บทบาทของ G6PD Mahidol ต่อการป้องกันมาลาเรียในเอเชียตะวันออกเฉียงใต้

นางสาว ชาลิสา หลุยเจริญ

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A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA

Miss Chalisa Louicharoen

A Dissertation Submitted in Partial Fulfillment of the Requirements

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By	Miss Chalisa Louicharoen
Field of Study	Biomedical Sciences
Advisor	Associate Professor Issarang Nuchprayoon, M.D., Ph.D.
Co-Advisor	Assistant Professor Anavaj Sakuntabhai, M.D., D.Phil.
	Richard Paul, D.Phil.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... .Dean of Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Professor Apiwat Mutirangura, M.D., Ph.D.)

.....Advisor
(Associate Professor Issarang Nuchprayoon, M.D., Ph.D.)

.....Co-Advisor
(Assistant Professor Anavaj Sakuntabhai, M.D., D.Phil.)

.....Co-Advisor
(Richard Paul, D.Phil.)

.....Examiner
(Professor Vorasuk Shotelersuk, M.D.)

.....Examiner
(Assistant Professor Kanya Suphapeetiporn, M.D., Ph.D.)

.....External Examiner
(Associate Professor Vip Viprakasit, M.D., D.Phil.)

ชาลิสา หลุยเจริญ : บทบาทของ G6PD Mahidol ต่อการป้องกันมาลาเรียในเอเชียตะวันออกเฉียงใต้ (A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. นพ. ดร. อิศรางค์ นุชประยูร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. นพ. ดร. อนวัช ศกุนตาภัย, Richard Paul, D.Phil.214 หน้า.

ภาวะพร่องเอ็นซัยม์กลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนสเป็นสาเหตุหลักของการเกิดดีซ่านในทารกและโลหิตจางในประชากรสี่ร้อยล้านคนทั่วโลก จากการรายงานก่อนหน้านี้พบว่าภาวะพร่องเอ็นซัยม์กลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนส มีผลต่อการป้องกันมาลาเรียแต่ยังไม่เป็นที่ทราบแน่ชัดว่าการป้องกันทางคลินิกดังกล่าวสามารถป้องกันมาลาเรียอันเกิดจากเชื้อ *Plasmodium falciparum* และ/หรือ *Plasmodium vivax* ด้วยเหตุนี้ข้าพเจ้าจึงได้ศึกษาการกลายพันธุ์ของยีนกลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนสที่พบมากในประชากรเอเชียตะวันออกเฉียงใต้ คือการกลายพันธุ์แบบมหิดลที่ก่อให้เกิดภาวะพร่องเอ็นซัยม์กลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนส และศึกษาผลของการกลายพันธุ์ดังกล่าวต่อการป้องกันการติดเชื้อ *P. vivax* และ/หรือ *P. falciparum*

จากผลการศึกษาชี้ให้เห็นว่าการกลายพันธุ์แบบมหิดลมีภาวะการถูกเลือกให้เกิดขึ้นในเชิงบวกอย่างชัดเจนและรวดเร็วในประชากรเอเชียตะวันออกเฉียงใต้ จากการคำนวณพบว่าการกลายพันธุ์ดังกล่าวเกิดขึ้นเมื่อ 1,500 ปีก่อนและมีค่าภาวะการถูกเลือกเท่ากับ 0.23 นอกจากนี้ยังพบว่าเชื้อ *P. vivax* ได้รับผลกระทบจากภาวะการถูกเลือกของการกลายพันธุ์แบบมหิดล โดยปริมาณเชื้อ *P. vivax* ในกระแสเลือดของคนไข้ลดลงอย่างมีนัยสำคัญ ขณะที่ปริมาณเชื้อ *P. falciparum* ในกระแสเลือดของคนไข้กลับไม่มีการเปลี่ยนแปลง จากการศึกษาดังกล่าวสนับสนุนคำกล่าวที่ว่าเชื้อ *P. vivax* ได้คุกคามต่อสุขภาพของมนุษย์อย่างน้อยในประชากรเอเชียตะวันออกเฉียงใต้มาช้านาน

สาขาวิชาชีววิทยาศาสตร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : GLUCOSE-6-PHOSPHATE DEHYDROGENASE/ G6PD MAHIDOL/ POSITIVE SELECTION/ SOUTHEAST ASIA/ MALARIA

CHALISA LOUICHAROEN : A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA. ADVISOR : ASSOC. PROF. ISSARANG NUCHPRAYOON, M.D., Ph.D., CO-ADVISOR : ASST. PROF. ANAVAJ SAKUNTABHAI, M.D., D.Phil, RICHARD PAUL, D.Phil, 214 pp.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency causes neonatal jaundice and hemolytic anemia and affects over 400 million people worldwide. The protective effect against malaria conferred by G6PD deficiency alleles has been proposed, yet the link with clinical protection from *Plasmodium falciparum* and *Plasmodium vivax* remains inconclusive. Here we investigated the impact on human survival of a common G6PD deficiency mutation in Southeast Asia – G6PD Mahidol^{487A} – from *vivax* and *falciparum* malaria.

Our results indicate that strong and recent positive selection has targeted G6PD Mahidol^{487A} mutation for the last 1,500 years with the selection intensity 0.23. In addition, we provide evidence that *Plasmodium vivax* has been the agent responsible for the strong selective advantage conferred by the G6PD-Mahidol^{487A}, because it reduces *vivax* parasite density, but not *falciparum*, in the host. These findings support the notion that *P. vivax* historically had a considerable impact on human health, at least in South-East Asia.

Field of Study: Biomedical Sciences Student's signature.....Chalisa Louicharoen.....

Academic year: 2008 Advisor's signature.....Issarang Nuchprayoon.....

Co-Advisor's signature.....

Co-Advisor's signature.....Richard Paul.....

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More importantly, to my mom, I want you to know that all of your unconditional love and supports to get me this far is playing off and I hope that you will be proud of my success.

CONTENTS

Page

Page

Page

Page

Page

Page

Page

Page

LIST OF TABLES

Table **Page**

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF ABBREVIATIONS

°C	degree Celsius
μg	microgram
μl	microlitre
μmol	micromole
2dG6P	2-deoxyglucose 6-phosphate
3'UTR	3' untranslated region
5'UTR	5' untranslated region
6PGD	6-phosphogluconate dehydrogenase
ACD	acid – citrate – dextrose
AD	Anno domini, after death in the year of our Lord
Arg	arginine
ATP	adenosine trophosphate
bp	base pairs
Cat	catalase
CHB	Han Chinese in Beijing, China
Chr	chromosome
cm	centimeter
CNSHA	chronic non-spherocytic hemolytic anemia
Cys	cysteine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	dATP, dTTP, dGTP, dCTP
ds	double stand
EDTA	ethylenediamine tetraacetic acid
EHH	extended haplotype homozygosity
EMSA	electrophoretic mobility shift assay
F	forward primer
FBAT	family base association test
FST	fluorescence spot test
g	gram (s)
G-6-P	glucose-6-phosphate
G6PD	glulose-6-phosphate dehydrogenase
Gly	glycine
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
H or Het	heterozygosity
HbS	hemoglobin S
HbC	hemoglobin C

HbE	hemoglobin E	mM	millimolar
HCl	Hydrochloric acid	mRNA	messenger ribonucleic acid
His	histidine	MRT	methylene blue reduction test
HMP	hexose monophosphate pathway	MTT	3-(4,5-dimethylthiazol-2-yl)-
HWE	Hardy-Weinberg Equilibrium		2,5-diphenyl tetrasodium
IBS	identity by state		bromide
ICSH	International Council for	N	number
	Standardization in Hematology	NADP	nicotinamide adenine
I.U.	international unit		dinucleotide phosphate
IVS	intervening sequence		(Oxidized form)
JPT	Japanese in Tokyo, Japan	NADPH	nicotinamide adenine
kb	kilobase		dinucleotide phosphate (reduced
KCl	potassium chloride		form)
kDa	kilodalton	ND	no defined, no data
Km	Michaelis constants	ng	nanogram
LD	linkage disequilibrium	nm	nanometer
LRH	long range haplotype	NPF	no parasites found
Leu	leucine	nt	nucleotide
M	molar	OD	optical density
MAF	minor allele frequency	*P. falciparum*	*Plasmodium falciparum*
Met	methionine	*P. vivax*	*Plasmodium vivax*
mg/dl	milligram/deciliter	PCR	polymerase chain reaction
$MgCl_2$	magnesium chloride	pH	power of hydrogen
min	minute	Phe	phenylalanine
ml	millilitre	pmol	picomole

PPP	pentose phosphate pathway	UV	ultraviolet
R	reward primer	Val	valine
RBCs	red blood cells	WBCs	white blood cells
REHH	relative extended haplotype homozygosity	WHO	World Health Organization
		YBP	year before present
RFLP	restriction fragment length polymorphism		
rpm	revolution per minute		
RTIC	The Rajanagarindra Tropical Diseases International Center		
SEA	Southeast Asia		
sec	second		
SD	standard deviation		
Ser	serine		
SNPs	single nucleotide polymorphisms		
TBE	tris-borate-EDTA		
TEMED	N,N,N',N'-yetramethylethylenediamine		
T_m	temperature		
Tris-HCl	tris-(hydroxymethyl)-aminoethane		
TPN	triphosphopyridine nucleotide		
U	unit		
UTR	untranslated region		

CHAPTER I

INTRODUCTION

1. Background and Rationale

Malaria is a major cause of human mortality worldwide and considered as one of the strongest known forces of evolutionary selection in the recent history of the human genome. Host genetic defense mechanisms have most likely evolved for resisting malaria infection in regions where the parasites have been historically prevalent. Among malaria-causing parasites, *Plasmodium falciparum* and *Plasmodium vivax* seem to have exerted selective pressure on the cellular phenotype of human erythrocytes – RBC Duffy negative, the hemoglobinopathies- HbS, HbC, HbE, thalassemias and other inherited blood disorders- Ovalocytosis, and Glucose-6-phosphate dehydrogenase deficiency (Carter and Mendis 2002).

Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked essential enzyme playing a key role in regulating cellular oxidative stress, particularly important in red blood cells. G6PD deficiency affects ~400 million people and causes neonatal jaundice and hemolytic anemia following infection notably by Hepatitis A & B and typhoid, as well as being responsible for favism. It has been proposed that the high overall population frequency of deficiency alleles result from their otherwise protective effect against malaria: the geographic distribution of G6PD deficiency largely overlaps with that of malaria (Allison 1960). Analyses of the naturally-occurring variation at the *G6PD* locus support the occurrence of local and recent positive selection targeting the G6PD deficient allele G6PD A-$^{202A/376G}$ in Africa starting 2,500-3,800 years ago (Tishkoff *et al.*, 2001; Sabeti *et al.*, 2002). These

observations are consistent with the signs of recent expansions apparent in African populations of *P. falciparum* (Joy *et al.,* 2003). From a clinical standpoint, however, the link between G6PD deficiency and malaria is more controversial. Although a clinical protective effect of G6PD deficiency from human lethal malaria, *P. falciparum*, in Africa has been shown (Bienzle *et al.,* 1979; Ruwende *et al.,* 1995; Guindo *et al.,* 2007), several other reports revealed lack of association (Powell and Brewer 1965; Martin *et al.,* 1979). In addition, the proposed underlying mechanism(s), via enhanced phagocytosis of infected red cells (Cappadoro *et al.,* 1998) and/or reduced parasite growth rate (Luzzatto *et al.,* 1969), are not reflected in *in vivo* parasite density (Bienzle *et al.,* 1972).

Most clinical, epidemiological and evolutionary studies relating G6PD deficiency and malaria protection have been focused on *P. falciparum* malaria, particularly in the African continent. Nevertheless, *P. vivax* imposes a considerable burden of disease on the human population and historically inflicted considerable mortality on and loss of fecundity in human populations (Carter and Mendis 2002). But, the role of G6PD deficient alleles in the susceptibility or resistance to *P. vivax* malaria has been received less attention. This is notably true for northern Europe where high frequencies of *G6PD* alleles are observed (Cavalli-Sforza 1994) and where *P. vivax* but not *P. falciparum* was historically prevalent. In Southeast Asia, both *P. falciparum* and *P. vivax* coexist, with *P. vivax* accounting for by over half of malarial cases. Moreover, there is increasing evidence supporting an ancient origin of *P. vivax* in Asia (Escalante *et al.,* 2005), where its presence most likely predates that of *P. falciparum* (Carter 2003). Such deep historical prevalence of *P. vivax* in greater Southeast Asia intimates a role for *P. vivax* in generating the higher number of G6PD deficient variants- G6PD Mahidol^{487A} and G6PD Viangchan^{871A}- mainly observed in

this region with respect to Africa, where a single deficiency variant – G6PD A-$^{202A/376G}$ – dominates (Mason *et al.*, 2007).

To this end, I have performed evolutionary and epidemiological studies to determine whether the frequency of G6PD deficiency variants- G6PD Mahidol^{487A} and G6PD Viangchan^{871A}- would increase survival chance of human population in Southeast Asia from *P. vivax* and *P. falciparum* malaria.

2. Research Questions

2.1 Which *Plasmodium* parasite could be responsible for the selection of G6PD deficiency in the Southeast Asian population?

2.2 Which *G6PD* mutation in Southeast Asian population is under selective pressure of *Plasmodium spp.*?

2.3 Which protective effect of *G6PD* mutation against *Plasmodium spp.*?

3. Objectives

3.1 To determine the influence of *Plasmodium spp.* on the selection of G6PD deficiency in the Southeast Asian population

3.2 To define the positive selection of *G6PD* mutation in Southeast Asia in protection against *Plasmodium spp.*

3.3 To assess the potential protective role of *G6PD* mutations which include G6PD Mahidol^{487A} and G6PD Viangchan^{871A} against *Plasmodium spp.*

4. Hypotheses

4.1 *P. vivax* and/ or *P. falciparum* have a potential role in selecting G6PD deficiency in the Southeast Asian population.

4.2 G6PD Mahidol^{487A} mutation and/ or G6PD Viangchan^{871A} are strongly under a positive selection of *Plasmodium spp.* in Southeast Asian population.

4.3 G6PD Mahidol^{487A} mutation reduces *P. vivax* parasite density and/ or number of clinical attacks.

5. Keywords

Glucose-6-phosphate dehydrogenase (G6PD)

G6PD Mahidol^{487A}

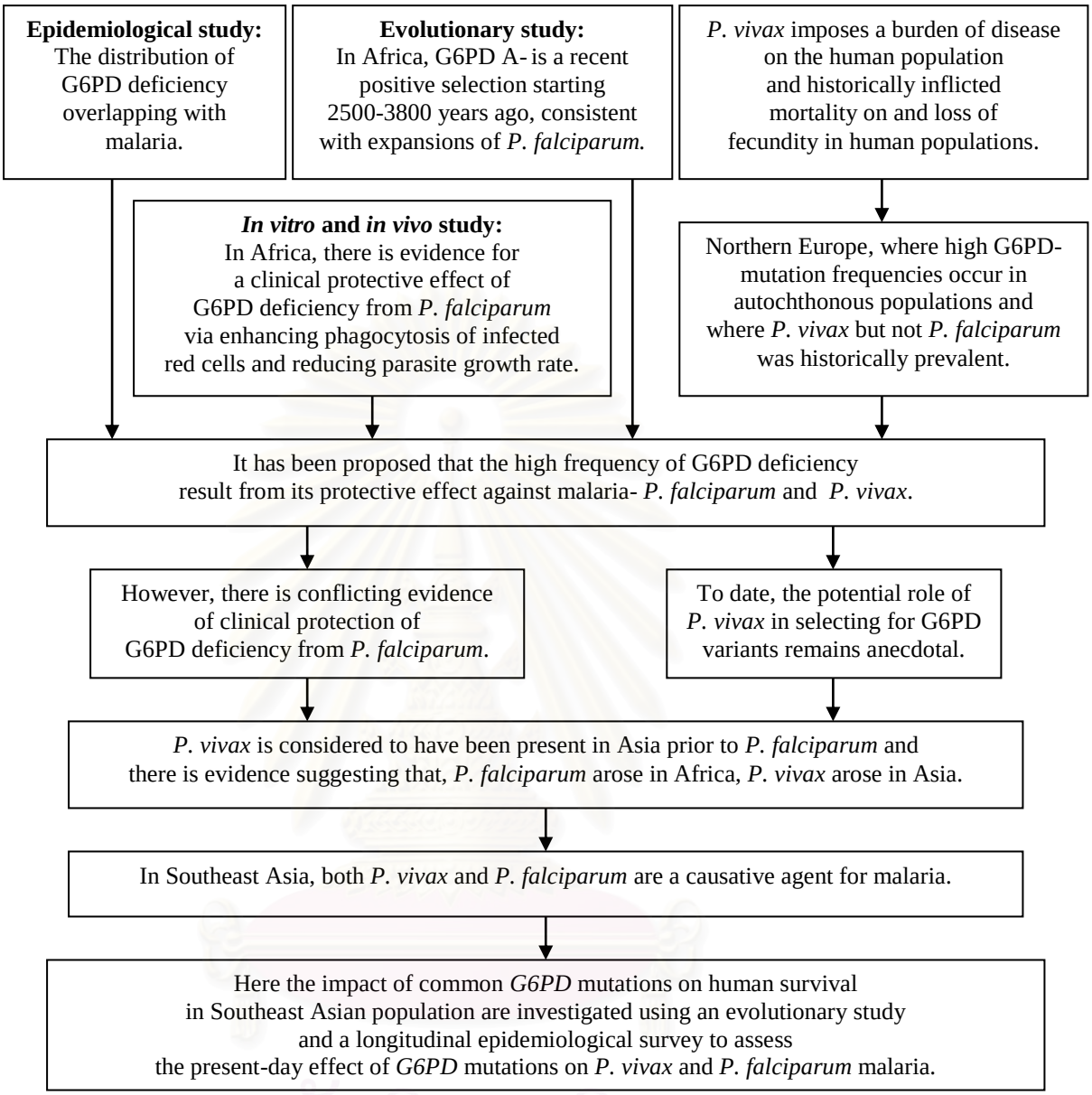
G6PD Viangchan^{871A}

Recent positive selection

Plasmodium falciparum (P. falciparum)

Plamodium vivax (P. vivax)

6. Conceptual Framework



7. Expected Benefits and Applications

7.1 To understand the evolutionary history of *G6PD* mutation and the spread of *Plasmodium spp.* in Southeast Asia.

7.2 To develop a malarial therapeutic strategy using weak points of *Plasmodium spp.* discovered from the protective effect of G6PD deficiency.

CHAPTER II

LITERATURE REVIEW

1. Malaria

Malaria is caused by protozoan parasite - *Plasmodium* spp. - infection of red blood cells, which are transmitted from one human to another by female *Anopheles.* Five species of *Plasmodium* genus infect humans - *P. falciparum, P. vivax, P. ovale, P. malariae,* and potentially *P. knowlesi* - (Cox-Singh *et al.*, 2008; White 2008), of which the first two are the most common and lethal malaria parasites (WHO Expert Committee on Malaria 2000) (Table 2.1). Nowadays, *P. falciparum* and *P. vivax* are very common in tropical, subtropical, and temperate regions due to the temperature limitations on its transmission by their mosquito.

Table 2.1 Distribution of four recognized species of malaria parasites of humans in the world today (Carter and Mendis 2002).

Species	Distribution of species (%) in following area (total no. of cases):							
	Sub-Saharan Africa		Asia	South Central	Western	Western	Central	South
	West and Central (858)	East and Southern (297)	(all) (863)	Asia and Middle East (14,539,081)	Pacific and Southeast Asia (86,461,294)	Pacific (Vanuatu) (1,708)	America and Caribbean (178,242)	America (859,480)
P. falciparum	88.2	78.8	4.2	19.8	51.4	43	12.9	29.2
P. vivax	1.2	9.8	95.6	80.2	48.6	56.1	87.1	70.6
P. malariae	2.2	3.0	0.0			0.9	0.0	0.2
P. ovale	8.4	8.4	0.2			0.0	0.0	0.0

1.1 Malaria Life Cycle

Anopheles mosquito is required for the transmission of malaria from one human host to another taking up gametocytes in the human blood meal. Appropriate development of the parasite in the mid-gut and salivary gland of the

mosquito is then necessary to complete the sporogonic cycle following injection of sporozoites during the human blood meal of Anopheles, parasites will develop in human liver stages (exo-erythrocytic cycle) and human blood stages (erythrocytic cycle).

