acute hemolytic anemia induced by food (favism), drugs, certain other chemicals, and infections (Sodeinde 1992). To date, at least 140 G6PD gene (*G6PD*) variants have been reported from various populations (Beutler and Vulliamy 2002). High frequency of some *G6PD* variants in particular populations was once attributed to heterozygous advantage against malaria (Luzzatto et al. 1969), and epidemiological evidence indicates that G6PD

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deficiency confers some resistance to *P. falciparum*, the primary human malaria (Ruwende et al. 1995). In fact, there is a global geographic correlation, in general, between the frequency of G6PD deficient variants and the local history of malarial disease (Allison 1960; Oppenheim et al. 1993). In other words, G6PD deficiency is a genetic witness of the past exposure to malaria in a population.

In southeast Asia, a large number of G6PD deficient variants have been reported from various populations (Iwai et al. 2001); however, there are still many ethnic groups whose G6PD status has not yet been established. Surveys of G6PD deficiency in Southern Thailand were reported from the Songkhla region (Panich et al. 1980; Laosombat et al. 2005). Malaria infection has been eradicated in Phuket Island in Southern Thailand (Fig. 1), which is inhabited by peoples with different cultural/ethnic origins. The so-called Sea Gypsies of the Andaman Sea—who are Austronesian speakers—are comprised of two ethnic groups, the Moken, and the Urak Lawoi, who are known to have lived on the boat. The Moken live in the Mergui Archipelago of Southern Myanmar and adjacent Thai territories, while the Urak Lawoi live on the west coast of the Malay Peninsula (Ivanoff 1997) (Fig. 1). Co-habiting in the southernmost Phuket Island, the G6PD status of these two ethnic groups has not been studied so far.

There is much in the literature on the culture and ethnography of the Moken and the Urak Lawoi; however, little is available about their genealogy and

physical characteristics. As a part of a population genetic study on the Sea Gypsies, we have surveyed the prevalence of G6PD deficiency among Phuket islanders and characterized the molecular basis of G6PD deficiency with special reference to the genealogy of the islanders.

Materials and methods

Subjects

A total of 345 healthy adult volunteers (123 males and 222 females) of a village in Southern Phuket, Thailand (Fig. 1) were the subjects of this study. So as to represent the population, they were recruited from various families in the village. They are the Moken ($n=41$) and those with Moken background ($n=76$), the Thais ($n=36$), with Thai background ($n=35$), with Moken and Thai background ($n=14$), the Urak Lawoi ($n=94$), and with Urak Lawoi background ($n=32$), and others ($n=17$) (Table 1). Their ethnic classification was based on their declaration including their parental lineage. This study was approved by the relevant Ethics Committee of Khon Kaen University and The University of Tokyo. Informed consent was obtained from the subjects prior to the survey and blood collection.

G6PD activity test

G6PD-deficient individuals were identified with the WST-8/1-methoxy PMS method (Tantular and Kawamoto 2003). To exclude a possible misdiagnosis of G6PD deficiency caused by a lower count of red blood cells (RBCs) in the blood, RBCs centrifuged at 1,000 g for 10 min were used. A 10 μ l sample of RBCs was diluted with 40 μ l saline. We prepared a reaction mixture containing 0.025 M Tris-HCl buffer (pH 8.0) with 2.5 mM $MgCl_2$, 1.25 mM D-glucose-6-phosphate diso-

Fig. 1 Location of Phuket Island, Thailand

Table 1 Prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency in Southern Phuket islanders

Group	Number tested		Number of severe (mild) deficiency	
	Male	Female	Male	Female
Moken	19	22	4	2 (3)
w/Moken	26	50	4	(5)
Thais	12	24		(4)
w/Thai ^a	14	21	2	(5)
w/Moken and w/Thai	5	9		(1)
Urak Lawoi	29	65	(1)	(1)
w/Urak Lawoi	11	21		(1)
Others ^b	7	10	1	1
Total	123	222	11(1)	3(20)

^aw/ denotes 'with ethnic background of'

^bMalay ($n=1$) and of unidentified origin ($n=16$)

dium salt, and 0.1 mM nicotinamide adenine dinucleotide phosphate oxidized form (Wako, Japan); 50 µl of this reaction mixture was mixed with 5 µl 5 mM WST-8/0.2 mM 1-methoxy PMS reagent (Seikagaku, Japan) and 5 µl diluted RBCs, and then incubated at 37°C for 45 min. For quantitative measurement, absorbance was measured at 450 nm. Relative values of absorbance for the normal G6PD were >1.0, whereas those of severe G6PD deficiency were ≤0.5. Mild G6PD deficiency exhibited intermediate values.