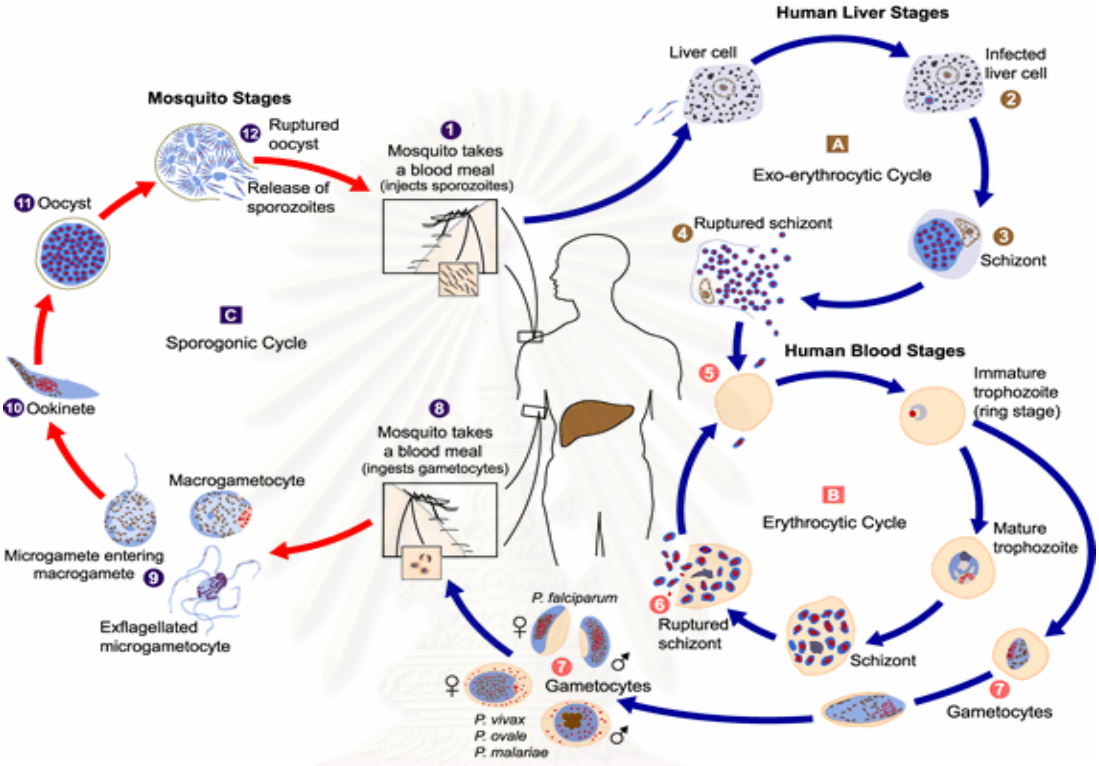


Figure 2.1 Diagram depicting the life cycle of human malaria (http://www.uni-tuebingen.de/modeling/Mod_Malaria_Cycle_en.html)

1.2 Clinical Manifestations of Malaria

The common symptoms of clinical form of malaria are fever, headache, body aches, general weakness, chills, sweats, myalgia, nausea, vomiting, diarrhea, and cough. The clinical manifestations of the severe malaria occurring from untreated *P. falciparum* infections are coma, severe anemia, renal failure, respiratory distress syndrome, hypoglycemia, shock, spontaneous hemorrhage, enlargement of the spleen, and convulsions (Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster 2000; Carter and Mendis 2002).

Most malaria clinical cases occur in Africa and out of an estimated 1.1–2.7 million deaths each year, over 90% are children under 5 years of age, mainly from cerebral malaria and anemia (WHO Expert Committee on Malaria 2000). The immunological status of a person is one of the factors that play an important role in determining the effect of malaria on human health and in the intensity of disease transmission. The antimalarial immunity is called “age dependent” or “duration of exposure dependent” taking time to achieve effective immunity to malaria under conditions of endemic infection (Carter and Mendis 2002). In endemic areas of malaria, children under 5-6 years old are those most at risk of acquiring severe malaria (Gupta and Day 1994) whereas in older children and adults who have partial immunity, the parasite density is observed to decrease with increasing age (Rogier *et al.*, 1996). In non-endemic areas, severe malaria affects adults and children as well as non-immune travelers and migrants. However, the effective antimalarial immunity of the individual, who is exposed to endemic malaria, is readily lost whenever they have an interval period- half a year to a year- without reinfection (Carter and Mendis 2002).

1.3 Malaria Burden in Southeast Asia (SEA) Region

Malaria is a major public health problem in the SEA region. All countries in the region except Maldives are endemic for malaria (Figure 2.2). Around 40% of the global population is threatened from malaria, which 8.5% of them live in SEA. Around 4.1% of global mortality due to malaria was reported in SEA (http://www.searo.who.int/EN/Section10/Section21/Section340_4018.htm).

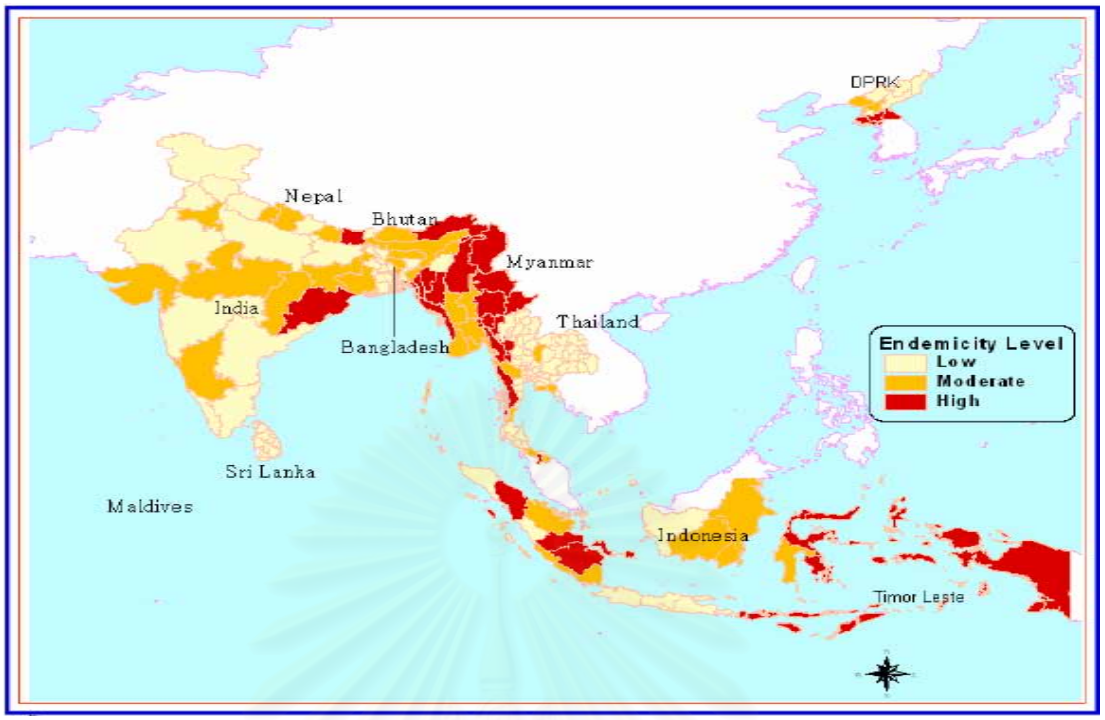


Figure 2.2 Malaria endemicity map of Southeast Asia region in 2006 (http://www.searo.who.int/EN/Section10/Section21/Section340_4018.htm)

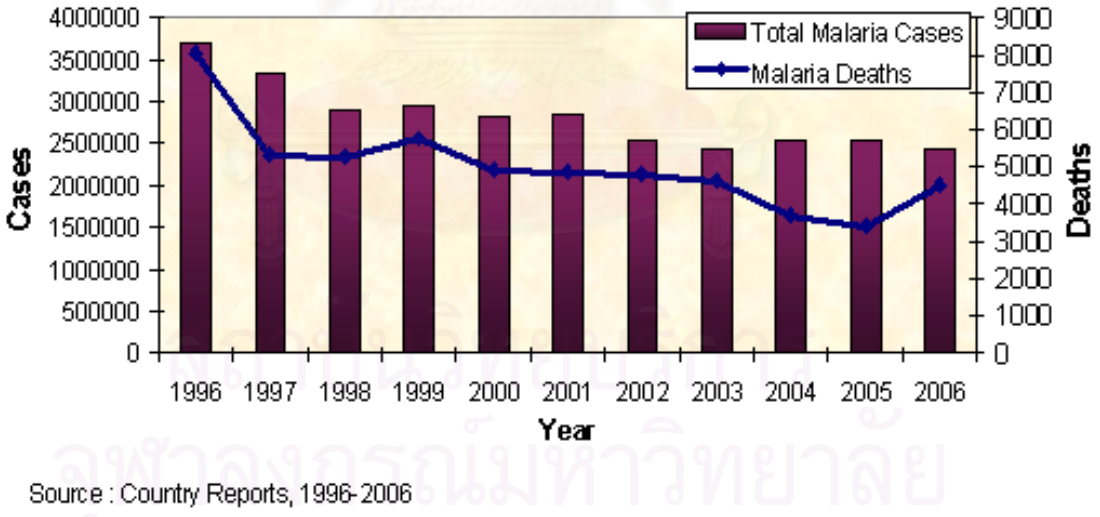


Figure 2.3 Trends of malaria cases and deaths in Southeast Asia during 1996-2006 (http://www.searo.who.int/EN/Section10/Section21/Section340_4018.htm)

During 2000-2006, malaria incidence remains static around 2.43 -2.77 millions (Figure 2.3). In Thailand, the number of malaria cases (morbidity) has been markedly reduced, as it has in Bhutan, and Sri Lanka. However, the mortality rate in

Thailand in 2006 (2.5%) increased slightly as compared to the mortality rate in 2005 (1.6%). Most malaria cases are found at the border of Thailand especially Thai-Myanmar and Thai-Cambodia border but the endemic areas are in the hilly or forested areas only. The presence of efficient vectors and the frequency of population movement make malaria transmission in forested areas intense (Ministry of Public Health 2002), and the major parasites are *P. falciparum* and *P. vivax*. Malaria chemoprophylaxis is not recommended in Thailand because of high level of *P. falciparum* resistance to antimalarial drugs including chloroquine, mefloquine, sulfadoxine pyrimethamine combination, and the serious, diminished sensitivity to quinine. But, the treatment regimens using mefloquine or its combination with artimisinin derivatives remain effective.

1.4 Malaria Diagnosis Using Microscopic Examination

1.4.1 Examining Thick Smear

Since the erythrocytes (RBCs) are lysed and the parasites are more concentrated, the thick smear is useful for parasites screening and for detecting mixed infections. An initial reading in thick smear in made for tentative species determination and then the thin smear is examined to determine the species present. Most often, thin smear is the appropriate sample for species identification. For determination of "No Parasites Found" (NPF): for malaria diagnosis, WHO recommends that at least 100 fields, each containing approximately 10 leucocytes (WBCs), be screened before calling a negative thick smear. Assuming an average WBC count of 8,000 per microliter of blood, this gives a threshold of sensitivity of 4 parasites per microliter of blood. In nonimmune patients, symptomatic malaria can occur at lower parasite densities, and screening more fields (e.g., 200, 300, or even the

whole smear) might be warranted, depending on the clinical context and the availability of laboratory personnel and time**.**

1.4.2 Examining Thin Smear

Thin smear is useful for species identification of parasites already detected on thick smears; screening for parasites if adequate thick smears are not available, and a rapid screen while a thick smear is still drying.

1.4.3 Quantifying Parasites

In some malaria cases, quantification of parasites yields clinically useful information. If this information is needed by a physician, malarial parasites can be quantified against blood elements such as RBCs or WBCs. To quantify malarial parasites against RBCs, the parasitized RBCs are counted from 500-2,000 RBCs on thin smear and express the results as % parasitemia.

% parasitemia = (parasitized RBCs / total RBCs) × 100

If the parasitemia is high (e.g., > 10%), 500 RBCs are examined. If it is low (e.g., <1%), 2,000 RBCs (or more) are examined. Asexual blood stage parasites and gametocytes are counted separately.

To quantify malarial parasites against WBCs, on the thick smear, the parasites are counted against WBCs, 500 parasites or 1,000 WBCs, whichever comes first. The result is expressed as parasites per microliter of blood, assuming 8,000 WBCs per microliter of blood.

Parasites/ µl blood = (parasites/WBCs) × WBC count per µl (or 8,000)

Result in % parasitized RBCs and parasites per microliter of blood can be interconverted if the WBC and RBC counts are known or otherwise by assuming 8,000 WBCs and $4x10^{6}$ RBCs per microliter of blood.

2. Glucose-6-Phosphate Dehydrogenase

2.1 Function of Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step of the hexose monophosphate pathway (HMP) or pentose phosphate pathway (PPP), changing glucose-6-phosphate (G-6-P) to 6-phosphogluconolactone and converting cofactor nicotinamide-adenine dinucleotide phosphate (NADP) to NADPH (Luzzatto 2006), powerful in reducing cellular oxidation (Figure 2.4).

Erythrocytes have only one pathway (HMP) to produce NADPH because they have no mitochondria, energy producing organelle (Luzzatto et al 2001). Furthermore, HMP also produce the ribose sugar, necessary for nucleotide synthesis. Normally, Red blood cells (RBCs) are exposed to two oxidative situations. First, oxygen radicals are produced from hemoglobin form to methemoglobin form. Second, the exogenous oxidizing agents such as glycosides from fava bean and phagocytosis granulocytes directly expose to erythrocytes (Luzzatto 2006). Therefore, HMP mainly protects RBCs and their hemoglobin from the oxidative stress via producing NADPH, which serves as a proton donor to regenerate the reduced glutathione (GSH) using glutathione reductase (GR), actively synthesized and highly concentrated in RBCs. Glutathione peroxidase (GPx), converting reduced glutathione (GSH) to oxidized glutathione (GSSG) for removing peroxide and oxygen radicals from the RBCs in the process. Indeed, the reduced form of glutathione is essential for the maintenance of hemoglobin and other RBC proteins in the reduced state (Luzzatto et al 2001) (Figure 2.4). NADPH also serves as an electron donor for other enzymatic reactions, involved in reductive biosynthesis.

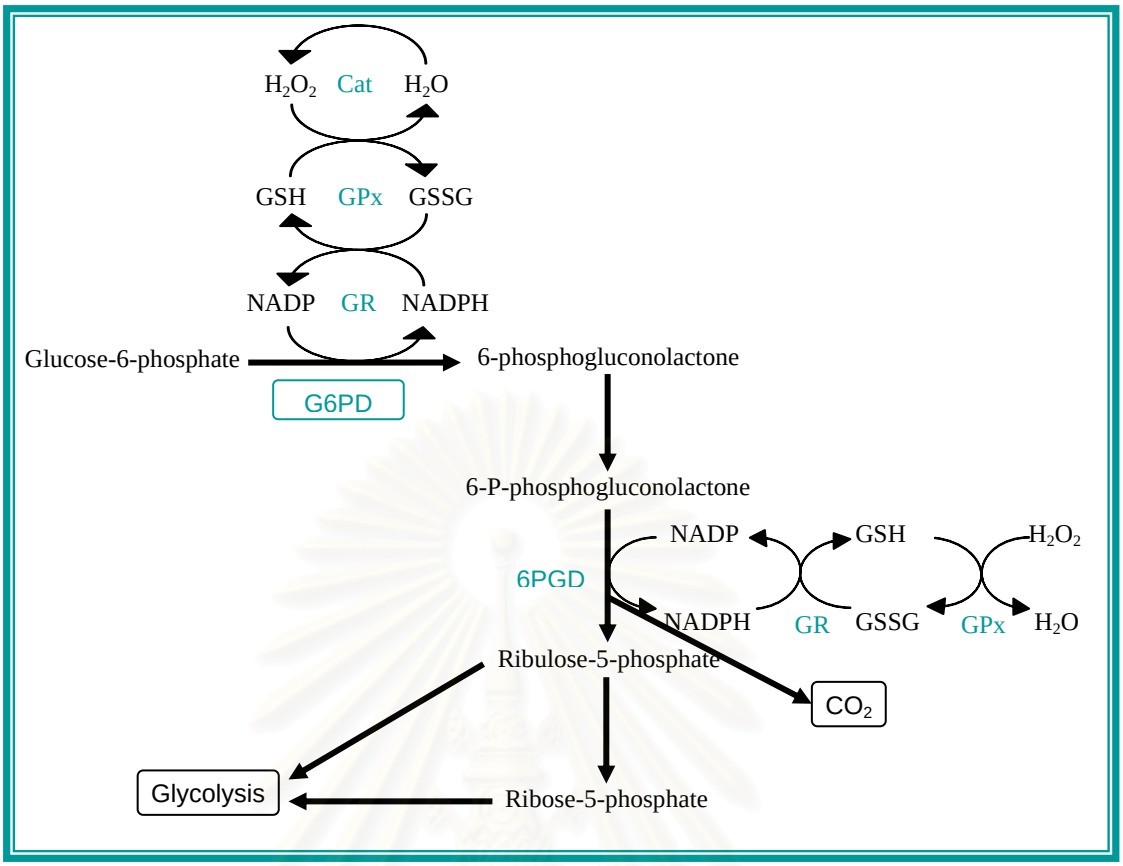


Figure 2.4 Hexose monophosphate pathway (HMP) (Cappellini and Fiorelli 2008) Cat = catalase, GPx = glutathione peroxidase, GR = glutathione reductase, G6PD = glucose-6-phosphate dehydrogenase, 6PGD = 6-phosphogluconate dehydrogenase, GSH = reduced glutathione, GSSG = oxidized glutathione.

The housekeeping enzyme, G6PD is present in all cell types. However, its concentration depends on type of tissues (Battistuzzi *et al.*, 1985). In normal RBCs, enzyme maintains its concentration at only 1-2% of its maximum potential or amounts the 0.03% of total cellular protein (Toniolo *et al.*, 1984), affects from ordinary circumstances;

(1) The amount of G-6-P and NADP are less than the saturating levels.

(2) NADPH and ATP inhibit the enzyme.

(3) Most of NADP is not free but bound to catalase

Oxidative stress leading to increased oxidation of NADPH coincidentally increases the level of NADP and releases enzyme inhibition and finally activity of G6PD increases proportionately. Consequently, the capacity in reducing the oxidation stress of normal RBCs have enough potential to deal with very significant levels of oxidation. This can explain that why a major reduction in G6PD activity has little clinical effect under normal circumstances. As under G6PD deficient RBCs, it may lead to pathophysiological features after failure in defense to oxidative stress (Gaetani *et al.*, 1974).

Since RCBs have no protein synthesis, the activity of G6PD diminishes as the cell ages. Under normal G6PD situation, the activity in reticulocytes has five times more than in the oldest, present only 1-2% of red cells. During hemolysis, the oldest cells will be a first candidate to be destroyed. As in G6PD deficient cases, the older red cells are even more severely deficient than younger ones. Although G6PD deficiency effects every cell in the body, it firstly effects hematological because the red cell has no other sources of NADPH. While, the other types of cells are protected by additional enzyme systems, generating NADPH in the deficient G6PD activity. Furthermore, RBCs have long period of non-nucleated, contain proteases that prefer to degrade the mutant enzyme much more than the proteases of other tissues (Beutler 1991).

2.2 Structure of G6PD

The G6PD active form in mammalian cells functions as dimer or tetramer subunits, in a pH dependent equilibrium condition. The G6PD monomer consists of 515 amino acids with 59 kilodalton (kDa) molecular weight (De Flora *et al.*, 1974; Morelli *et al.*, 1976). There are highly conserved regions of the molecule, aligned for evolution study, have been identified their functions. Moreover, the three dimensional structure of G6PD has recently been discovered (Figure 2.5, 2.6). The molecule consists of 2 domains: the N-terminal domain, covers amino acid 27 to amino acid 200, contains a coenzyme binding site or amino acids GASGDLA (amino acids 38-44); and a second domain, β+α domain, cover amino acid 199 to amino acid 515, contains a G-6-P binding site or amino acid RIDHYLGKE (amino acids 198-206) and an antiparallel nine-stranded sheet site, cover amino acid 380 to amino acid 425). The dimer interface site takes on a barrel arrangement, in this second domain of the G6PD molecule (Naylor *et al.*, 1996). Two domains are linked using an α-helix, functions as a substrate binding site or amino acid 198 to amino acid 206 (Mason 1996; Au *et al.*, 2000). NADPH is required in forming inactive monomers into active dimer and tetramer forms (Kirkman and Hendrickson 1962). Study G6PD molecule at 0.3 nm resolution NADP molecule, in every subunit of the tetramer, distant from the active site but close to the dimer interface (Au *et al.*, 2000). Therefore, G6PD requests NADPH in forming active molecule and in substrate metabolism (De Flora *et al.*, 1974; De Flora *et al.*, 1974; Canepa *et al.*, 1991; Beutler 2008).