G6PD variant analysis

When severe or mild G6PD deficiencies were found, the underlying molecular basis was investigated by PCR-direct sequencing procedures or PCR-restriction enzyme fragment length polymorphism (RFLP) assays. Genomic DNA was extracted from the whole blood of 345 subjects, and a cDNA for *G6PD* was constructed using total RNA extracted from ten available Epstein–Barr virus immortalized lymphoblastoid cells with G6PD deficiency [Gen Elute™ Mammalian Total RNA Mini-prep Kit (Sigma–Aldrich, St. Louis, MO)].

G6PD consists of 13 exons and 12 introns, with the coding region located in exons 2–13. To minimize molecular screening steps, ten cDNA samples from G6PD-deficient individuals were subjected to nucleotide sequence analysis. Selected primers spanning nt653–nt1510 (exons 4–9) (Table 2) were used for PCR, and the nucleotide sequences of the resulting fragments were determined using an ABI prism 3100 Genetic Analyzer/Avant (Applied Biosystems, Foster City, CA) following the manufacturer's recommendations. Based on the results of this sequence analysis, which identified G6PD Mahidol (487G>A) and G6PD Viangchan/Jammu (871G>A), additional PCR reactions for RFLP analysis with *Alu* I (Tang et al. 1992) and *Xba* I (Nuchprayoon et al. 2002) were performed with appropriate primer sets (487 and 871 in Table 2), and the remaining G6PD-deficient samples were tested. Fragments were resolved on polyacrylamide gels and visualized by ethidium bromide staining after electrophoresis. To distinguish G6PD Viangchan from G6PD Jammu, we performed PCR-direct sequencing through exons 11, 12 (Table 2); G6PD Viangchan is defined by additional 1311C>T in exon 11 and T>C in intron 11 (IVS11 93T>C) mutations (Beutler et al. 1992). For undetermined G6PD deficient samples, PCR-direct sequencing was performed for each exon with an appropriate primer set (Table 2).

Detection of *Plasmodium* DNA

Using isolated DNAs, infection of *P. falciparum* and *P. vivax* was also screened using a nested PCR method (Snounou et al. 1993). A pair of genus-specific primers designed against the small subunit ribosomal RNA gene, rPLU (Table 2) was used for the first amplification.