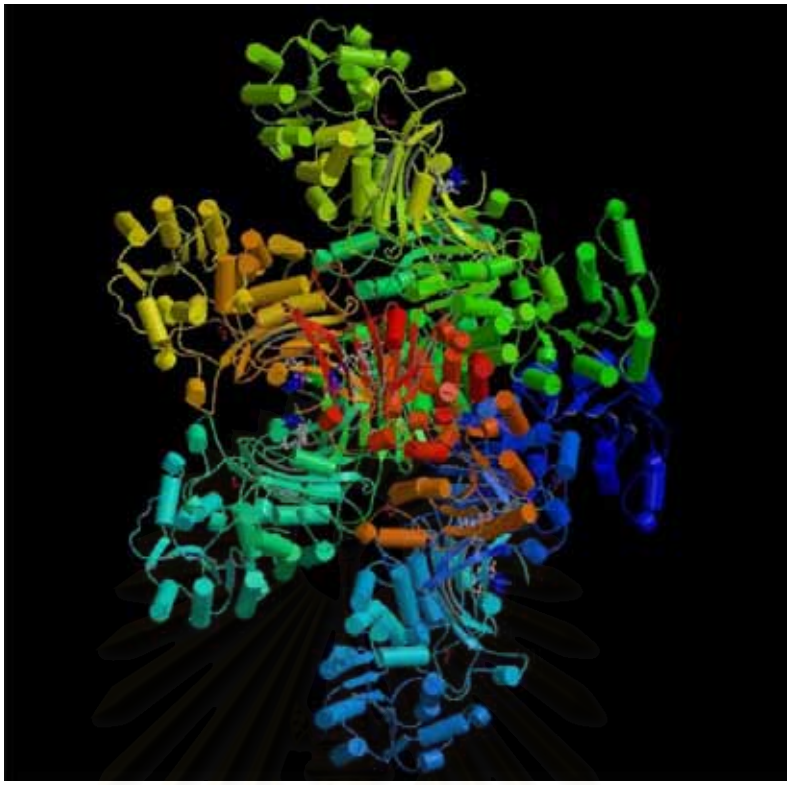


Figure 2.5 The structure of active tetrameric G6PD Canton^{1376T} (www.rscb.org).

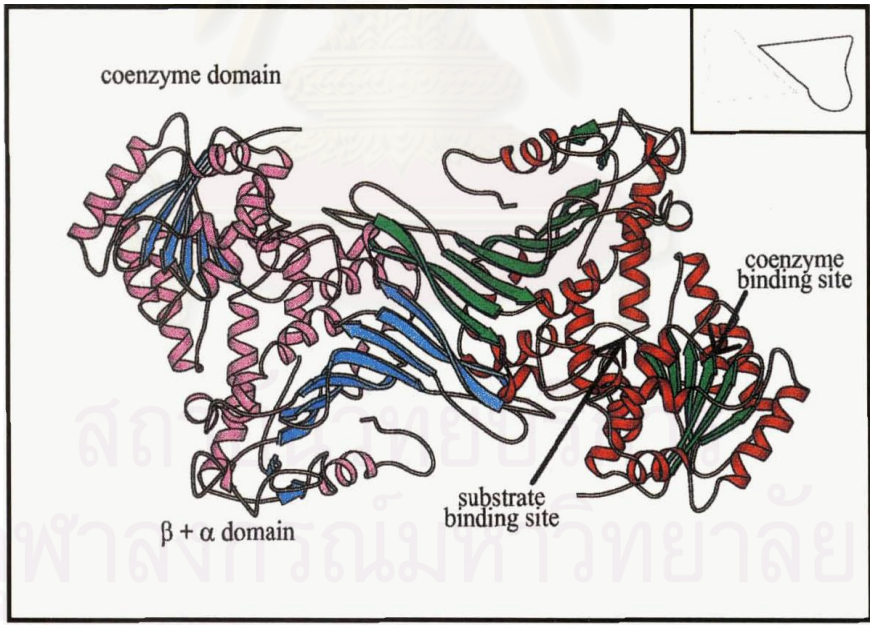


Figure 2.6 The human active dimeric G6PD three dimension structure. The figure at upper right serves as a monomeric shape (Naylor *et al.*, 1996).

3. Glucose-6-Phosphate Dehydrogenase Deficiency

3.1 Clinical Manifestation of Glucose-6-Phosphate Dehydrogenase Deficiency

G6PD deficiency is a common human genetic disorder, inherited in an X linked Mendelian transmission pattern (Beutler *et al.,* 1990). G6PD deficiency caused by mutations in the *G6PD* gene, resulting in variants of protein with alternative levels of enzyme activity, affects the clinical phenotypes. Normally, G6PD deficient individuals are asymptomatic throughout their life span. It does not affect the quality of life, the life expectancy, and the activity of deficient individuals (Hoiberg *et al.,* 1981; Cocco *et al.,* 1998). However, G6PD deficiency can causes hemolytic anemia, favism, chronic non-spherocytic hemolytic anemia (CNSHA), and neonatal jaundice, which can lead to kernicterus and death or spastic cerebral palsy. It can lead to life-threatening hemolytic crises in childhood and in later ages when interacting with oxidative inducing drugs, infection, and intake of fava beans (favism). The frequency and severity of manifestation is alternatively influenced by extrinsic and cultural factors and by genetic factors (Beutler 1994). Fatigue, back pain, anemia, and jaundice, clinical symptoms of acute hemolysis, caused from G6PD deficiency, helpful in characterization of G6PD deficiency. In addition, increased unconjugated bilirubin, lactate dehydrogenase, and reticulocytosis are good markers of the disorder.

3.1.1 Neonatal Jaundice

Neonatal jaundice, means total bilirubin > 15 mg/dl, is one of the most life and health-threatening affected from of G6PD deficiency, and consequent to kernicterus in these infants (Brown and Boon 1968; Gibbs *et al.,* 1979; Beutler 2008). Jaundice is usually obvious by 1 to 4 days of age. Kernicterus may generate

permanent neurological damage if treatment is incorrect. The inability of the liver to adequately conjugate bilirubin in G6PD deficient infants is the principle cause of neonatal icterus. The risks leading to neonatal jaundice depend on the nature of the variant, the level of G6PD activity in the liver, the genetic background of patient, exogenous factors such as the method of feeding and the maturity of the infant, the exposure of the newborn to environmental agents, the consumption of hemolytic agents such as fava beans, specific drug, or herbal treatments by pregnant heterozygotes (el-Hazmi and Warsy 1989). Form incident rate, about a third of all male newborn babies with neonatal jaundice have G6PD deficiency. However, the deficiency is less common in female newborns with jaundice (Kaplan *et al.,* 2001).

3.1.2 Favism

This term is used to describe an acute hemolytic reaction in a G6PD deficient individual after ingestion of fava beans (*Vicia faba)* (Figure 2.7).

Figure 2.7 Fava bean (*Vicia faba)*(www. vc/bs.org/beansaboutbeans.htm).

Patients with favism are always G6PD deficient, but not all G6PD deficient individuals, who ingest fava beans, develop hemolysis. Although the same individuals may have an unpredictable response, suggesting that some other risk factors, probably genetic (Stamatoyannopoulos *et al.,* 1966) and the amount of ingested fava beans, affect development of the symptom. Both dried and frozen beans ingestion can leads to favism, especially in period of bean harvest (Meloni *et al.,* 1983). Twenty four hours after beans ingestion, favism will present an acute

hemolytic anemia. Breastfed babies, whose mothers have eaten fava beans, show a tendency for hemolysis. In Mediterranean countries, Middle East, the far East, and north Africa, where the growth and consumption of fava bean was widespread, favism was widely found (Kattamis *et al.*, 1969). The mutation G6PD Mediterranean^{563T} is frequently associated with favism.

Fava beans contain high amount of vicine and convicine (up to 6.7 g/100g dry weight), which have been identified as candidate toxins (Arese *et al.*, 1981). Vicine and convicine are β-glucosides of pyrimidine compounds, are changed using β-glucosidases to aglycones; divicine and isouramil respectively. These compounds form reactive semiquinoid-free radicals and able to produce active oxygen species by inducing oxidization of GSH to GSSG in red cells. Consequently, the formation of ferrylhemoglobin, methemoglobin, and inactivation of various enzymes in G6PD deficient red cell (Figure 2.8). The reaction varies and remains unpredictable (Arese and De Flora 1990).

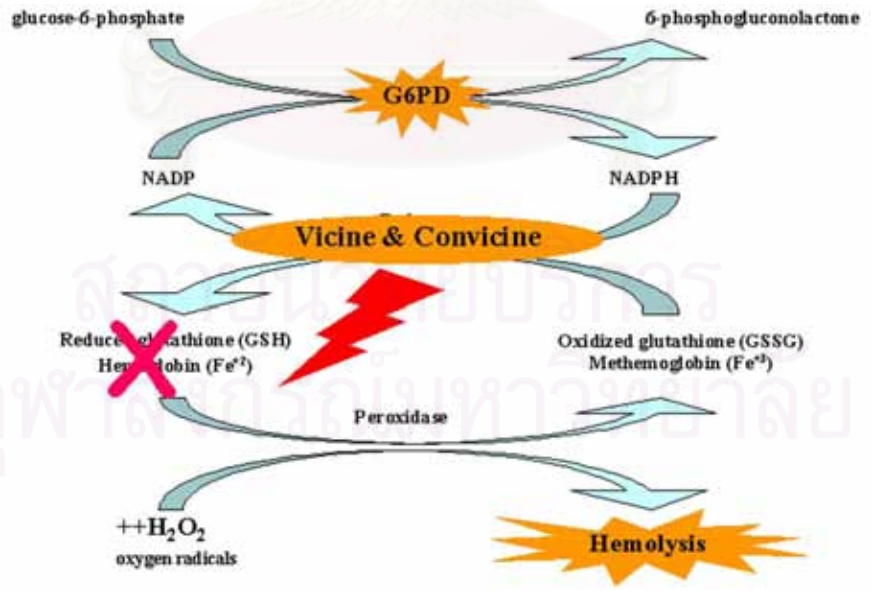


Figure 2.8 Oxidative stress mechanism of fava bean.

3.1.3 Acute Hemolytic Anemia

3.1.3.1 Drug-Induced Hemolysis

G6PD was first discovered by investigating the development of hemolysis after patients had received primaquine (Beutler 1959). Primaquine and several other drugs shorten RBC life span in G6PD-deficient individuals. (Beutler 1994) (Table 2.2) (Figure 2.9).

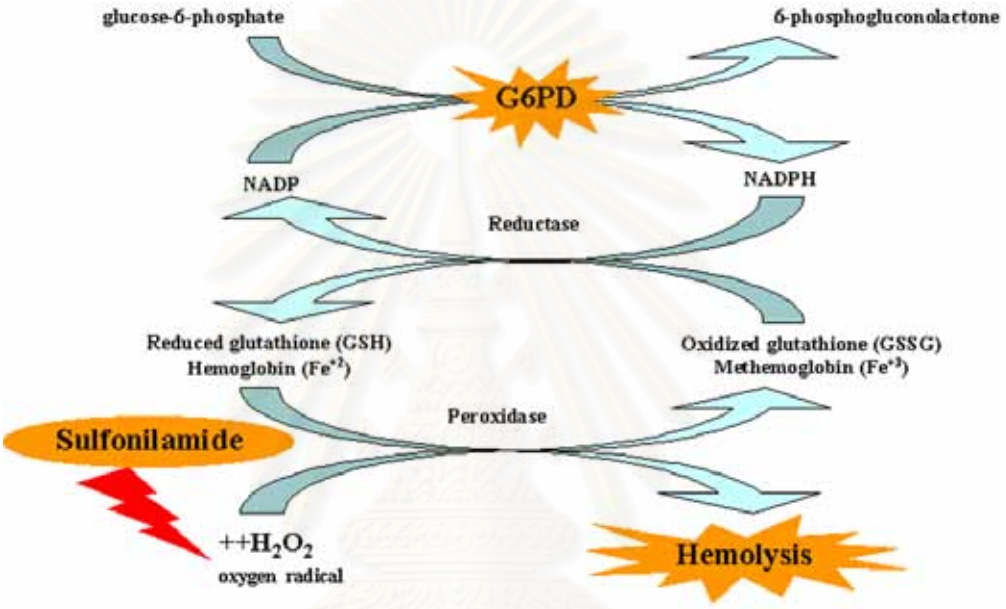


Figure 2.9 Oxidative stress mechanism of sufanilamide and drugs.

However, identification of specific drugs that cause hemolysis in G6PD-deficient patients is difficult because there are many risk factors that affect individual susceptibility to, and severity of, drug-induced oxidative hemolysis (Dern *et al.*, 1954): these include

- **Inherited**
  - Metabolic integrity of the erythrocyte
  - Precise nature of enzyme defect
  - Pharmacogenomics of individual

- Acquired

- Age and age distribution of RBC population
- Dose, absorption, metabolism, and excretion of drug
- Presence of additional oxidative stress; infection
- Effect of drug or metabolite on enzyme activity
- Pre-existing hemoglobin concentration

Table 2.2 Drugs and chemicals associated with substantial hemolysis in G6PD deficient patients (Luzzatto et al 2001)**.**

	Definite association	Possible association	Doubtful association
Antimalarials	Primaquine Pamaquine	Chloroquine	Mepacrine Quinine
Sulfonamides	Sulfanilamide Sulfacetamide Sulfapyridine Sulfamethoxazole	Sulfadimidine Sulfasalazine Glibenclamide	Aldesulfone Sulfadiazine Sulfafurazole
Sulfones	Dapsone	-	-
Nitrofurantoin	Nitrofurantoin	-	-
Antipyretic or Analgesic	Acetanilide	Aspirin	Paracetamol Phenacetin
Other drugs	Nalidixic Acid Niridazole Methylthionium Phenazopyridine Co-trimoxazole	Ciprofloxacin Chloramphenicol Vitamin Kanalogues Ascorbic acid Mesalazine	Aminosalicytic Acid Doxorubicin Probenedd Dimercaprol
Other Chemicals	Naphthalene 2,4,6-trinitrotoluene	Acalypha indica extract	

Acute hemolysis presents within 1 or 2 days of drug administration, then will worsen until day 7 to day 8. After drug cessation, the concentration of hemoglobin begins to recover by 8 to 10 days. The red cell destruction mechanism is via denaturation and precipitation of hemoglobin to form heinz bodies that adhere to the red cell membrane. They appear in the early stages of drug administration and disappear as hemolysis progresses. Heinz bodies lead the red cells to be trapped in the spleen. The reaction may vary from transient mild anemia to rapidly progressing

anemia with back and abdominal pain, jaundice and hemoglobinuria (Figure 2.10), and transient splenomegaly.

Figure 2.10 Comparing dark urine (hemoglobinuria) from G6PD deficient patient and normal urine.

3.1.3.2 Infection-Induced Hemolysis

Infection is the most typical cause of hemolysis in G6PD deficient individual. Although the mechanism by which this occurs is not clear, it probably happen during phagocytosis whereby leukocytes attack erythrocytes by discharging active oxygen species during phagocytosis (Baehner *et al.,* 1971). Hepatitis viruses A and B, cytomegalovirus (Siddiqui and Khan 1998), pneumonia (Tugwell 1973), and typhoid fever are potentially infective inducers. The severity of hemolysis depends on many factors such as concomitant drug administration, liver function, and age of patient.

3.1.4 Chronic Non-Spherocytic Hemolytic Anemia (CNSHA)

G6PD deficient individuals, resulting from inherited rare mutations and designated class 1, are threatened by this symptom (Table 2.3). Class 1 variants severely affect the G6PD molecule on the putative NADP-binding site or the glucose-6-phosphate binding site. Most G6PD variants causing congenital non-spherocytic hemolytic anemia sporadically arise (Fiorelli *et al.,* 2000) are not usually propagated

in populations (Luzzatto 2001). The RBCs of class 1 variant patients may have residual G6PD activity as high as 35% of normal RBCs (Engstrom and Beutler 1970). The functional impairment leads to shortening of the RBC life span (Yoshida 1973). The disorder is usually suspected during infancy or childhood. CNSHA caused by G6PD deficiency mostly results in neonatal jaundice, reticulocytosis, gallstones, splenomegaly, and chronic anemia exacerbated by oxidative stress and requires blood transfusions. Moreover, the levels of bilirubin and lactose dehydrogenase increase, and hemolysis is mainly extravascular.

3.2 Diagnosis of G6PD Deficiency

When patients have acute hemolysis triggered by exposure to an oxidative drug, infection, or ingestion of fava beans, G6PD deficiency diagnosis is preferred. In addition, members of families especially male in which jaundice, splenomegaly or cholelithiasis, should be tested for G6PD deficiency (Fiorelli *et al.*, 2000). In 1967, WHO recommended the method for diagnosis of G6PD deficiency as follows:

3.2.1 Quantitation of G6PD Activity in Erythrocytes

The diagnosis is based on the measurement of NADPH production rate from NADP using quantitative spectrophotometric analysis (Beutler 1984). This reaction takes place when two electrons are transferred from G6P to NADP in the reaction catalysed by G6PD (Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group 1967). In cases of high level of reticulocytes false negative results for G6PD deficiency may occur because of the higher activity in young erythrocytes relative to more mature cells (Ringelhahn 1972).

3.2.2 Screening for G6PD Deficiency

For rapid screening, several semiquantitative methods, suitable for field study, have been applied, notably the dye-decolouration test (Motulsky, Campbell-Kraut et al. 1961). The principle of the test uses the reduction of NADPH, invisible to the naked eye, linked to the reduction of the visible dye (brilliant cresyl blue) (Motulsky, Campbell-Kraut et al. 1961). Other tests use other photometric substances, include MTT tetrasodium, dichloroindophenol, methemoglobin or methylene blue reduction test (MRT) were developed (Beutler 1967). Methemoglobin is formed through the reaction of nitrite on the RBCs. Then, in the presence of methylene blue, methemoglobin is reduced through the oxidative pathway, and the rate of reduction is proportional to the G6PD activity of the cell (Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group 1967). Blood film examination or Heinz body test after dye decolouration is helpful for the diagnosis (Bernstein 1963). In addition, the fluorescent spot test uses the detection by fluorescence when there is NADPH reduction, instead of linking the dye with reduced pyridine nucleotide (Beutler 1966).

None of the biochemical tests can vigorously detect heterozygous females, because the mosaicism of chromosome X leads to partial deficiency. The variably of X inactivation in heterozygous females can result in normal G6PD activity. Only molecular analysis enables the detection of heterozygotes. Molecular analysis uses for example polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), direct sequencing, and denaturing gradient gel electrophoresis and allows detection of specific, rare, and novel mutations (Mason 1996).

3.3 Class of G6PD Deficiency

Based on enzyme activity and clinical manifestations, WHO categorized G6PD deficiency into 5 classes (Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group 1989) (Table 2.3).

Table 2.3 Classes of G6PD deficiency (Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group 1989).

Class	Enzyme Activity and Clinical Manifestation
Class I	Severely deficient, associated with chronic non-spherocytic hemolytic anemia
Class II	Severely deficient (1-10% residual activity), associated with acute hemolytic anemia
Class III	Moderately deficient (10-60% residual activity)
Class IV	Normal activity (60-150%)
Class V	Increased activity (>150%)

At present, more than 400 biochemical variants G6PD deficiency have been grouped into 5 classes (Xu *et al.*, 1995). The G6PD variants have been characterised according to their biochemical properties as measured by residual enzyme activity and electrophoretic mobility, physicochemical properties which included thermostability and chromatographic behaviour, as well as kinetic variables that are defined by the concentration of substrate needed for an enzymatic reaction at half the maximum speed; these include [Michaelis constants (Km)] for G-6-P, Km for NADP, relative rate of utilization of 2dG6P, pH dependence, use of substrate analogies. Biochemical and physical characteristics of G6PD variants have been previously described (Beutler *et al.*, 1968). The kinetic properties of G6PD variants are shown in Table 2.4. Furthermore, variants can be placed into two groups; sporadic or polymorphic (Luzzatto et al 2001). G6PD enzyme deficit can be caused by a reduction

in the number of enzyme molecules, a structural difference in the enzyme causing a qualitative change, or both. Most G6PD variants display enzymatic instability, implying amino acid substitutions in different locations that can destabilise the enzyme molecule.

Table 2.4 Kinetic properties of G6PD variants

***G6PD* mutation**	**RBC enzyme activity % of normal**	**Electrophoretic mobility % of normal**	**K_m G-6-P uM**	**K_m TPN uM**	**2dG-6-P utilization % of G-6-P**	**DeaminoTPN utilization % of TPN**	**pH optimum**	**Thermal stability**
G6PD B*	100	100	68	4.67	<4	60	8.5	normal
G6PD Viangchan^{871A}*	3	100	105	12	27	45	9.5	normal
G6PD Mediterranean^{563T}**	0-7	100	19-26	1.2-1.6	23-27	350	-	low
G6PD Canton^{1376T}**	4-24	105	17.7-38.3	-	1.2-20.8	-	-	low
G6PD Union^{1360T}**	<3	107	8-12	3.6-5.2	180	400	-	low
G6PD A-$^{202A/376G}$**	8-20	110	normal	normal	<4	50-60	normal	normal
G6PD Jammu^{871A}*	5	99	53	9	<4	57	normal	normal
G6PD Mahidol^{487A}**	5-32	100	22.9-52.6	-	1.6-5.6	42.9-79.8	normal	slightly high

***** (Poon *et al.*, 1988; Beutler *et al.*, 1991)

** (Panich *et al.*, 1972)

3.4 Geographical Distribution and Frequency

G6PD deficiency is an important public health issue with an estimated total number of individuals with G6PD deficiency of all types of about 400 million. The highest prevalence 15-26% has been reported in Africa, the Middle East, Southeast Asia, and the central and southern Pacific islands. Because of migration, deficient alleles are more prevalent in North and South America and in parts of northern Europe (Frank 2005). Approximately 7.5% of the world's population carry one or two mutations leading to G6PD deficiency. It is believed that because *G6PD* is X-linked. G6PD deficiency usually affects only males. Homozygous deficient females contribute only about 10% of those genetically G6PD deficiency. In addition, about 10 % of heterozygous females may exhibit effectively G6PD deficient due to unequal inactivation of their X-chromosomes, thus about 3.4% of the world population are at risk for complications of G6PD deficiency. With about 130 million births annually, it is expected that 4.5 million G6PD deficient children particularly vulnerable to neonatal jaundice and acute hemolytic crises are born every year. The distribution of G6PD deficiency around the world is shown in Figure 2.11.

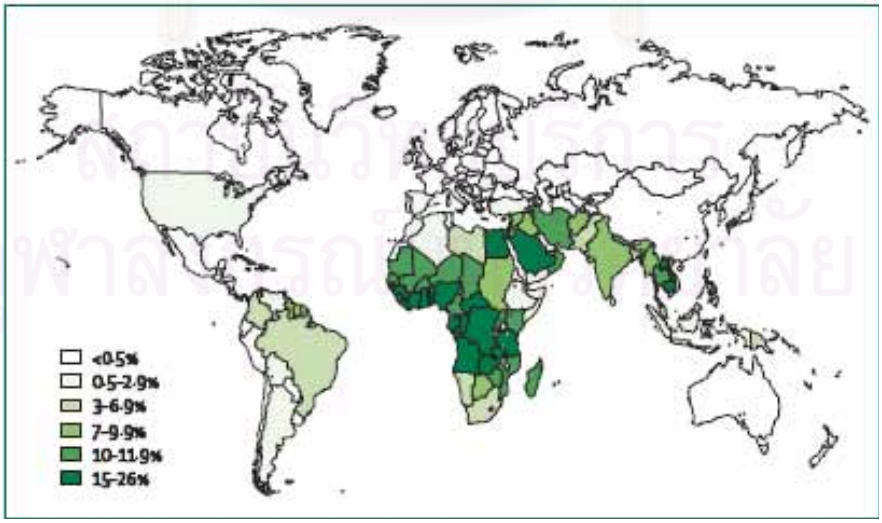


Figure 2.11 World map distribution of G6PD deficiency (Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group 1989).

In Southeast Asia, the high prevalence of G6PD deficiency in Thai (11.1% of 350 males) is analogous with other ethnic groups in Thailand and neighbouring countries in Southeast Asia, where malaria was historically and often currently endemic. Thus frequencies are 7.3-11% in Burmese or Burman (Iwai *et al.,* 2001; Matsuoka *et al.,* 2004; Nuchprayoon *et al.,* 2008), 10.8% in Shan (Iwai *et al.,* 2001), 6.7-12% in Mons (Iwai *et al.,* 2001; Nuchprayoon *et al.,* 2008), 7.1% in Danu (Iwai *et al.,* 2001), 6.3% in Kachin (Iwai *et al.,* 2001), 12.6-26.1% in Cambodian (Table 2.4) (Louicharoen and Nuchprayoon 2005; Matsuoka *et al.,* 2005), 2.7-7.2% in Malays (Ainoon *et al.,* 2003), 7.2% in Laos (Iwai *et al.,* 2001), and 9.8% in southern Phuket islanders (Ninokata *et al.,* 2006).

Previously, prevalence of G6PD deficiency was studied in Thai, Cambodian, Lao, and Burmese (Table 2.5). From 215 Cambodian blood samples, we found G6PD deficiency in 26.1% of Cambodian male (31 of 119) and 3.1% of females (3 of 96). Among Cambodian neonates, 21 of 56 males and two of 51 females were G6PD deficient. Among Cambodian adults, 10 of 63 males and one of 45 females were G6PD deficient (Louicharoen and Nuchprayoon 2005). One hundred and sixty two Lao subjects consisting of 84 males and 78 females were analyzed for G6PD deficiency. Twenty (23.8% of 84) males and three (7.7% of 78) females were G6PD deficient. One hundred and ninety eight Thai males and one hundred thirty five Thai females were screened G6PD activity. Thirty three (16.7% of 198) males and eleven (8.1% of 135) females were defined as G6PD deficient. For Burmese subjects, who had been collected in 2002-2003, there are one hundred and thirty one subjects consisting of 72 males and 59 females are screened for G6PD activity. Seven (9.7% of 72) males and one (1.7% of 59) females were defined as G6PD deficient.

Table 2.5 Prevalence of G6PD deficiency in Cambodian, Lao, Thai, and Burmese (2002-2003)

Ethnic group	Case (N)	Sex (N)	G6PD deficiency (N)	Prevalence
Cambodian	215	M 119	31	0.26*
		F 96	3	0.03*
Lao	162	M 84	20	0.24
		F 78	6	0.08
Thai	333	M 198	33	0.17
		F 135	11	0.08
Burmese (2002-2003)	131	M 72	7	0.10
		F 59	1	0.02

* (Louicharoen and Nuchprayoon 2005)

The finding supports Haldane's theory that suggests malaria was the evolutionary driving force for selecting for G6PD variants in tropical population (Haldane 1949).

3.5 Genetics and Molecular Basis of G6PD Deficiency

The inheritance of G6PD deficiency has an X-linked pattern, with higher incidence rate in males than in females and transmission generally from mother to son. Males carry a single copy of *G6PD* or are hemizygous and either have normal *G6PD* gene expression or G6PD deficiency. As females have two copies of the *G6PD* gene one on each X chromosome, they will be homozygous or heterozygous. The putative heterozygotes often have intermediate degrees of glutathione stability. Since heterozygous females are genetic mosaics as a result of X-chromosome inactivation, females are susceptible to the same pathophysiological phenotype as males if the normal *G6PD* is inactivated (Beutler *et al.,* 1962). Normally, heterozygous females have less severe clinical symptom than G6PD deficient males, although some may develop severe acute hemolytic anemia.

G6PD gene has been located at the telomeric region of the long arm of the X chromosome (band Xq28), close to the genes for congenital dyskeratosis,

hemophilia A and color blindness (Figure 2.12) (Szabo *et al.*, 1984; Trask *et al.*, 1991).

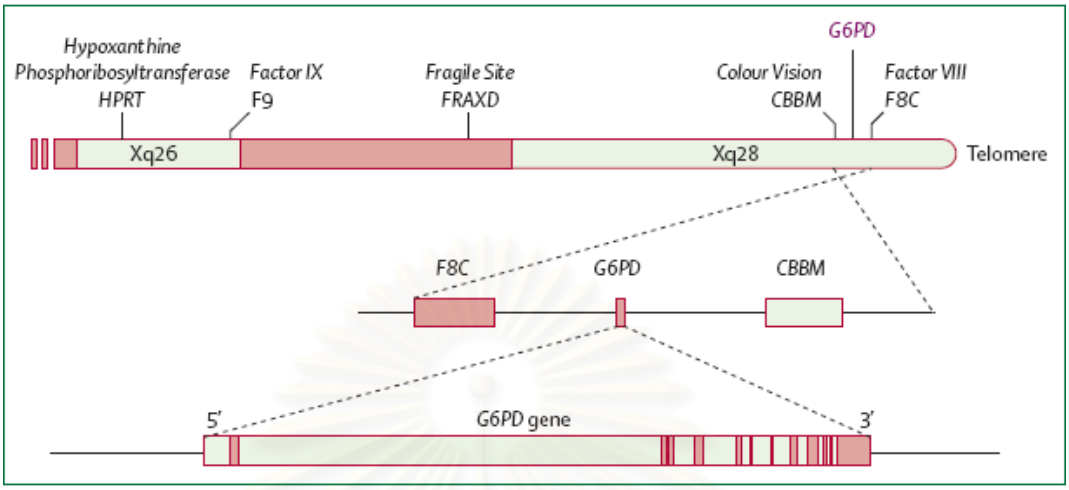


Figure 2.12 Location of *G6PD* on Chromosome X (Cappellini and Fiorelli 2008).

The gene spanning approximate 18 kb of genomic DNA consists of a GC-rich (more than 70%) promoter region, 13 exons and 12 introns. All introns are smaller than 300 bp except intron 2, which extends for about 11 kb (Martini *et al.*, 1986) (Figure 2.13). The 5' untranslated region (5'UTR) of the mRNA corresponds to exon I and part of exon II. The initiation codon has been located in exon II (Poggi *et al.*, 1990; Chen *et al.*, 1991; Beutler 1994). The mRNA encodes 515 amino acids. The 2,269 nucleotides of *G6PD* mRNA consists of 5' UTR (69 nucleotides), coding region (1,545 nucleotides) which divided into 12 segments ranging in size from 12-236 bp, and 3'UTR (655 nucleotides).

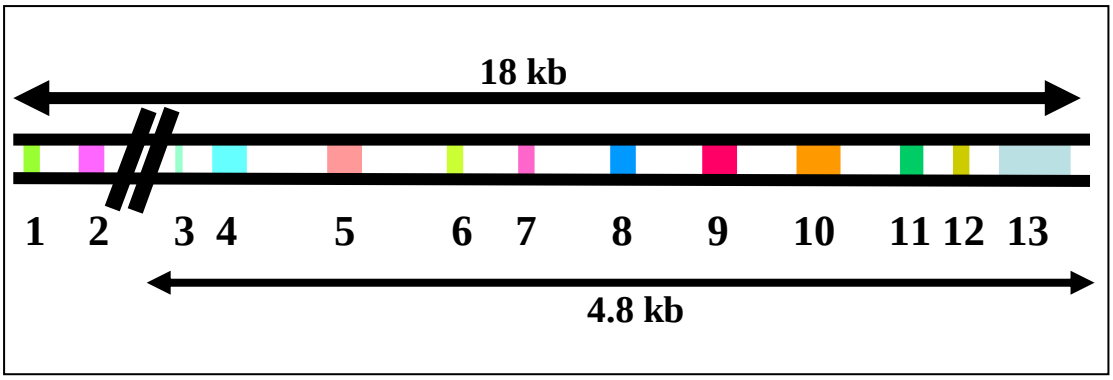


Figure 2.13 Diagram of *G6PD* gene

There are several binding sites for the transcription factor SP1—GGCGGG and CCGCCC sequences in the promoter region, similar to promoters of other housekeeping genes (Toniolo *et al.*, 1991).

Wild-type *G6PD* is referred to G6PD B. It has been reported that all mutations of the *G6PD* resulting in enzyme deficiency affect the coding sequence (Figure 2.14) (Bulliamy *et al.*, 1997; Cappellini and Fiorelli 2008).

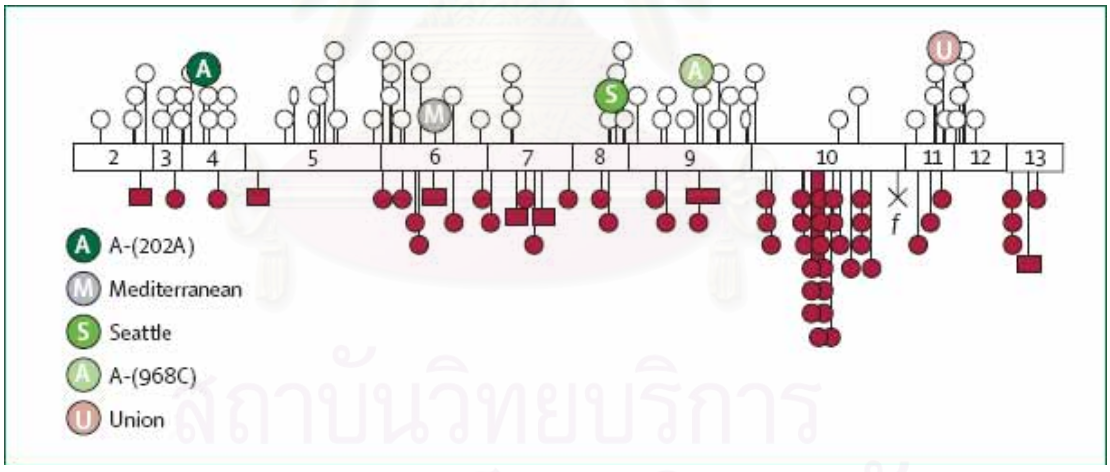


Figure 2.14 Most common mutations along coding regions of *G6PD*. Exons are shown as open numbered boxes. Filled circles are sporadic mutations giving rise to severe variants or class I. Open circles represent mutations causing classes II and III variants. Open ellipses are mutations causing class IV variants. Filled squares represent small deletions. Cross is a nonsense mutation. f represents a splice site

mutation. 202A and 968C are the two sites of base substitution in G6PD A-$^{202A/376G}$ (Cappellini and Fiorelli 2008).

Compared to the biochemical variants, mutation variants are produced in a number of different ways. Therefore, many different biochemical variants may turn out to be caused by mutations of the same gene, or one variant may turn out to be the result of several gene mutations (Beutler 1991). From molecular characterization, 140 mutations have been reported. Most of mutations are single base substitutions or missense mutations leading to amino acid replacements. These affect the three-dimensional structure of G6PD, especially in the NADP-binding site or glucose-6-phosphate binding site, associated with an altered enzyme phenotype (D'Urso *et al.*, 1988; Beutler 1993; Vulliamy *et al.,* 1993). Rarely, a second mutation is present in cis (Town *et al.,* 1992; Hirono *et al.,* 2002). Using electrophoretic mobility shift assays (EMSA) and systematic mutagenesis of the promoter region of *G6PD,* no mutations have yet been reported in the human promoter (Franze *et al.,* 1998), although findings from a mouse model showed that mutations of GC boxes can affect transcriptional activity (Philippe *et al.,* 1994). Point mutations spreading throughout the entire coding region result in variants that produced chronic hemolytic anemia. These mutations have been found to be clustered either between nt 563 to nt 844, including the putative glucose-6-phosphate binding site or a cluster of mutations, in exon 10 and 11 (amino acids 380–430, close to the dimer interface), that cause a severe phenotype (class I, chronic non-spherocytic hemolytic anemia). Analysis of the three-dimensional model of human G6PD enzyme, obtained from the study of crystallized protein (Naylor *et al.,* 1996; Au *et al.,* 2000), indicate that the NADP+ binding site is located closely to the N terminus with highly conserved amino acid in 23 species. Arg72 plays a direct part in coenzyme binding (Hirono *et al.,* 1989; Scopes *et al.,* 1998). The cluster of

mutations around exon 10 and 11 designates the subunit interface, which interacts with other important residues located elsewhere when they are brought close to this domain by protein folding. Almost all mutations in and around this domain cause variants of G6PD deficiency associated with chronic non-spherocytic hemolytic anemia (class I), and affect both hydrophobic and charge–charge interactions or salt bridges, which are weak ionic bonds. *In vitro* studies show that all variants caused by mutations found in this area exhibit a striking reduction in thermal stability. In addition to missense mutation, a nonsense mutation in which a base substitution at nt1,284 C→A created a stop codon, "G6PD Georgia", produce truncated protein (Xu *et al.,* 1995). Only 3 deletion mutations have been reported; 1) deletions of 3 bases in exon 2 "G6PD Stony Brook", 2) "G6PD Sunderland" (MacDonald *et al.,* 1991; Vulliamy *et al.,* 1993) which is a deletion 6 bases of nt724 to 729 in exon 7 affecting deletion of 242 glycine and 243 threonine (Xu *et al.,* 1995), and 3) "G6PD Nara" which is a deletion of 24 bases (Hirono *et al.,* 1993). Significantly, all deletions that have been reported occur in the multiples of 3 resulting in the triple codon deletion rather than a frameshift. Furthermore, 3' acceptor splice site mutation in *G6PD* gene has also been reported (Xu *et al.,* 1995). The deletion of the invariant dinucleotide ApG at the 3' acceptor splice site in the highly conserved sequence between intron 10 and exon 11 is known as "G6PD Varnsdoft". Its effect on the position of polypeptide chain has not been found but this type of mutation produces chronic hemolytic anemia. Interestingly, results from the absence in the *G6PD* gene of larger deletions or frameshift mutations that would completely abolish the function of the protein suggests that a complete absence of the G6PD enzyme is incompatible with life (Vulliamy *et al.,* 1992).

Distributions of *G6PD* mutations are found among the people of various ethnic groups. Normally, the type of *G6PD* mutations are restricted to specific geographical regions (Brown and Boon 1968). However, there are many single point mutations that have been recorded repeatedly in different parts of the world, which suggest that their origin is unlikely to be from a common ancestor and that they are, therefore, probably new mutations that have arisen independently (Mason *et al.,* 1995; Hirono *et al.,* 1997; Vulliamy *et al.,* 1998).

In Africa, G6PD A–$^{202A/376G}$ is a common type accounting for about 90% of G6PD deficiency. G6PD A–$^{202A/376G}$ is also found frequently in North and South America, in the West Indies, and in areas where people of African origin are present. Furthermore, G6PD A–$^{202A/376G}$ is quite prevalent in Italy (Cappellini *et al.,* 1994; Martinez di Montemuros *et al.,* 1997), the Canary Islands (Pinto *et al.,* 1996), Spain, and Portugal and in the Middle East, including Iran, Egypt, and Lebanon (Beutler *et al.,* 1989). The second most common variant is G6PD Mediterranean^{563T}. This variant is present in all countries surrounding the Mediterranean Sea. It is also widespread in the Middle East, including Israel, India, and Indonesia. In Israel it accounts for almost all G6PD deficiency in Kurdish Jews (Kurdi-Haidar *et al.,* 1990; Karimi *et al.,* 2003); (Oppenheim *et al.,* 1993). In several populations, such as the countries around the Persian Gulf, G6PD A–$^{202A/376G}$ and G6PD Mediterranean^{563T} coexist at polymorphic frequencies (Bayoumi *et al.,* 1996).

In Asia, G6PD Canton^{1376T} has been found to be the most common mutation among the Chinese in Taiwan (Tang *et al.,* 1992; Lo *et al.,* 1994), Malaysian Chinese (42.3-84%) (Ainoon *et al.,* 1999; Ainoon *et al.,* 2004), Singapore chinese (45%) (Saha *et al.,* 1994), southern Chinese (40-75%) (Chiu *et al.,* 1991; Chen *et al.,* 1998; Du *et al.,* 1999; Yang *et al.,* 2001), and Thai (10%) (Nuchprayoon *et al.,* 2002).

Other *G6PD* mutations found in Chinese include G6PD Kaiping^{1388A}, G6PD Union^{1360T}, G6PD Chinese-5^{1024T} (Tang *et al.,* 1992; Lo *et al.,* 1994; Saha *et al.,* 1994; Xu *et al.,* 1995; Nuchprayoon *et al.,* 2002). G6PD Union^{1360T} and G6PD Chinese-5^{1024T} were found in Malaysian Chinese (0.8% and 1.5%) (Ainoon *et al.,* 2004) and Thai (each was 2.6%) (Nuchprayoon *et al.,* 2002).