Table 2 List of primers for PCR assays

Primer	Sequence
For cDNA	5'-TCTGCCCCGAAAACACCTT-3' 5'-CCCCATCCCACCTCTCAT-3'
For genomic DNA	
487 ^a	5'-GCGTCTGAATGATGCAGCTCTGAT-3' 5'-CTCTGCAGGTCCCTCCCGAAGGGC-3'
871 ^b	5'-TGGCTTTCTCTCAGGTCTAG-3' 5'-GTCGTCCAGGTAGGGTTTGGGG-3'
Exon 2	5'-AGGGGCTAACTTCTCAATGC-3' 5'-GGGAGGAGGAGCTCAACTTA-3'
Exon 3, 4	5'-TGAGTAGTGCCCAGATCACCA-3' 5'-GCAGGAGAGGAGGAGAGCATC-3'
exon 5	5'-CCCCTGGGGCAGAACACA-3' 5'-CCGGACACGCTCATAGAGTG-3'
exon 6	5'-GGGAGGGCGTCTGAATGA-3' 5'-ACCTTGGGCCTCTGTGGTG-3'
exon 7	5'-GGGTGACCCCTCACATGTGG-3' 5'-GAGGAGCTCCCCAAGA-3'
exon 8	5'-CATGCCCTTGAACCAGGTGA-3' 5'-GCATGCACACCCAGCTC-3'
exon 9 ^c	5'-ACCCAAGGAGCCCATTC-3' 5'-TGCCTTGCTGGGCCTCG-3'
exon 10	5'-AGACACTACGCACCCGTCCA-3' 5'-CCACTGCCTGCCACCAT-3'
exon 11, 12	5'-GGACCTGACCTACGGCAACA-3' 5'-CTCGGCTGGAGAGTGACGG-3'
exon 13	5'-TGCCTCTCCTCCACCCGTCA-3' 5'-GTCAATGGTCCCGGAGTC-3'
For <i>Plasmodium</i> detection	
rPLU ^d	5'-CCTGTTGTTGCCTTAAACTTC-3' 5'-TTAAAATTGTTGCAGTTAAACG-3'
rFAL ^d	5'-TTAAACTGGTTTGGGAAAACCAAA TATATT-3' 5'-ACACAATGAACTCAATCATGACTAC CCGTC-3'
rVIV ^d	5'-CGCTTCTAGCTTAATCCACATAACT GATAC-3' 5'-ACTTCCAAGCCGAAGCAAAGAAAGT CCTTA-3'

^aTang et al. (1992)

^bNuchprayoon et al. (2002)

^cHirono et al. (1994)

^dSnounou et al. (1993)

PCR products of genus-specific amplification confirmed by gel electrophoresis were subjected to a second round of PCR for species-specific amplification. Species-specific primer sets for *P. falciparum* (rFAL) and *P. vivax* (rVIV) were used (Table 2). The sizes of PCR products indicating *P. falciparum* and *P. vivax* were 205 bp and 120 bp, respectively.

Results

The prevalence of G6PD deficiency in each ethnic group is shown in Table 1. In all, 14 and 21 individuals showed severe and mild G6PD deficiency, respectively. Among 123 males and 222 females, 11 and 3 individuals showed severe deficiency, respectively. Mild G6PD deficiency was found in 1 male and 20 females. The overall

prevalence of G6PD deficiency in each group differed; high prevalences were observed for those with Moken (15.4%) or Thai (15.5%) ethnic background, while other groups exhibited low prevalence (Table 1).

Among the 35 deficient cases observed, the types of G6PD mutation could be identified for the following 29 cases, including all cases with severe deficiency, but no mutation in the coding region of the *G6PD* was seen in the remaining six cases with mild deficiency. The most common G6PD mutation, G6PD Mahidol (487G > A) ($n=14$) was followed by G6PD Viangchan (871G > A) ($n=11$), which was confirmed with the presence of 1311C > T and IVS11 93T > C. G6PD Gaohe (95A > G) ($n=2$: Moken females), G6PD Kaiping (1388G > A) ($n=1$: a Thai female) and G6PD Kerala-Kalyan (949G > A) ($n=1$: an Urak Lawoi male) were also identified (Table 3). The Moken and the Thais including those who have their ethnic traits showed a variety of G6PD mutations (Table 3).

Using a PCR-based diagnostic method, *P. falciparum* and *P. vivax* infection was not detected in any of the subjects.

Discussion

We have identified G6PD deficiency in 9.8% of males ($n=123$) and 10.4% of females ($n=222$) in southern Phuket islanders. The frequency of G6PD deficiency in males in Thailand's neighboring countries where malaria is endemic was 11.0% in Myanmar (Matsuoka et al. 2004), 12.6% in Cambodia (Matsuoka et al. 2005), and 2.7–7.2% in Malaysia (Ainoon et al. 2003). Although no malarial infection was observed, the prevalence of male G6PD deficiency in Southern Phuket (9.8%) was comparable to these latter values. This indicates that these groups in Phuket Island showing G6PD deficiency have experienced malaria endemics and that G6PD deficiency has been maintained as an advantageous genetic trait in these populations.