The distribution of G6PD variants in Southeast Asia vary with ethnic groups. G6PD Viangchan^{871A} is a dominant mutation among Thai (31-54%) (Nuchprayoon *et al.,* 2002; Laosombat *et al.,* 2005), Lao (100%) (Hsia *et al.,* 1993; Iwai *et al.,* 2001), Cambodian (82.4-97.9%) (Louicharoen and Nuchprayoon 2005; Matsuoka *et al.,* 2005), and the ethnic groups in Vietnam (Kinh, K'Ho, and Nung) (31.6%) (Matsuoka *et al.,* 2007). Moreover, G6PD Viangchan^{871A} has been also reported among Lao in Hawaii, Chinese in South China (Poon *et al.,* 1988; Xu *et al.,* 1995). G6PD Mahidol^{487A} is a common mutation in Burmese (Iwai *et al.,* 2001; Matsuoka *et al.,* 2004; Nuchprayoon *et al.,* 2008), Mon (Nuchprayoon *et al.,* 2008), Shan, Danu, Kachin, Lisu (Iwai *et al.,* 2001), Malaysian (Iwai *et al.,* 2001) and Thai (Nuchprayoon *et al.,* 2002). Summarized data of *G6PD* mutations in Southeast Asia are shown in Table 2.6.

Table 2.6 *G6PD* mutations in East and Southeast Asian.

Mutation	Nucleotide Substitution	Amino acid Substitution	WHO Class	Ethnic Group
G6PD B	-	-	-	various
Viangchan Jammu	871G→A	Val 291 Met	2	Laotian, Thai Indian
Mahidol	487G→A	Gly 163 Ser	3	Burmese, Thai, Taiwanese
Chinese-5	1024C→T	Leu 342 Phe	?	Chinese, Thai
Union	1360C→T	Arg 454 Cys	2	Chinese,Thai
Canton Taiwan-Hakka	1376G→C	Arg 459 Leu	2	South Chinese Thai,Taiwanese
Kaiping	1388G→A	Arg 463 His	2	Chinese, Thai, Taiwanese

Previously, *G6PD* mutations were defined in Thai, Cambodian, Lao, and Burmese (Table 2.7). G6PD Viangchan^{871A} was the most common in Cambodians (82.4% of 34) (Figure 2.15) (Louicharoen and Nuchprayoon 2005), Lao (46.2% of 26), and Thai (47.7% of 44) but not identified in Burmese. By contrast G6PD Mahidol^{487A} was a dominant mutation in Burmese (62.5% of 8) but rarely found in Thai (4.5% of 44) but not identified in Cambodians and Lao. G6PD Canton^{1376T} was found in one Burmese (12.5% of 8), Lao (3.8% of 26), and one Thai (2.3% of 44). G6PD Union^{1360T} was found in one case of Cambodian (2.9% of 34) and Lao (3.8% of 26). G6PD Kaiping^{1388A} was found in two Lao (7.7% of 26) and seven Thai (15.9% of 44). G6PD Chinese-5^{1024T} was found in one Thai (2.3% of 44). G6PD Coimbra^{592T} was found in one Cambodian (2.9% of 34). There are 25 G6PD deficient subjects of Burmese, Cambodian, Thai, and Lao individuals that had unidentified mutations. The G6PD activity in each variant is shown in Table 2.7.

Table 2.7 *G6PD* mutations in each ethnic groups and G6PD variants activity

Mutation	Base Change	Cases (N (%))	Ethnic group (N(%)) Burmese	Cambodian	Lao	Thai	Sex (N)	G6PD level (IU/g Hb)(SD)
Viangchan	871A	61(54.5)	0	28(82.4)*	12(46.2)	21(47.7)	M 49	0.42(0.120)
							F 12	0.51(0.592)
Mahidol	487A	7(6.3)	5(62.5)	0	0	2 (4.5)	M 6	0.62(0.454)
							F 1	1.1
Canton	1376T	6(5.4)	1(12.5)	0	4(3.8)	1(2.3)	M 5	0.56(0.449)
							F 1	0.62
Union	1360T	2(1.8)	0	1(2.9)*	1(3.8)	0	M 2	0.72(0.318)
							F 0	-
Kaiping	1388A	9(8.0)	0	0	2(7.7)	7(15.9)	M 8	0.22(0.314)
							F 1	0
Chinese-5	1024T	1(0.9)	0	0	0	1(2.3)	M 1	0.95
							F 0	-
Coimbra	592T	1(0.9)	0	1(2.9)*	0	0	M 1	0.00
							F 0	-
Unknown		25(22.3)	2(25)	4(11.7)*	7(26.9)	12(27.3)	M 20	0.43(0.454)
							F 6	0.59(0.564)
Total		112(100)	8(100)	34(100)*	26(100)	44(100)	M 91	
							F 23	

* (Louicharoen and Nuchprayoon 2005)

Figure 2.15 Distribution of *G6PD* mutations in Cambodian. (Louicharoen and Nuchprayoon 2005)**.**

There are many single nucleotide polymorphisms (SNPs) that may not affect the phenotype (D'Urso *et al.,* 1988). The restriction fragment length polymorphisms (RFLPs) have shown to be in marked linkage disequilibrium with the polymorphic mutations in G6PD variants (Tishkoff *et al.,* 2001). For SNP polymorphisms in Asia, there are two common SNPs consist of exon11 1311C (-) →T (+) and IVS11 93T (-) →C (+). Polymorphism 1311T (+) (*Bcl*I polymorphism) has been found in G6PD Viangchan^{871A} in Cambodians, Thai, Malay, and Kinh (Xu *et al.,* 1995; Hamel *et al.,* 2002; Nuchprayoon *et al.,* 2002), G6PD Mediterranean^{563T} (Kurdi-Haidar *et al.,* 1990; Filosa *et al.,* 1993), some cases in G6PD B (Panich *et al.,* 1972; De Vita *et al.,* 1989; Kurdi-Haidar *et al.,* 1990; Arambula *et al.,* 2000) and G6PD Canton^{1376T} (Xu *et al.,* 1995). Polymorphism 93C (+) (*Nla*III polymorphism) has been found in G6PD B and G6PD Viangchan^{871A} (Xu *et al.,* 1995; Arambula *et al.,* 2000; Hamel *et al.,* 2002). Haplotype 1311C, 93T (-/-) (was the most common in both G6PD Mahidol^{487A} and G6PD-normal Mon and Burmese (Nuchprayoon *et al.,* 2008). In the literature, haplotype 1311T, 93C (+/+) has never been reported in G6PD Canton^{1376T}, G6PD Union^{1360T}, and G6PD Chinese-5^{1024T}.

IVS5 611C (-) →G (+) (*Pvu*II polymorphism), was previously identified in African populations (100%) of G6PD A-$^{202A/376G}$, 20% of G6PD A^{376G} (Beutler and Kuhl 1990; Fey *et al.,* 1990; Xu *et al.,* 1995; Arambula *et al.,* 2000; Saunders *et al.,* 2002). This allele has not been found in the European, Asia and Middle Eastern populations. IVS7 175C (-) →T (+) (*Sca*I polymorphism) has been found in African populations (100% of G6PD A-$^{202A/376G}$, 20% of G6PD A^{376G} (Tishkoff *et al.,* 2001; Saunders *et al.,* 2002). IVS8 163C (+) →T (-) (*Bsp*HI polymorphism) has been found in African populations (30% of G6PD B, 10% of G6PD A^{376G}) (Tishkoff *et al.,* 2001; Saunders *et al.,* 2002). Exon10 1116G (+) →A (-)

(*Pst*I polymorphism) was found in G6PD B in Tsumkwe in Namibia (Tishkoff *et al.,* 2001; Saunders *et al.,* 2002). All SNPs haplotypes previously been defined are summarized in Table 2.8.

Six SNPs consisting of 611G (+), 175T (+), 163T (-), 1116A (-), 1311T (+), and 93C (+) were analyzed in all male G6PD deficient and normal subjects. Polymorphic haplotypes in *G6PD* mutation are summarized in Table 2.9. 611G (+), 175T (+), 163T (-), and 1116A (-) were not found in our Southeast Asian population. Only 1311T (+), and 93C (+) were found in our samples. There are 4 haplotypes detected, with haplotype 1311C, 93T (-/-) accounting for 70% of all G6PD B. Haplotype 1311T, 93C (+/+) was the most frequent in G6PD Viangchan^{871A}. G6PD Mahidol^{487A}, G6PD Canton^{1376T}, G6PD Union^{1360T}, G6PD Kaiping^{1388A}, and G6PD Chinese-5^{1024T} harbored haplotype 1311C, 93T (-/-).

Table 2.8 SNPs haplotypes of G6PD A-$^{202A/376G}$, A^{376G} and B in various ethnic (Beutler and Kuhl 1990; Fey *et al.,* 1990; Kurdi-Haidar *et al.,* 1990; Beutler *et al.,* 1991; Filosa *et al.,* 1993; Xu *et al.,* 1995; Arambula *et al.,* 2000; Hamel *et al.,* 2002; Saunders *et al.,* 2002)

Mutation	Nucleotide Substitution	Ethnic	Haplotype					
			*Pvu*II	*Sca*I	*BspH*I	*Pst*I	*Bcl*I	*Nla*III
G6PD B	-	African	-	-	+	+	-	NR
G6PD B	-	African	-	-	+	+	+	NR
G6PD B	-	African	-	-	+	-	-	NR
G6PD B	-	White, Black Amazonian	-	NR	NR	+	NR	-
G6PD B	-	Black	-	NR	NR	-	NR	NR
G6PD B	-	Mexican	-	NR	NR	+	+	-
G6PD B	-	Mexican	-	NR	NR	+	+	+
G6PD B	-	Mexican	+	NR	NR	+	-	-
G6PD B	-	Mexican	+	NR	NR	+	-	+
G6PD A	376G	African	-	-	+	+	-	NR
G6PD A	376G	African	-	-	-	+	-	NR
G6PD A	376G	African	+	+	-	+	-	NR
G6PD A	376G	Black	-	NR	NR	+	NR	NR
G6PD A	376G	Mexican	-	NR	NR	+	+	+
G6PD A	376G	Mexican	+	NR	NR	+	-	+
G6PD A-	202A/376G	African	+	+	-	+	-	NR
G6PD A-	202A/376G	Black, Mexican Puerto Rican White US, Spanish	+	NR	NR	+	NR	NR
G6PD A-	202A/376G	Canary Island	+	NR	NR	+	-	+
G6PD A-	376G/680T	Black	+	NR	NR	+	NR	NR
G6PD A-	376G/968C	Spanish, Black	+	NR	NR	+	NR	NR
G6PD A-	376G/968C	Canary Island	-	NR	NR	+	-	+
G6PD A-	376G/968C	Canary Island	-	NR	NR	+	-	-
G6PD Viangchan	871A	Laotian,Chinese	-	NR	NR	+	+	+
G6PD Jammu	871A	Indian	NR	NR	NR	NR	-	NR
G6PD Ananindeua	871A/376G	Amazonian	-	NR	NR	+	-	+
G6PD Canton	1376T	South Chinese	-	NR	NR	+	-	-
G6PD Med	563T	Mediterranean	NR	NR	NR	NR	+	NR
G6PD Med	563T	Mediterranean	NR	NR	NR	NR	-	NR

NR represents no report

Table 2.9 Haplotypes of the *G6PD* mutations in Southeast Asian population.

Haplotype						*G6PD* mutation (N (%))							
611G	**175T**	**163T**	**1116G**	**1311T**	**93C**	**G6PD B**	**871A**	**487A**	**1376T**	**1360T**	**1388A**	**1024T**	**UK***
-	-	+	+	-	-	141 (69.8)	0	6	5	2	8	1	9 (47.4)
-	-	+	+	-	+	13 (6.4)	0	0	0	0	0	0	1 (5.3)
-	-	+	+	+	-	5 (2.5)	3 (6.1)	0	0	0	0	0	0
-	-	+	+	+	+	43 (21.3)	46 (93.9)	0	0	0	0	0	9 (47.4)

* UK represents unknown mutation

Since there are mutations that lead to enzyme deficiency, several polymorphic sites in introns have been identified, which is enable the definition of G6PD haplotypes (Vulliamy *et al.,* 1991; Maestrini *et al.,* 1992). These haplotypes have been used to understand the evolutionary history of the *G6PD* gene. By looking at linkage disequilibrium in haplotypes themselves and with coding sequence polymorphisms, dating of the most common mutations and estimation of the timeframe of malaria selection has been possible (Luzzatto 2006).

4. G6PD Deficiency and Malaria Hypothesis: Evolutionary Selection by Malaria

During the course of human evolution in regions where malaria was prevalent, naturally occurring genetic defense mechanisms have evolved for resisting infection by *Plasmodium.* Most of the human genes that are thought to provide reduced risk from malarial infection are expressed in red blood cells (Brown and Boon 1968); erythrocyte cell surface oligoproteins (blood groups), globin (HbS, HbC, HbE, thalassemias), enzymes in oxidative stress (G6PD deficiency).

The following evidence supporting the hypothesis that the G6PD deficiency was selected by malaria can be summarized as follows.

4.1 Epidemiological Evidence

Several hypotheses have been proposed to explain why G6PD deficiency has been selected in different populations (Sodeinde 1992). The geographical correlation between the distribution of these polymorphic deficiency variants and historical endemicity areas of *P. falciparum* malaria suggests that G6PD deficiency has frequently risen through positive selection by malaria (Allison 1960). The geographical distribution of G6PD deficiency cannot only be attributable to gene flow. Indeed, the presence of diverse G6PD variants arisen independently and reaching polymorphic frequencies in geographically disparate areas supports the occurrence of natural selection of this condition. This hypothesis is further supported by mapping studies within relatively restricted geographical areas such as Kenya (Allison 1960; Stamatoyannopoulos *et al.,* 1966), Greece (Stamatoyannopoulos *et al.,* 1966) and Sardinia (Siniscalco *et al.,* 1966). These results demonstrated that a similarly remarkable geographical correlation between altitude and the distribution of G6PD deficiency with the lower altitude (<1000 m) areas, known to have more intense malaria transmission, clearly associated with higher frequencies for G6PD deficiency.

4.2 *In Vitro* - *In Vivo* Study Evidence

Previous studies have shown that *P. falciparum* parasite preferentially invade and develop in younger red-blood cells that have relatively higher G6PD activity than deficient older erythrocytes (Kruatrachue *et al.,* 1962; Luzzatto *et al.,* 1969). This evidence has led to a hypothesis that G6PD deficient erythrocytes confer protection against malaria by inhibiting erythrocyte invasion or intracellular development of the malaria parasite. Since then, there have been several independent studies reporting impaired growth of *P. falciparum* in G6PD deficient erythrocytes

(Roth *et al.*, 1983; Miller *et al.*, 1984). Intracellular parasite schizogenesis, rather than invasion of RBC, is affected by G6PD deficient RBCs (Miller *et al.*, 1984), where is oxidative injury of the parasite can happen. Furthermore, heterozygous females have different proportions of normal and deficient cells because of the mosaic of X-chromosome inactivation, the degree of parasite growth inhibition is proportional to the percentage of presented deficient cells (Roth *et al.*, 1983). It has been shown that RBCs taken from *G6PD* A-$^{202A/376G}$ heterozygous females, have 2–80 times more parasitic growth than G6PD deficient RBCs (Luzzatto *et al.*, 1969). Although there is growth inhibition in G6PD deficient erythrocytes, it is now clear that after a few growth cycles the parasite can overcome the inhibition (Roth and Schulman 1988), and it was suggested that the parasite achieved this by producing its own G6PD enzyme (Hempelmann and Wilson 1981; Usanga and Luzzatto 1985). An ingenious mechanism based on the premise that expression of parasite G6PD enzyme is determined by *G6PD* genotype of the host erythrocyte was then put forward (Usanga and Luzzatto 1985) as a possible mechanism to account for the results of a previous study that had indicated that G6PD deficiency protection against malaria was the sole prerogative of female heterozygotes (Bienzle *et al.*, 1972). Hence in uniformly deficient RBCs such as those found in deficient hemizygous males or deficient homozygous females the parasite's own G6PD enzyme would compensate for the lack of the host's enzyme. However, in female heterozygotes, who necessarily have mixed populations of deficient and non-deficient erythrocytes, parasite adaptation would be compromised, and thus the parasite growth and multiplication could be impaired by repeatedly switch on and off its own enzyme as it moved from deficient to non-deficient host red blood cell. While confirming the phenomenon of adaptation, subsequent studies have found that the parasite G6PD levels do not appear to be

affected by the host red cell genotype (Ling and Wilson 1988; Roth and Schulman 1988; Kurdi-Haidar and Luzzatto 1990). Although parasites may have adapted to produce their own G6PD to survive in the G6PD deficient host cells, the host immune system can screening by differentiate G6PD deficient and normal parasite infected cells. Indeed G6PD deficient red cells infected with parasites undergo phagocytosis by macrophages at an earlier stage of parasite maturation than do normal red cells with parasitic infection. This could be a further protective mechanism against malaria (Cappadoro *et al.,* 1998).

Finally, the available literature on G6PD deficiency and malaria show extremely conflicting evidence regarding the protective role of G6PD deficiency against malaria infection (Allison and Clyde 1961; Kruatrachue *et al.,* 1962; Powell and Brewer 1965; Gilles *et al.,* 1967; Luzzatto *et al.,* 1969; Bienzle *et al.,* 1972; Martin *et al.,* 1979; Kar *et al.,* 1992), as summarized in Table 2.10.

Table 2.10 Summary of studies on G6PD deficiency and malaria (Ruwende and Hill 1998).

Study indicating protective role of G6PD deficiency				
Population	**Evidence**	**N**	**Year**	**Reference**
East African	Lower parasite rates and densities in deficient male children Reduced levels similar to sickle trait children both indices	532	1961	Allison and Clyde 1961
Nigeria	Reduced frequency of deficient males and females in cases with very high parasite counts (>100,000@mm3) compared to controls	100	1967	Gilles, Fletcher et al. 1967
Nigeria	Greater rates of parasitisation of non-deficient erythrocytes in mixed erythrocytes of female heterozygotes with acute malaria	-	1969	Luzzatto, Usanga et al. 1969
Nigeria	Reduced frequency of female heterozygotes only in children Reduced parasite densities in same group	702	1972	Bienzle, Ayeni et al. 1972
Vietnamese	Lower incidence of malaria in non-immune deficient adult African-American males compared to their non-deficiencies	277	1973	
Indian	Significantly reduced parasitisation rates in deficient India males and females	708	1992	Kar, Seth et al. 1992
Study supporting no protective role of G6PD deficiency				
Population	**Evidence**	**N**	**Year**	**Reference**
Thai	Increased malaria in deficient males (1–3 years old) compared to non-deficient males in same age group	203	1962	Kruatrachue, Charoenlarp et al. 1962
American	No difference in parasitisation and parasitaemia levels in infected deficient and non-deficient non-immune adult African-American male prison inmates	16	1965	Powell and Brewer 1965
Nigeria	No protection against cerebral malaria for any of the deficiency genotypes	68	1979	Martin, Miller et al. 1979

It appears that the protective role of G6PD deficiency in malaria is difficult to verify. There are several reasons that could explain this (Ruwende and Hill 1998). Moreover, there is considerable genetic heterogeneity associated with G6PD deficiency, and in some populations more than one deficiency variant is present.

Moreover, it is known that fitness of the deficiency phenotype is decreased significantly only under a limited number of specific circumstances and therefore on average very little. Lastly, G6PD deficiency may interact with other genetic factors and specific non-malaria environmental factors; e.g. diet, to modify the net fitness of the carrier (Martin 1994). Several clinical studies have attempted to define various genotypes from phenotypic measurements of enzyme levels, electrophoretic mobility and cytochemical staining patterns (Gilles *et al.,* 1967; Bienzle *et al.,* 1972; Martin *et al.,* 1979). This is difficult because overlapping levels of enzyme activity are seen among genotypes, related partly to variable inactivation of X chromosomes in female heterozygotes and partly to altered rates of erythrocyte turnover in acute malaria. In 1995, there are the studies on G6PD deficiency and malaria carried out in East and West Africa, which are malaria endemic regions. G6PD A–$^{202A/376G}$, G6PD A^{376G} and G6PD B genotype frequencies were measured in over 2000 DNA samples collected from children under 10 years (Ruwende *et al.,* 1995). The results demonstrated female heterozygotes were significantly protected against both severe malaria (46%) and mild malaria (41%), whereas male hemizygotes were only significantly protected against severe malaria (58%). These data strongly suggest that the G6PD A–$^{202A/376G}$ allele is associated with substantial resistance to severe malaria in hemizygous males as well as in heterozygous females. The results also suggest that the degree of enzyme deficiency is a key to the protective mechanism of G6PD deficiency against malaria. However, there are several mechanisms which might explain this phenomenon at the molecular level as summarized in Figure 2.16.

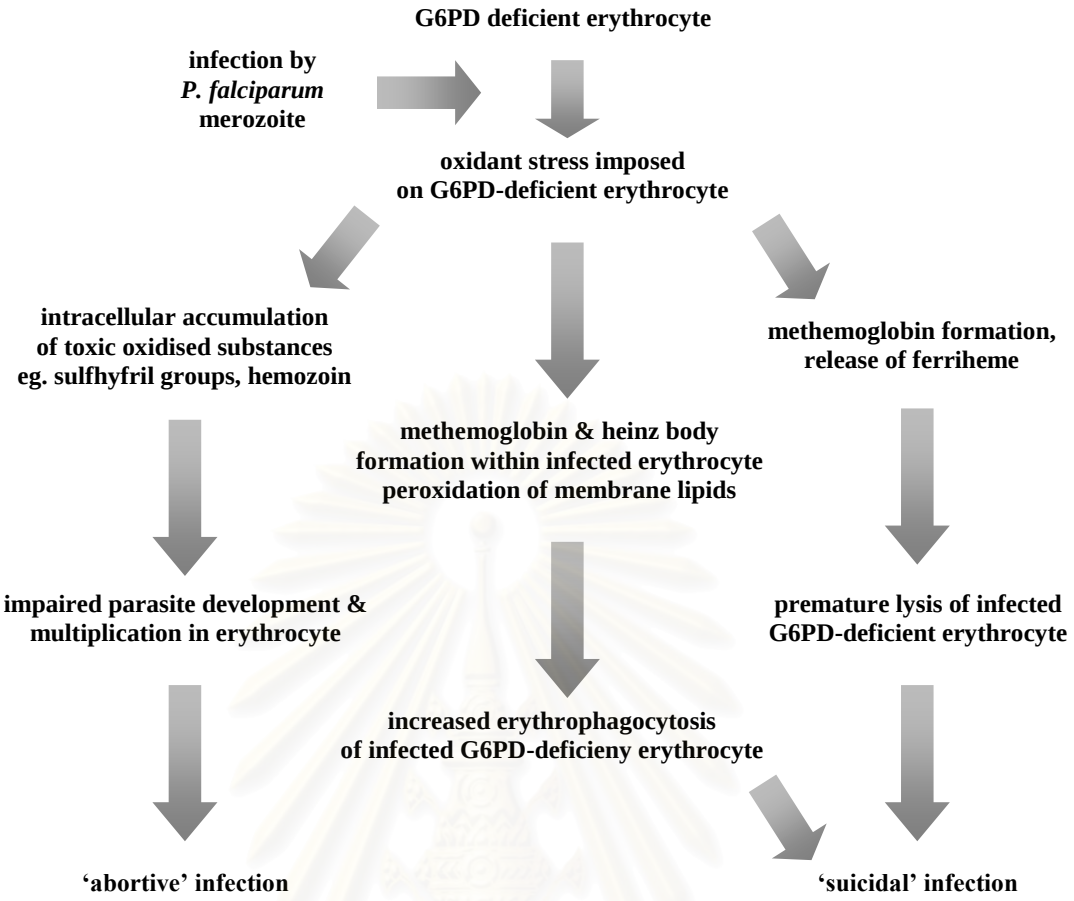


Figure 2.16 Possible protective mechanisms of G6PD deficiency against severe malaria.

Reduced parasite multiplication in deficient erythrocytes is probably caused by an accumulation of intracellular toxic oxidized substances (Miller *et al.*, 1984; Golenser *et al.*, 1988; Turrini *et al.*, 1993). Moreover, there is supporting evidence where infected deficient erythrocytes are prone to hemolyse through increased methemoglobin and release of ferriheme (Janney *et al.*, 1986). Furthermore, after methemoglobin producing heinz body formation and membrane damage in infected erythrocyte, result in increased phagocytosis by the reticuloendothelial system (Turrini *et al.*, 1993). Since the estimated degree of malaria resistance afforded to female heterozygotes is little different from that of male hemizygotes, the overall fitness of female heterozygotes is likely to be greater than that of hemizygotes

and homozygotes. This situation makes up a balanced polymorphism (Motulsky 1960).

4.3 Evolutionary Study

From evolutionary selection of *G6PD* allele by malaria, there are 2 major points. First, the selective pressure is very strong; G6PD Mahidol^{487A} allele has risen to high frequencies in malaria-endemic Southeast Asia. Second, the evolutionary responses to malaria occur at both a global and a local level; different populations have evolved different genetic variants to protect against malaria (Kwiatkowski 2005). The estimation of age and time of origin of *G6PD* mutations, useful for deriving explanations on the evolutionary history of malaria selection of G6PD deficiency, can be inferred by analysis of the strong linkage disequilibrium of genetic variant with its neighboring genetic markers (Miller 1994; Tishkoff *et al.*, 2001; Sabeti *et al.*, 2002; Saunders *et al.*, 2002; Verrelli *et al.*, 2002; Kwiatkowski 2005; Saunders *et al.*, 2005)

In 2001, haplotype analysis and statistical modeling of an African malaria-resistance allele based on intra-allelic microsatellite variability on *G6PD* locus estimated a force of selection for G6PD A-$^{202A/376G}$ as 0.044, with an estimated age 3,840-11,760 years (Tishkoff *et al.*, 2001). In 2002, the strong LD and high extended haplotype homozygosity (EHH) of G6PD A-$^{202A/376G}$ allele in West Africa are consistent with recent positive selection, suggesting an origin of G6PD A-$^{202A/376G}$ at around 10,000 years ago (Sabeti *et al.*, 2002; Saunders *et al.*, 2002). In 2005, intra-biallelic variability and extended haplotype analyses among G6PD A-$^{202A/376G}$ suggests that the age of G6PD A-$^{202A/376G}$ allele is ~2,500-3,750 years (~100 generations, assuming a 25 year generation time) with given a selection coefficient ~0.1 (Saunders *et al.*, 2005).

These observations coincide with the estimations of recent expansions apparent in African populations of *P. falciparum* around 4,000-10,000 years ago, that resulted in a cascade of changes in human behavior and mosquito transmission (Joy *et al.*, 2003). However, the selection of G6PD deficiency may be influenced by *P. vivax.* In north Holland, *P. vivax* is the most likely malaria parasite to have had a selective impact on G6PD deficiency and is consistent with the presence of elevated frequencies of G6PD deficiency for at least 500 years (Cavalli-Sforza 1994; Carter and Mendis 2002). In Southeast Asia, over half of malarial cases were caused by *P. vivax.* Moreover, the investigation of phylogenetic relationships of 10 species of primate *Plasmodium* found that *P. vivax* was derived from a species that inhabited macaques in Southeast Asia, and where origin estimated at between 45,680-81,607 years ago, supporting an ancient origin of *P. vivax* in Asia (Escalante *et al.*, 2005). Although *P. vivax* imposes a burden of disease on the human population and historically threaten the productivity of human populations, most epidemiological and evolutionary genetics studies of malaria relating with G6PD deficiency have, however, been focused on *P. falciparum.*

CHAPTER III

MATERIALS AND METHODS

1. Samples Preparation

1.1 Population Samples

1.1.1 Karen

1.1.1.1 Study Location

Tanaosri subdistrict, Suan Phung district, Ratchaburi province, is located near Thai-Myanmar border surrounded by Tanaosri Mountain on the western side and was selected as a study site. There are 5,368 residents and the majority ethnic group is Karen. The subdistrict is located at the southern part of Suan Phung and is an endemic area of malaria. The incidence of malaria is moderately seasonal, and the peak of malaria transmission occurs during April to June. Most of the people have been infected by either *P. falciparum* or *P. vivax.*

The Rajanagarindra Tropical Diseases International Center (RTIC) (Figure 3.1) situated near Tanaosri subdistrict is managed by the Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University. The RTIC provides free diagnostic services for malaria infection and free medications for malaria prevention and treatment. The RTIC has more than 10,000 patients per year coming from the villages in the Tanaosri subdistrict and other neighboring areas in Suan Phung district.

1.1.1.2 Family Data

The studied site comprises 3,481 villagers of all ages living in 7 hamlets in a total of 482 households. The objectives of project were explained to the villagers and informed consent was individually obtained from all study participants or their parents or guardians. The ethical permission for the study was approved by the Ethical Committee of the Ministry of Public Health of Thailand. In construction of familial pedigree, 2,545 participants were recruited using a questionnaire interviewing each individual to generate both demographic information such as birth date, age, sex, and genetic relationships between children, their parents, and their grandparents or non-relatives in the same household, and between households. All pedigrees comprised of 2,545 individuals including absent or dead relatives forming 238 families, which consists of 3 to 959 inhabitants per family. Family structures were constructed by interviewing and verified by Identity by State (IBS) sharing of each relative pair from genotyping results of 400 microsatellite markers used during genome screening using IBS_check program (Heath, unpublished). There was <5 % discordance including labelling errors. The family pedigrees were drawn using HAPLOPAINTER pedigree drawing software (Thiele and Nurnberg 2005). Multiple marriages are common in every hamlet resulting in a large number of half siblings. The family structures were confirmed by the PEDCHECK software (O'Connell and Weeks 1998).

All two thousand five hundred forty five samples were performed for epidemiological study. In evolutionary study, *G6PD* mutations were defined in nine hundred twenty two samples. Three hundred eighty four unrelated individuals were next typed for 28 SNPs located on chromosome X for defining the long range haplotype.

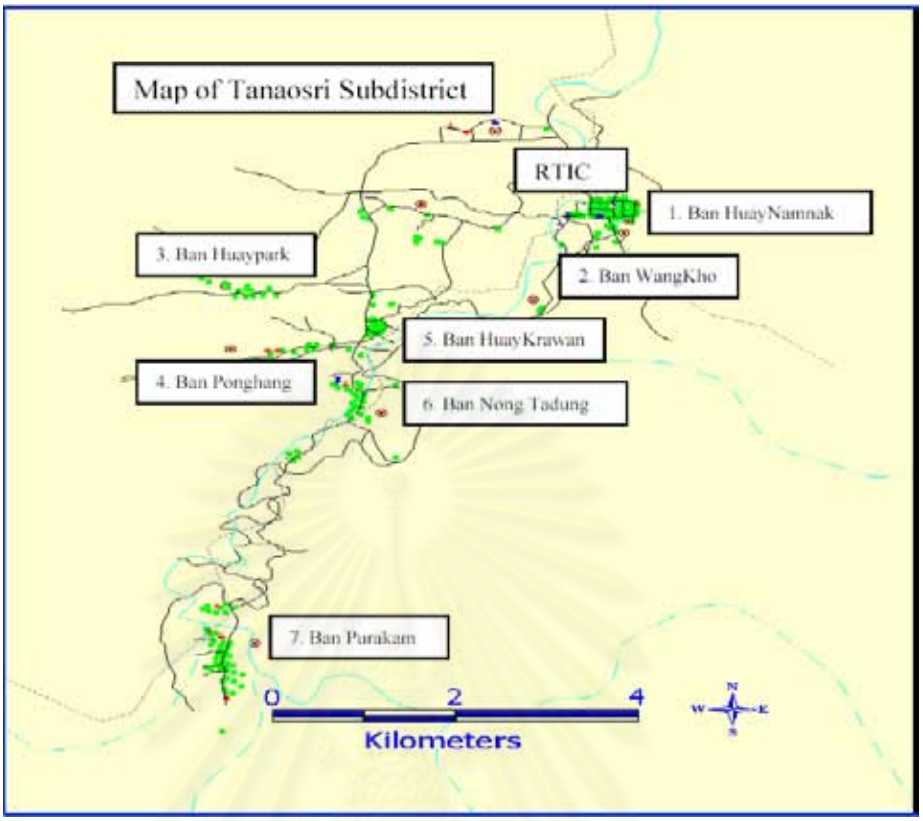


Figure 3.1 Map of studied 7 hamlets location in Tanaosri subdistrict.

Both DNA samples and genotypic data from Thai, Cambodian, Lao, and Burmese (year 2002-2003) were brought from my previous study. For the identification of Thai, Burmese, Cambodian, Laotian, and Mon ethnic groups, the volunteers were questionnaire interviewed (Appendix). Informed consent was taken from all participant individuals or their parents or guardians. The ethical permission was approved by The Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

1.1.2 Thai

One hundred and seven Thai DNA samples, consisting of 92 normal controls and 15 G6PD deficient subjects, were all newborns admitted to the King Chulalongkorn Memorial Hospital, Bangkok, Thailand during June 2001 to March 2002 or the Buriram Hospital, Buriram province, Thailand during April to May 2003.

1.1.3 Burmese

Sixty four DNA samples, consisting of 38 normal and 26 G6PD deficient subjects were immigrant workers, who lived in Chanthaburi province during March 2002 or who were admitted to Samutsakhon Hospital during February to June 2003. In 2004-2005, one hundred seventy eight samples had been collected from immigrant worker males, who had a physical examination in the work permit program at Samutsakhon Hospital.

1.1.4 Cambodian

Seventy three DNA samples, consisting of 44 normal controls and 29 G6PD deficient subjects were newborn subjects, who had been admitted to Buriram Hospital during April to May 2003 or who were immigrant workers living in Chanthaburi province during March 2002.

1.1.5 Lao

Fifty eight DNA samples, consisting of 37 normal controls and 21 G6PD deficient subjects were newborn subjects admitted to Buriram Hospital during April to May 2003 or who were immigrant workers living in Chanthaburi province during March 2002.

1.1.6 Mon

One hundred sixty two samples were collected from immigrant worker males, who had a physical examination in the work permit program at Samutsakhon Hospital in 2004-2005.

1.2 Specimen Collection

1.2.1 Karen Specimens

RTIC collected blood samples from participants, who were five years old or elder. Samples from subjects less than 5 years old were collected by buccal swab.

1.2.2 Thai, Burmese, Cambodian, Lao, and Mon Specimens

Three milliliters (ml) of blood was collected in 5 ml of Vacutainer™ tube containing 1.25 ml of acid – citrate – dextrose (ACD) for G6PD activity assay using the standardized method. In addition, 2.7 ml of blood was collected in 3 ml of Vacutainer™ tube containing 0.3 ml of ethylene diaminetetraacetic acid (EDTA) for DNA extraction. Samples were stored at 4°C.

2. Phenotypic Study

2.1 G6PD Deficiency Study

2.1.1 G6PD Deficiency Screening Test

Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand screened G6PD deficiency in 846 Karen samples using fluorescence spot test (FST), as recommended by International Council for Standardization in Hematology (ICSH) (Beutler *et al.*, 1979). The principle is the measurement of the fluorescence intensity after G6PD metabolizes its substrate, which is tagged with fluorescence. Ten microliters (μl) of blood, is incubated with 200 μl of the reagent mixture and spotted to the filter paper. Fluorescence intensity was measured at the 0, 5, 10, and 20 minutes after incubation of blood with reagent mixture. There are 4 stages of G6PD activity, which depend on fluorescence

intensity; deficiency (no fluorescence), partial deficiency (light fluorescence), normal/ partial deficiency (moderate fluorescence), and normal (high fluorescence).

2.1.2 Standardized Method for G6PD Assay of Hemolysates

Mon, Burmese, Thai, Cambodian, and Lao samples were measured for G6PD activity using standardized method for G6PD assay of hemolysates. The G6PD activity method was modified from technical report series of World Health Organization (WHO) (Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group 1967). Whole blood was centrifuged at 3,000 rpm for 1 minute, and then plasma and buffy coat were removed. Red blood cells (RBCs) were washed in 0.9% saline (Chulalongkorn Memorial Hospital), then centrifuged at 3,000 rpm for 1 minute, and the suspended saline removed. This was repeated three times to remove the residual buffy coat, which interferes with G6PD activity measurement of RBC. The hematocrit and hemoglobin concentration of washed RBCs was measured using an automated complete blood count (CBC) (Technicon H*3, Bayer, New York, USA). Fifty µl of washed RBCs was mixed well with 950 µl of distilled water (Chulalongkorn Memorial Hospital) in 5 ml tube and then frozen at -20^{o}C for 10 minutes for hemolysis. Next, they were centrifuged at 8,000 rpm for 20 minutes to precipitate the membrane proteins. Then, 50 µl of the supernatant (hemolysate) was mixed well with 850 µl of nucleotide nicotine-adenine dinucleotide (TPN) in buffer (2 mM NADP, 1 M Tris-HCl pH 8.0, 1 M $MgCl_2$) (SIGMA) in 2.5 ml of cuvette. The mixture was incubated at room temperature for 5 minutes. Then, 100 µl of 6 mM G6P (SIGMA) was added in the mixture to start the reaction of G6PD. The reaction was followed by spectrophotometer for 5 minutes; the optical density (International Unit) was measured with a 1-cm light path at 340 nm and at 22^{o}C. One unit of G6PD is the

quantity of enzyme that reduces 1 μmol of NADP per minute. One μmol/ml of reduced NADP has an absorbance of 6.22 in a light path of one centimeter. The formula of enzymatic activity is:

$$\text{Activity (IU)/g Hb} = \frac{\text{O.D/ min}}{\text{Hb gm\%}} \times 6.44 \times 10^3 \times 1.66 \qquad \text{-(equation 3.1)}$$

Where 1.66 : Temperature factor 22^{o}C

The ranges of G6PD activity were showed; normal male 7.39 ± 2.57 I.U/g Hb, normal female 6.94 ± 2.51 I.U/g Hb, G-6-PD deficiency < 1.5 I.U/g Hb.

2.2 Epidemiological Study

The epidemiology of malaria in this site has been described elsewhere (Phimpraphi *et al.,* 2008). The installation of a health clinic enabled passive case detection of malaria episodes and a record of non-malaria presentation. Clinical malaria episodes were defined as measured fever (temperature > 37.5 ºC) or fever-related symptoms including headache, back pain, chills, myalgia, nausea and vomiting associated with a slide positive for blood-stage trophozoite *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* parasites at any density. To determine the correct number of clinical episodes, consecutive presentation at the clinic with blood-stage malaria parasite of the same species within 30 days after treatment of the initial infection was considered as the same episode. Consecutive presentation with non-malaria fever (or aforementioned symptoms) within 7 days following first presentation was likewise considered as the same episode. In addition, initial parasite negative visits were considered as part of a malaria episode if followed by a parasite-positive visit within the subsequent two days. All positive malaria cases were treated with appropriate antimalarial treatment according to the recommendation of the Malaria Division,

Ministry of Public Health, as previously described (Phimpraphi *et al.*, 2008). Self-treatment is considered to be rare in the study area, because the only other access to treatment is a government clinic with which the study has good communication concerning malaria treatment of the study site population. In all cases parasite positivity was established as follows. Thick and thin blood films were prepared and stained by 3% Giemsa stain. Blood films were examined under an oil immersion objective at x1000 magnification by the trained laboratory technicians and 200 thick film fields were examined before films were declared negative. Parasite species were identified on thin films and densities (per µl) were calculated from thick film by establishing the ratio of parasites to white blood cells (WBC) after at least 200-500 WBCs had been counted and then multiplying the parasite count by 8,000, the average WBC count per µl of blood.

Two categories of malaria phenotypes were considered in two thousand five hundred forty five Karen individuals.

A. Clinical phenotypes: the number of clinical visits that were positive for *P. falciparum* or *P. vivax* were studied.

B. Parasite biological phenotypes: For each species, the maximum trophozoite density experienced by any individual were studied.

3. Genotypic Study

3.1 DNA Extraction

3.1.1 Phenol-Chloroform DNA Extraction Method

Laboratoire de la Génétique de la réponse aux infections chez l'homme, Institut Pasteur extracted DNA from three milliliters of EDTA bloods of

922 Karen samples or from buccal swabs and heparinized bloods in the capillary tubes of those whom I could not take venous blood using Phenol-Chloroform. All samples were frozen at -80 °C until extract. The Phenol-Chloroform DNA extraction process consists of 4 steps.