Among the 35 deficient cases, 29 were confirmed to harbor known G6PD variants; however, six cases with mild deficiency had no mutation in the coding region of the *G6PD*. This might be because our diagnostic criterion for mild deficiency G6PD using the WST-8/1-

methoxy PMS method did not quantify absolute enzyme activities and thus some normal cases were regarded as mild deficiency. Alternatively, cryptic mutations in non-coding regions such as the promoter region may play a role in decreasing G6PD activity.

G6PD variants found among the Moken were G6PD Mahidol (487G > A), G6PD Viangchan (871G > A), and G6PD Gaohe (95A > G) (Table 3). G6PD Gaohe (95A > G) was found exclusively in Chinese (Iwai et al. 2001), whereas G6PD Mahidol (487G > A) and G6PD Viangchan (871G > A) was the most common variant in Myanmar (91.3%) (Matsuoka et al. 2004), and in Lao-tian (100%) (Hsia et al. 1993) and Cambodia (97.9%) (Matsuoka et al. 2005), respectively. The Moken have been recorded in the literature as having had contact with traders from the continental countries such as Myanmar and China (Ivanoff 1997). These traders merged perfectly into some nomadic groups and, moreover, frequently married Moken women. Thus G6PD mutant alleles were introduced into the Moken community. Our study, which shows heterogeneous G6PD variants in the Moken population, supports the historical records that the Moken have been influenced by the Chinese and Burmese. Moreover, we propose that they share some genetic background in part with Lao-tian and/or Cambodian populations, probably via the Thais. The relatively high frequency of G6PD deficiency in the Moken suggests that they have in the past been under the presence of malarial pressure.

Among the Thais, we found G6PD Mahidol (487G > A), G6PD Viangchan (871G > A), and G6PD Kaiping (1388G > A) (Table 3). G6PD Kaiping (1388G > A) was also exclusively found in Chinese (Iwai et al. 2001). A quite similar distribution of the variants was reported in a previous study, which showed the leading three common variants in Southern Thailand facing the Gulf of Siam to be G6PD Viangchan (871G > A) (31.3%), G6PD Kaiping (1388G > A) (20.1%), and G6PD Mahidol (487G > A) (17.2%) (Laosombat et al. 2005), suggesting that the genetic components of Southern Thailanders facing the Gulf of Siam and those of the Andaman Sea are similar.

One of the interesting results in this study is the first description of G6PD Kerala-Kalyan (949G > A) out of India, among the Urak Lawoi, who are Austronesian-

Table 3 Type and distribution of *G6PD* variants

Group	<i>G6PD</i> variant type n (male:female)					
	Gaohe	Kaiping	Kerala-Kalyan	Mahidol	Viangchan	Total
Moken	2 (0:2)			3 (1:2)	4 (3:1)	9 (4:5)
w/Moken				8 (0:8)		8 (0:8)
Thais		1 (0:1)		1 (0:1)	1 (0:1)	3 (0:3)
w/Thais					6 (2:4)	6 (2:4)
w/Moken and w/Thais						0
Urak Lawoi			1 (1:0)			1 (1:0)
w/Urak Lawoi						0
Others				2 (1:1)		2 (1:1)
Total	2 (0:2)	1 (0:1)	1 (1:0)	14 (2:12)	11 (5:6)	29 (8:21)

speaking descendants. This mutation—the second most common variant (24.9%) in India—has not been reported in any other population (Sukumar et al. 2004). Although the carrier was thought to be pure Urak Lawoi from his family record, the presence of the Indian oriented mutation postulates a possible gene flow from Indian into the Urak Lawoi in the past.

Ethnic variety of the Southern Phuket islanders is obvious from their physical and cultural appearance and we tentatively classified them into several groups; however, in terms of the heterogeneity of the *G6PD*, their origins are more complicated than expected. At least five *G6PD* variants exist in the Southern Phuket islanders; even a small ethnic group such as the Moken had three *G6PD* variants. Our results suggest that several groups of peoples of the Asian Continent, such as Burmese, Laotian or Cambodian, Thai and Chinese, participated in the establishment of ethnic identity of the current ethnic groups of Phuket Island. Our study has revealed the ethnic complexity of Southern Phuket islanders, and will contribute to the tracing their origins.

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