3.1.1.1. Red Cells Lysis

Four millilitres of lysis buffer (0.32M sucrose, 10mM Tris-HCl pH 7.5, 5mM MgCl2, 1% Triton X-100) were added to 1 ml of whole blood. Then, RBCs were lysed on ice for 5 min. Next, lysed RBCs tubes were centrifuged for 10 min, 1000g at 4°C. After that, the supernatant was discarded and the pellet was washed with 5 ml lysis buffer. Next, tubes were centrifuged for 10 min, 1000g at 4°C. If the supernatant was still dark, the sample was washed with lysis buffer again. Finally, all supernatant was removed and the pellet kept.

3.1.1.2. Proteinase K Digestion

The pellet was resuspended well with 1 ml of SE buffer (0.075M NaCl, 0.024M EDTA pH8.0), and 4 ml of Proteinase K digestion buffer (27% sucrose, 1XSSC, 1mM EDTA, 1%SDS) and 150 µl Proteinase K 20mg/ml were added to the pellet. Next, tubes were incubated at 50°C overnight.

3.1.1.3. Phenol-Chloroform Protein Extraction

One volume (5 ml) of phenol-chloroform-isoamyl alcohol (25:24:1) was added to tubes containing PK digestion solution. Then, the sample was incubated for 30 min to 2hrs at room temperature on a tube rotator (slow shaking). Next, tubes were centrifuged for 10 min, 2500 rpm. After that, the upper aqueous phase was transferred to a new tube without disturbing the interphase, which contains proteins. This was repeated by adding the same volume of phenol-chloroform-isoamyl alcohol and incubated 30 min. to 2hrs at room temperature on a tube rotator. Next, the sample

tube was centrifuged for 10 min, 2500 rpm. Finally, the upper aqueous phase was transferred to a new tube.

3.1.1.4. DNA Precipitation

The transferred upper aqueous phase was added to the same volume of isopropanol (2-propanol) and mixed gently by inverting the tube. At this step, the DNA precipitant should be visible like white threads. Then, tube was centrifuged for 10 min, 4000 rpm. For tubes where no DNA precipitate was visible, tube was placed for DNA precipitated for 30 min at room temperature, and then centrifuged for 30 min, 4000 rpm. Then, the supernatant was removed carefully. Next, the DNA pellet was washed with 5 ml. of 70% ethanol and mixed to resuspend the pellet. After that, tube was centrifuged for 10 min, 4000 rpm. All supernatant was removed, and the tube air dried at room temperature to evaporate ethanol traces. Finally, the DNA was resuspended in 200 µl 1x TE -4 (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH8.0) or less if the DNA precipitate was not visible.

3.1.2 Commercial DNA Extraction kit

Genomic DNA was extracted from blood samples of Thai, Burmese, Mon, Cambodian, and Lao using QIAamp® DNA blood mini kit (QIAGEN, Germany). The method was modified from Mini kit handbook. Two hundred µl blood sample was mixed well with 20 µl of protease, 200 µl of AL buffer, 200 µl of 100% ethanol in 1.5 ml microtube. The mixture was incubated at 56^{o}C for 10 min. and placed into the spin column (2 ml collection tube) and centrifuged at 8,000 rpm for 1.5 min. Then the spin column was placed in new clean 2 ml collection tube and the tube containing the filtrate discarded. Next, 500 µl of AW1 buffer was added and the column centrifuged at 8,000 rpm for 1.5 min. After that 500 µl of AW2 buffer was added and centrifuged at 12,000 rpm for 5 min. Then the spin column was moved into

1.5 ml microtube. Two hundreds of AE buffer was added into the spin column, incubated at room temperature for 5 min, and centrifuged at 8,000 rpm for 1.5 min.

3.2 DNA Quantification

Picogreen is a fluorescence stain used to label double-stranded DNA in solution. The Molecular Probes Picogreen dsDNA Quantitation reagent is an ultrasensitive fluorescence stain used for the quantitation of double-stranded DNA in solution. Picogreen reagent stocked in DMSO (Invitrogen, Molecular Probes Cat. No. P-7581) was diluted 1:200 in 1X TE-3 buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) in a plastic tube protected from light. This diluted reagent can be stored several weeks at 4°C protected from light. For the 96-well plate [Maxisorp polystyrene clear plates 96 wells (flat-bottom) Nunc439454], 50 μl of diluted reagent is needed per well. Then, Lambda standard (100 μg/ml in TE buffer) was diluted 1:50 in 1X TE-3 buffer. For standard curve, 2 μg/ml Lambda DNA stock in 1X TE-3 buffer, and 6 further 1:2 dilutions of this stock (1,000 ng/ml, 500 ng/ml, and 250 ng/ml. etc.) were prepared. The final quantity in each well (50 μl) was be 100, 50, 25, 12.5, 6.25, 3.12, 1.56 ng. TE-3 buffer was used as a blank. In quantification of DNA, 50 μl of TE-3 buffer, DNA standard dilutions, control DNA or samples were pipetted into the well of a 96-well plate. DNA samples were diluted 1:50 in 1X TE-3 buffer (1μl of sample and 49 μl of TE-3 buffer). Then, 50 μl of diluted Picogreen reagent was added. The solution was mixed well at room temperature for 2-5 minutes protected from light. Next, the DNA sample in each well was measured using MFX Dynex fluorometer.

3.3 Adjustment of DNA Concentration

DNA concentration in each sample was adjusted to be 10 ng/μl and then diluted to be 1 ng/μl for Genotyping test. TE-4 buffer was used to dilute DNA.

3.4 Identification of *G6PD* Mutations and rs Number of SNPs on Chromosome X

3.4.1 Type of *G6PD* Mutations

Nine hundred twenty two Karen subjects were first genotyped for the four most common *G6PD* mutations and one SNP reported in Southeast Asia- G6PD Mahidol^{487A}, G6PD Viangchan^{871A}, G6PD Canton^{1376T}, and silent mutation^{1311T} (rs2230037). Genotyping used TaqMan SNP genotyping assay (ABI Prism-7000 Sequence Detection System), and polymorphism 93C (+) using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Unidentified mutations in G6PD deficient and partial deficient Karen were sequenced for all coding region.

Thai, Cambodian, Mon, Burmese, and Lao deficient subjects revealed 10 *G6PD* mutations and one SNP- G6PD Mahidol^{487A}, G6PD Viangchan^{871A}, G6PD Canton^{1376T}, 1311T (+) (rs2230037), G6PD Union^{1360T}, G6PD Kaiping^{1388A}, G6PD Chinese-5^{1024T}, G6PD Coimbra^{592T}, G6PD Gaohe^{95G}, G6PD Chinese-4^{392T}, and 93C (+). All unidentified mutation samples were sequenced in all coding regions.

3.4.1.1 G6PD Viangchan^{871A}

For PCR-RFLP approach, G6PD Viangchan^{871A} creates an *Xba*I restriction site (TCTAGG→T^CTAGA) (Fermentas) (Table 3.3). Exon 9 was amplified using a pair of primer 871F and 9R, which have been previously described (Huang *et al.,* 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2.

After digestion, the normal and mutant fragments are 126 bp and 106+20 bp, respectively (Table 3.2). TaqMan® SNP genotyping assay is described in topic 3.4.4 and table 3.5.

3.4.1.2 G6PD Mahidol^{487A}

For PCR-RFLP approach, G6PD Mahidol^{487A} creates a *Hind*III restriction site (AGGCTT→A^AGCTT) (Biolabs) (Table 3.3). Exon 6 was amplified using a pair of primer 487F and 487R, which have been previously described (Huang *et al.*, 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments are 104 bp and 82+22 bp, respectively (Table 3.2). TaqMan® SNP genotyping assay is described in topic 3.4.4 and table 3.4.

3.4.1.3 G6PD Canton^{1376T} and G6PD Union^{1360T}

For PCR-RFLP approach, G6PD Canton^{1376T} creates an *Afl*III restriction site (CGTAAG→C^TTAAG) and G6PD Union^{1360T} removed a *Hha*I restriction site (GCG^C→GTGC) (Biolabs) (Table 3.3). Exon 11, 12 were amplified using a pair of primer 1360F and 1360R, which have been previously described (Huang *et al.*, 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments of G6PD Canton^{1376T} are 214 bp and 194+20 bp, respectively (Table 3.2). While, G6PD Union^{1360T} normal and mutant fragments are 142+45+27 bp and 187+27 bp, respectively. TaqMan® SNP genotyping assay of G6PD Canton^{1376T} is described in topic 3.4.4 and table 3.4.

3.4.1.4 G6PD Kaiping^{1388A}

For PCR-RFLP approach, G6PD Kaiping^{1388A} creates a *Nde*I restriction site (CGTATG→CA^TATG) (Biolabs) (Table 3.3). Exon 12 was amplified using a pair of primer 1360F and 1388R, which have been previously described

(Huang *et al.,* 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments of G6PD Kaiping^{1388A} are 227 bp and 206+21 bp, respectively (Table 3.2).

3.4.1.5 G6PD Chinese-5^{1024T}

For PCR-RFLP approach, G6PD Chinese-5^{1024T} mutation creates a *Mbo*II restriction site (Biolabs) (Table 3.3). Exon 9 was amplified using a pair of primer 1024F and 1024R, which have been previously described (Huang et al. 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments of G6PD Chinese-5^{1024T} are 187 bp and 150+37 bp, respectively (Table 3.2).

3.4.1.6 G6PD Coimbra^{592T}

For PCR-RFLP approach, G6PD Coimbra^{592T} creates a *Pst*I restriction site (CCGCAG→CTGCA^G) (Biolabs) (Table 3.3). Exon 6 was amplified using a pair of primer 592F and 592R, which have been previously described (Huang et al. 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments of G6PD Coimbra^{592T} are 157+83 bp and 157+63+20 bp, respectively (Table 3.2).

3.4.1.7 G6PD Gaohe^{95G}

For PCR-RFLP approach, G6PD Gaohe^{95G} creates a *Mlu*I restriction site (ACACGT→A^CGCGT) (Biolabs) (Table 3.3). Exon 2 was amplified using a pair of primer 95F and 95R, which have been previously described (Huang et al. 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments of G6PD Gaohe^{95G} are 198 bp and 174+24 bp, respectively (Table 3.2).

3.4.1.8 G6PD Chinese-4^{392T}

For PCR-RFLP approach, G6PD Chinese-4^{392T} removes a *BstE*I restriction site (G^GTNACC→TGTNACC) (Biolabs) (Table 3.3). Exon 5 was amplified using a pair of primer 392F and 392R, which have been previously described (Huang et al. 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments of G6PD Chinese-4^{392T} are 188+15 bp and 203 bp, respectively (Table 3.2).

3.4.1.9 Unknown Mutation

G6PD deficient and partial deficient individuals showing an absence of those mutations were directly sequenced in all coding regions by modified primer sets from previous described (Nuchprayoon *et al.,* 2008) (Table 3.1). The conditions for sequencing are described in 3.4.3.

3.4.10 G6PD Rajanagarindra^{477C}

Novel mutation G6PD Rajanagarindra^{477C} was confirmed using PCR-RFLP technique, which the mutation removes *Nla*III restriction site (CATG^→CATC). Exon 5 was amplified using a pair of primer Ex5F and Ex5R shown in table 3.1. The PCR-RFLP condition was described in 3.4.2. The PCR fragment size is 320 bp. The normal and mutant fragments are 135+102+83 bp and 135+185 bp, respectively. Nine hundred twenty two Karen were defined G6PD Rajanagarindra^{477C}.

3.4.1.11 Exon 11 1311C→T

For PCR-RFLP approach, 1311T (+) removes a *Bcl*I restriction site (CGATCA→T^GATCA) (Biolabs) (Table 3.3). Polymorphism 1311 was amplified using a pair of primer R3F and R3Md, which have been previously described (Vulliamy *et al.,* 1991) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2.

After digestion, the normal and mutant fragments are 207 bp and 184+23 bp, respectively (Table 3.2). TaqMan® SNP genotyping assay of 1311T (+) is described in topic 3.4.4 and table 3.4.

3.4.1.12 IVS 11 nt 93T→C

For PCR-RFLP approach, 93C (+) creates an *Nla*III restriction site (TATG^→CATG^) (Biolabs) (Table 3.3). Polymorphism 93 were amplified using a pair of primer 1360F and 1360R, which have been previously described (Huang *et al.*, 1996) (Table 3.1). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments are 214 bp and 172+42 bp, respectively (Table 3.2).

The position of primers on *G6PD* locus was presented in figure 3.2.

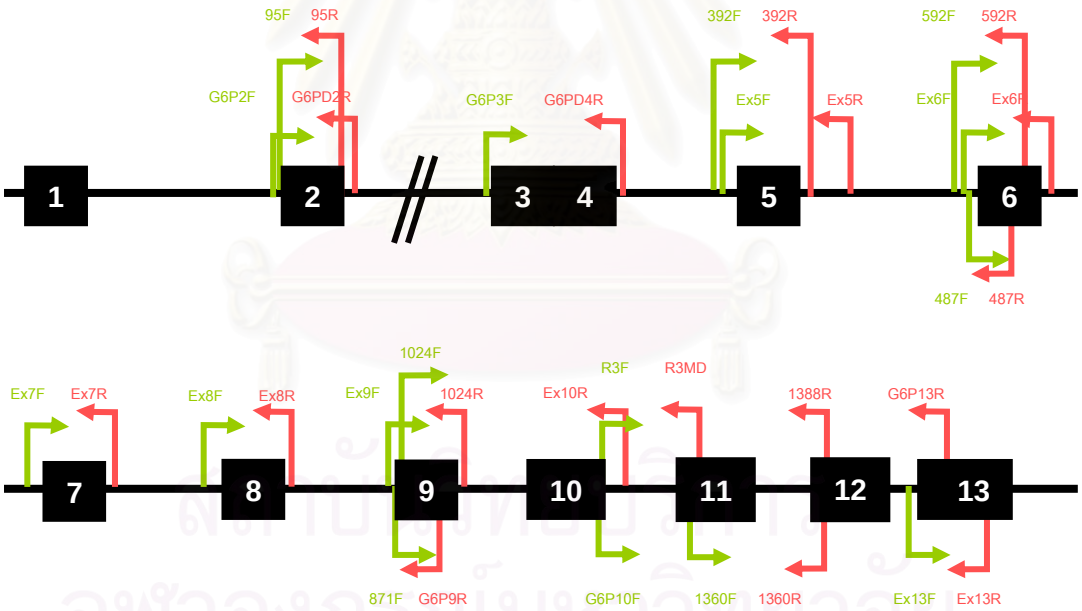


Figure 3.2 Primer positions for *G6PD* mutations genotyping.

3.4.2 rs number of SNPs on Chromosome X

For long range haplotype (LRH), twenty eight SNPs with a minor allele frequency (MAF) of more than 10% in Chinese (CHB) and/or Japanese (JPT) from HapMap were included in the study. Experimental design of long range SNPs is shown in Figure 3.3. Clustering of SNPs was dense on the core region G6PD Mahidol^{487A} and wide spread at the proximal sites to investigate the decay of LD from the core. All genotyped markers are shown in Table 3.5. Markers distal of *G6PD* could not be extended more than 200 kb due to subtelomeric region of chromosome X.

Three hundred eighty four unrelated individual Karen were recruited to define LRH of G6PD Mahidol^{487A} region.

Table 3.1 Primers for *G6PD* mutations and SNPs detection.

(Vulliamy *et al.*, 1991; Tang *et al.*, 1992; Huang *et al.*, 1996; Ninokata *et al.*, 2006; Nuchprayoon *et al.*, 2008)

Name	Sequence	Description	Name	Sequence	Description
871F	5'-TGGCTTTCTCTCAGGTCTAG-3'	G6PD Viangchan^{871A}	G6P3F	5'-AGGATGATGTAGTAGGTCG-3'	Exon3-4
9R	5'-GTCGTCCAGGTACCCTTTGGGG-3'		G6P4R	5'-CCGAAGTTGGCCATGCTGGG-3'	
487F	5'-GCGTCTGAATGATGCAGCTCTGAT-3'	G6PD Mahidol^{487A}	EX5F	5'-GTGTGTCTGTCTGTCCGTGTC-3'	Exon5
487R	5'-CTCCACGATGATGCGGTTCAAGC-3'		EX5R	5'-CACGCTCATAGAGTGGTGGG-3'	
1360F	5'-ACGTGAAGCTCCCTGACGC-3'	G6PD Union^{1360T}	EX6F	5'-GGGAGGGCGTCTGAATGA-3'	Exon6
1360R	5'-GTGAAAATACGCCAGGCCTTA-3'	93C	EX6R	5'-ACCTTGGGCCTCTGTGGTG-3'	
1376F	The same as for primer 1360F	G6PD Canton^{1376T}	Ex7F	5'-TCCACCTTGCCCCTCCCTGC-3'	Exon7
1376R	The same as for primer 1360R		Ex7R	5'-CCAGCCTCCCAGGAGAGAGG-3'	
1388F	The same as for primer 1360F	G6PD Kaiping^{1388A}	EX8F	5'-CATGCCCTTGAACCAGGTGA-3'	Exon8
1388R	5'-GTGCAGCAGTGGGGTGAACATA-3'		EX8R	5'-GCATGCACACCCCAGCTC-3'	
1024F	5'-GTCAAGGTGTTGAAATGCATC-3'	G6PD Chinese-5^{1024T}	EX9F	5'-TTCTCTCCCTTGGCTTTCTC-3'	Exon9-10
1024R	5'-CATCCCACCTCTCATTCTCC-3'		EX10R	5'-CACACTGCTCCTTCTCTGTA -3'	
592F	5'-GAGGAGGTTCTGGCCTCTACTC-3'	G6PD Coimbra^{592T}	G6P10F	5'-GAAGCCGGGCATGTTCTTCAAC-3'	Exon11
592R	5'-TTGCCCAGGTAGTGGTCGCTGC-3'		1360R	5'-GTGAAAATACGCCAGGCCTTA-3'	
95F	5'-CTCTAGAAAGGGGCTAACTTCTCA-3'	G6PD Gaohe^{95G}	1360F	5'-ACGTGAAGCTCCCTGACGC-3'	Exon12
95R	5'-GATGCACCCATGATGATGAATACG-3'		G6P13R	5'-CCAGGGCTCAGAGCTTGTG -3'	
392F	5'-GGACTCAAAGAGAGGGGCTG-3'	G6PD Chinese-4^{392T}	EX13F	5'-TGCCTCTCCTCCACCCGTCA-3'	Exon13
392R	5'-GAAGAGGCGGTTGGCCGGTGAC-3'		EX13R	5'-GTCAATGGTCCCGGAGTC-3'	
G6P2F	5'-CTCTAGAAAGGGGCTAACTTCTCAA-3'	Exon2	R3F	5'-TGTTCTTCAACCCCGAGGAGT-3'	1311T
G6P2R	5'-GGAATTCCTGGCTTTTAAGATTGGG-3'		R3MD	5'-AAGACGTCCAGGATGAGGTGATC-3'	

Table 3.2 Restriction enzymes and product sizes.

G6PD Mutations	Restriction Enzymes	Result: Product Sizes (bp)
G6PD Viangchan^{871A}	*Xba*I	N 126, M 106+20
G6PD Mahidol^{487A}	*Hin*dIII	N 104, M 82+22
G6PD Union^{1360T}	*Hha*I	N 142+45+27, M 187+27
G6PD Canton^{1376T}	*Afl*II	N 214, M 194+20
G6PD Kaiping^{1388A}	*Nde*I	N 227, M 206+21
G6PD Chinese-5^{1024T}	*Mbo*II	N 187, M 150+37
G6PD Coimbra^{592T}	*Pst*I	N 157+83, M 157+63+20
G6PD Gaohe^{95G}	*Mlu*I	N 198, M 174+24
G6PD Chinese-4^{392T}	*BstE*II	N 188+15, M 203
Exon 11 1311 C>T (rs2230037)	*Bcl*I	N 207, M 184+23
93C	*Nla*III	N 214, M 172+42
rs3752409 G>A	*Hinf*I	N 129+345, M 129+153+192

Note bp represents base pair (size of PCR product)

N represents normal allele. M represents mutant allele.

3.4.2.1 rs3752409

rs3752409 is located on chromosome X [151188483 (HapMap database HG16)] 1,042 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is G, as the mutant allele is A. This intronic SNP is in UCHL5 interacting protein or three prime repair exonuclease 2 gene (*UCHL5IP* or *TREX2)*. The MAF in CHB and JPT is 0.412 and 0.409 respectively. For PCR-RFLP approach, rs3752409 G→A allele creates a *Hinf*I restriction site (→) (Biolabs) (Table 3.3). rs3752409 were amplified using a pair of primer UCHL5F and UCHL5R (SIGMA). The PCR-RFLP conditions are described in 3.4.2. After digestion, the normal and mutant fragments are 129+345 bp and 129+153+192 bp, respectively (Table 3.2).

3.4.2.2 rs5970283

rs5970283 is located on chromosome X [150132248 (HapMap database release 16)] 2,151 kb from upstream of G6PD Mahidol^{487A}. The ancestral

allele of this region is T, as the mutant allele is C. This intronic SNP is in Gamma-aminobutyric acid (GABA) A receptor, alpha 3 gene (*GABRA3*). The MAF in CHB and JPT is 0.235 and 0.288 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__29615743_10 are described in 3.4.4 and table 3.5.

3.4.2.3 rs10218139

rs10218139 is located on chromosome X [150231600 (HapMap database release 16)] 2,051 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is A. This intergenic SNP is in Gamma-aminobutyric acid (GABA) A receptor, alpha 3 gene (*GABRA3*). The MAF in CHB and JPT is 0.364 and 0.485 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c____357104_10 are described in 3.4.4 and table 3.5.

3.4.2.4 rs4828596

rs4828596 is located on chromosome X [150339211 (HapMap database release 16)] 1,944 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is A, as the mutant allele is G. This SNP locates on intergenic region. The MAF in CHB and JPT is 0.301 and 0.258 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c___2774818_10 are described in 3.4.4 and table 3.5.

3.4.2.5 rs5924753

rs5924753 is located on chromosome X [150435621 (HapMap database release 16)] 1,847 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is C, as the mutant allele is T. This intronic SNP is in Gamma-aminobutyric acid (GABA) receptor, theta gene (*GABRQ*). The MAF in CHB and JPT

is 0.353 and 0.318 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__29610748_10 are described in 3.4.4 and table 3.5.

3.4.2.6 rs2515847

rs2515847 is located on chromosome X [150525789 (HapMap database release 16)] 1,757 kb from upstream of G6PD Mahidol[487A]. The ancestral allele of this region is T, as the mutant allele is C. This intronic SNP is in Melanoma antigen family A gene, 12 or chondrosarcoma associated gene 1 (*MAGEA12* or *CSAG1*). The MAF in CHB and JPT is 0.368 and 0.242 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c____329919_10 are described in 3.4.4 and table 3.5.

3.4.2.7 rs5970389

rs5970389 is located on chromosome X [150638709 (HapMap database release 16)] 1,644 kb from upstream of G6PD Mahidol[487A]. The ancestral allele of this region is C, as the mutant allele is T. This intronic SNP is in NAD(P) dependent steroid dehydrogenase-like gene (*NSDHL*). The MAF in CHB and JPT is 0.441 and 0.288 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c___2594825_10 are described in 3.4.4 and table 3.5.

3.4.2.8 rs5925261

rs5925261 is located on chromosome X [150737182 (HapMap database release 16)] 1,546 kb from upstream of G6PD Mahidol[487A]. The ancestral allele of this region is G, as the mutant allele is T. This SNP locates on intergenic region of Zinc finger protein 185 (LIM domain) gene (*ZNF185*). The MAF in CHB and JPT is 0.471 and 0.435 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c____334132_10 are described in 3.4.4 and table 3.5.

3.4.2.9 rs4145541

rs4145541 is located on chromosome X [150830982 (HapMap database release 16)] 1,452 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is G, as the mutant allele is A. This SNP locates on intergenic region. The MAF in CHB and JPT is 0.471 and 0.485 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__26031827_10 are described in 3.4.4 and table 3.5.

3.4.2.10 rs5924813

rs5924813 is located on chromosome X [150942649 (HapMap database release 16)] 1,251 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is C, as the mutant allele is T. This SNP locates on intergenic region. The MAF in CHB and JPT is 0.441 and 0.492 respectively. For TaqMan® SNP genotyping assay, the condition and probes c_29271617_10 were described in 3.4.4 and table 3.5.

3.4.2.11 rs3213466

rs3213466 is located on chromosome X [151042320 (HapMap database release 16)] 1,151 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is C, as the mutant allele is T. This SNP locates on intergenic region of Zinc finger protein 275 (*ZNF275*). The MAF in CHB and JPT is 0.426 and 0.470 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__11778692_1 are described in 3.4.4 and table 3.5.

3.4.2.12 rs2285034

rs2285034 is located on chromosome X [151282277 (HapMap database release 16)] 948 kb from upstream of G6PD Mahidol^{487A}. The ancestral

allele of this region is T, as the mutant allele is C. This intronic SNP is in ATPase, Ca++ transporting, plasma membrane 3 gene (*ATP2B3*). The MAF in CHB and JPT is 0.434 and 0.318 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__25473739_10 are described in 3.4.4 and table 3.5.

3.4.2.13 rs3761534

rs3761534 is located on chromosome X [151375455 (HapMap database release 16)] 855 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is C, as the mutant allele is T. This SNP locates on intergenic region of Dual specificity phosphatase 9 gene (*DUSP9*). The MAF in CHB and JPT is 0.420 and 0.320 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__27478723_10 are described in 3.4.4 and table 3.5.

3.4.2.14 rs4898437

rs4898437 is located on chromosome X [151483857 (HapMap database release 16)] 746.7 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is C. This SNP locates on intergenic region of ATP-binding cassette, sub-family D (ALD), member 1 gene (*ABCD1*). The MAF in CHB and JPT is 0.470 and 0.379 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__27936457_10 are described in 3.4.4 and table 3.5.

3.4.2.15 rs635

rs635 is located on chromosome X [151577265 (HapMap database release 16)] 653 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is A, as the mutant allele is G. This SNP locates on intergenic region. MAF in

CHB and JPT is 0.235 and 0.197 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c____341590_10 are described in 3.4.4 and table 3.5.

3.4.2.16 rs3027869

rs3027869 is located on chromosome X [151679442 (HapMap database release 16)] 551 kb from upstream of G6PD Mahidol[487A]. The ancestral allele of this region is G, as the mutant allele is A. This SNP locates on intergenic region of Host cell factor C1 (VP16-accessory protein) or Renin binding protein gene (*HCFC1* or *RENBP*). The MAF in CHB and JPT is 0.206 and 0.3227 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__15765137_10 are described in 3.4.4 and table 3.5.

3.4.2.17 rs17435

rs17435 is located on chromosome X [151779884 (HapMap database release 16)] 450.7 kb from upstream of G6PD Mahidol[487A]. The ancestral allele of this region is T, as the mutant allele is A. This intronic SNP is in Methyl CpG binding protein 2 (Rett syndrome) gene (*MECP2*). The MAF in CHB and JPT is 0.176 and 0.167 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c___2597094_20 are described in 3.4.4 and table 3.5.

3.4.2.18 rs1573656

rs1573656 is located on chromosome X [151876788 (HapMap database release 16)] 353.8 kb from upstream of G6PD Mahidol[487A]. The ancestral allele of this region is G, as the mutant allele is A. This intergenic SNP is in Opsin 1 (cone pigments), long-wave-sensitive (color blindness, protan) gene (*OPN1LW*). The MAF in CHB and JPT is 0.279 and 0.172 respectively. For TaqMan® SNP

genotyping assay, the conditions and probes c___7492723_10 are described in 3.4.4 and table 3.5.

3.4.2.19 rs2266894

rs2266894 is located on chromosome X [152016799 (HapMap database release 16)] 214 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is C, as the mutant allele is T. This intronic SNP is in Transketolase-like 1 gene (*TKTL1*). The MAF in CHB and JPT is 0.426 and 0.379 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__16177133_30 are described in 3.4.4 and table 3.5.

3.4.2.20 rs5945185

rs5945185 is located on chromosome X [152059378 (HapMap database release 16)] 171.2 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is G. This intergenic SNP is in Filamin A, alpha (actin binding protein 280) gene (*FLNA*). The MAF in CHB and JPT is 0.190 and 0.286 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__30138155_10 are described in 3.4.4 and table 3.5.

3.4.2.21 rs2283762

rs2283762 is located on chromosome X [152100111 (HapMap database release 16)] 130.5 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is G. This intronic SNP is in Tafazzin (cardiomyopathy, dilated 3A (X-linked); endocardial fibroelastosis 2 or Barth syndrome); small nucleolar RNA, H/ACA box 70 or Ribosomal protein L10 or Deoxyribonuclease I-like 1 gene (*TAZ* or *SNORA70* or *RPL10* or *DNASE1L1*). The MAF in CHB and JPT is 0.059 and 0.136 respectively. For TaqMan® SNP

genotyping assay, the conditions and probes c__15959557_10 are described in 3.4.4 and table 3.5.

3.4.2.22 rs3737557

rs3737557 is located on chromosome X [152127974 (HapMap database release 16)] 102.6 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is C. This intronic SNP is in ATPase, H+ transporting, lysosomal accessory protein 1 or GDP dissociation inhibitor 1 gene (*ATP6AP1* or *GDI1*). The MAF in CHB and JPT is 0.206 and 0.197 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c___2198310_1 are described in 3.4.4 and table 3.5.

3.4.2.23 rs7057286

rs7057286 is located on chromosome X [152181688 (HapMap database release 16)] 48.9 kb from upstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is C. This SNP locates on 3'untranslated region (UTR) of Ubiquitin-like 4A or Solute carrier family 10 (sodium/bile acid co-transporter family), member 3 or L antigen family, member 3 gene (*UBL4A* or *SLC10A3* or *LAGE3*). The MAF in CHB and JPT is 0.118 and 0.091 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c___2198331_10 are described in 3.4.4 and table 3.5.

3.4.2.24 rs743544

rs 743544 is located on chromosome X [152232717 (HapMap database release 16)] 2.5 kb from downstream of G6PD Mahidol^{487A}. The ancestral allele of this region is C, as the mutant allele is T. This intergenic SNP locates on Glucose-6-phosphate dehydrogenase gene (*G6PD*). The MAF in CHB and JPT is 0.449 and

0.455 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c___2479587_20 are described in 3.4.4 and table 3.5.

3.4.2.25 rs2472393

rs2472393 is located on chromosome X [152238847 (HapMap database release 16)] 8.6 kb from downstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is C. This intergenic SNP locates on Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma or Glucose-6-phosphate dehydrogenasegene (*IKBKG* or *G6PD*). The MAF in CHB and JPT is 0.074 and 0.182 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__16235463_10 are described in 3.4.4 and table 3.5.

3.4.2.26 rs4898389

rs4898389 is located on chromosome X [152295188 (HapMap database release 16)] 64.9 kb from downstream of G6PD Mahidol^{487A}. The ancestral allele of this region is G, as the mutant allele is A. This SNP locates on intergenic region. The MAF in CHB and JPT is 0.074 and 0.182 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__27946744_10 are described in 3.4.4 and table 3.5.

3.4.2.27 rs5987011

rs5987011 is located on chromosome X [152376383 (HapMap database release 16)] 146 kb from downstream of G6PD Mahidol^{487A}. The ancestral allele of this region is G, as the mutant allele is A. This intronic SNP locates on GRB2-associated binding protein 3 gene (*GAB3*). The MAF in CHB and JPT is 0.176 and 0.197 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__26709832_10 are described in 3.4.4 and table 3.5.

3.4.2.28 rs5945233

rs5945233 is located on chromosome X [152406876 (HapMap database release 16)] 176.6 kb from downstream of G6PD Mahidol^{487A}. The ancestral allele of this region is T, as the mutant allele is A. This intronic SNP locates on GRB2-associated binding protein 3 gene (*GAB3*). The MAF in CHB and JPT is 0.147 and 0.134 respectively. For TaqMan® SNP genotyping assay, the conditions and probes c__29988091_10 are described in 3.4.4 and table 3.5.

3.4.3 PCR-RFLP Condition

3.4.3.1 PCR Condition

The PCR reaction was carried out in a 10 μl reaction containing 1X PCR buffer (10X PCR buffer), 0.5 U of *Taq* polymerase (5 U/ μl) (Fermentas, Epicenter), 20 ng of each primer (50 ng/ μl), 1.5 mM $MgCl_2$ (50mM $MgCl_2$), 200 μM of each dNTPs, and approximate 10 ng DNA template. After incubation at 94°C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 0.45 min of denaturation, 56°C for 0.45 min of annealing, and 72°C for 0.45 min of extension. The final amplification cycle included an addition of a 15 min extension at 72°C.

For G6PD Rajanagarindra^{477C} and rs 3752409 G→A, the PCR reaction was carried out in a 15 μl reaction containing 5 μl of DNA at 1 ng/μl and 10 μl of mix PCR. The PCR mixture consisted of 1X PCR buffer (1.5 μl of 10X PCR buffer), 0.25 U of Platinum® *Taq* polymerase (0.05 μl of 5 U/ μl) (Invitrogen), 400 nM of each primer, 1.5 mM $MgCl_2$, and 200 μM of each dNTPs. Then, the mix was briefly centrifuge and covered with mineral oil. The PCR reaction was performed on the DNA Peltier thermal cycler (PTC-225 MJ Research). After incubation at 94°C for 5

min, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 0.45 min of denaturation, 56°C for 0.45 min of annealing, and 72°C for 0.45 min of extension. The final amplification cycle included an addition of a 15 min extension at 72°C.

3.4.3.2 RFLP Condition

Five microliters of PCR product was digested with 1 U of restriction enzyme according to manufacturer's protocols (New England Biolabs, Fermentas) (table 3.3), 1X reaction buffer (10X buffer), and sterile distilled water added to a final volume of 10 μl. The digestion mix was incubated at 37°C or 50°C or 60°C (depend on restriction enzyme) 2-4 hours.

Table 3.3 Restriction enzymes, recognition sites, buffers, and manufacturers. (Vulliamy *et al.*, 1991; Huang *et al.*, 1996; Nuchprayoon *et al.*, 2002)**.**

Enzymes	Recognition Site	Buffer	Temp (°C)	Manufacturer
*Xba*I	T^CTAGA	Buffer Y^+/Tango™	37	Fermentas
*Hind*III	A^AGCTT	Buffer 2	37	Biolabs
*Hha*I	GCG^C	Buffer 4	37	Biolabs
*Afl*II	C^TTAAG	Buffer 2	37	Biolabs
*Nde*I	CA^TATG	Buffer 4	37	Biolabs
*Mbo*II	GAAGA$(N)_8$^	Buffer 2	37	Biolabs
*Pst*I	CTGCA^G	Buffer 3	37	Biolabs
*Mlu*I	A^CGCGT	Buffer 3	37	Biolabs
*BstE*II	G^GTNACC	Buffer 3	60	Biolabs
*Bcl*I	T^GATCA	Buffer 3	50	Biolabs
*Nla*III	CATG^	Buffer 4	37	Biolabs
*Hinf*I	G^ANTC	Buffer 2	37	Biolabs, Fermentas

Note ^ represent the cleavage site of restriction enzyme.

3.4.3.3 Gel Electrophoresis

Polyacrylamide gel electrophoresis was used to detect *G6PD* mutations and SNPs that differentiate between DNA fragments at a 20 bp resolution [Mini-PROTEAN®3 Electrophoresis Cell (BIO-RAD Laboratories, USA)]. Twelve percentage of polyacrylamide gel (1 page) was freshly prepared as follows:

40% Acrylamide:Bisarylamide (BIO-RAD)	0.750 ml
5% TBE (Pacific Science)	1.000 ml
Distilled water	3.250 ml
10% ammonium persulphate	0.050 ml
TEMED	0.004 ml

After gel setting, five microliters of digestion's product was mixed with 1/6 volume of Loading Dye (0.25% bromophenol blue, 40% (w/v) sucrose in water), and loaded into gel slots in submarine conditions. Electrophoresis was performed at 110 volts for 1 hour. The DNA bands in the gel were visualized by staining with 2.0 μg/ml ethidium bromide and photographed under UV light at 302 nm.

For G6PD Rajanagarindra^{477C} and rs 3752409 G→A, 4% agarose gel was used to detect polymorphism. Gels consisted of 3% agarose GibcoBRL (Invitrogen) and 1% agarose Nusieve (Tebu-Bio) and 1X TBE (Sigma);

- 500 ml for large gel (15g GibcoBRL+5g Nusieve+3 drop ethidium bromide)
- 250 ml for medium gel (7.5g GibcoBRL+2.5g Nusieve+2 drop ethidium bromide)
- 100 ml for small gel (3g GibcoBRL+1g Nusieve+1 drop ethidium bromide)

Gel powder was mixed with 1X TBE and stirred well on a magnetic stirrer and melted in the microwave until boiling. Then, the gel mix was stirred again

on a magnetic stirrer to reduce the temperature before adding the ethidium bromide. The gel was poured, placed into the tank, the combs removed and covered with 1X TBE. After gel setting, ten microliters of digestion's product was mixed with 1/6 volume of Loading Dye (0.25% bromophenol blue, 40% (w/v) sucrose in water), and loaded into gel slots in submarine conditions. Electrophoresis was performed at 250 volts for 1 hour. The DNA bands in the gel were visualized under UV light at 302 nm.

3.4.4 Sequencing

PCR sequencing was performed by using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit Version 3.1 (Applied Biosystems, USA). The PCR reaction was carried out in a 10 μl reaction containing 4.0 μl of terminator ready reaction mix, 3.2 pmol of sequencing primer, and 50 ng DNA template. After incubation at 95°C for 2 min, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10 sec of denaturation, 50°C for 5 sec of annealing, and 60°C for 4 min of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate, and 2 volumes of absolute ethanol, and incubated at -20°C for 1 hour. After centrifugation at 12,000 rpm for 10 min, the pellet was washed with 500 μl of 70% ethanol, and air dried. The DNA pellet was resuspended in 10 μl Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer. The nucleotide sequences from the PCR products were compared against nucleotide sequences in database using ClustalX multiple alignment program for finding novel mutations.

For 96 well plate sequencing, another protocol was performed, consists of 4 procedures.

3.4.4.1 Sephadex Preparation

Sephadex is a trademark for cross-linked dextran gel. It is normally manufactured in a bead form and most commonly used for gel filtration columns. By varying the degree of cross-linking, the fractionation properties of the gel can be altered. These highly specialized gel filtration and chromatographic media are composed of macroscopic beads synthetically derived from the polysaccharide, dextran. The organic chains are cross-linked to give a three dimensional network having functional ionic groups attached by ether linkages to glucose units of the polysaccharide chains. Available forms include anion and cation exchangers, as well as gel filtration resins, with varying degrees of porosity; bead sizes fall in discrete ranges between 20 and 300 µm.

In a 2 liter cylinder, 100 gr. of G50 or P100 Sephadex ^{TM} Superfine powder was placed and completed to 2 l. with distilled water and transfer into a bottle and mixed to completely dissolve the Sephadex powder (10 -15 min), then left over night at 4 °C. The upper layer, which is water, was removed using a pipette or aspiration, leaving about 4 cm. of upper layer water to mix well and gently with Sephadex solution before use.

3.4.4.2 PCR Product Purification (Column Purification)

Sephadex P100 was mixed well in the bottle before use. Three hundred µl of P100 were pipetted per well on the filter of column plate (Multiscreen Plate MILLIPORE MAHVN4510), sandwiched with a waste eliminated plate. Next, this was centrifuged for 3 min at 500 g (1800 rpm) and the bottom plate containing the liquid waste discarded. Then, filter plate was placed on the top of PCR plate to recover purified PCR. Last, 15 µl of PCR product was added in the middle of the column without touching the Sephadex and centrifuged 4 min at 500g (1800rpm).

3.4.4.3 PCR Sequencing Reaction Preparation

In PCR plate, which contained 1 µl previously treated PCR product, was added 1 µl Terminator Ready Reaction Mix (Big dye terminator), 2 µl of Half-Dye Mix BIOLINE, 1 µl of Primer 10 µM, and 10 µl of H_2O. Mineral oil was not recommended so heated lid and reusable mat were used to cover the plate.

After incubation at 96°C for 5 min, amplification was carried out for 30 cycles with the following temperature cycling parameters; 96°C for 10 sec (ramping rate 1°C/sec) and 60°C for 4 min (ramping rate 1°C/sec).

3.4.4.4 Sequencing Reaction Purification

Sephadex G50 was mixed well in the bottle before use. Then, 300 µl of G50 were added per well on filter of column plate that was which sandwiched with the waste eliminated plate. Next, this was centrifuged for 3 min at 1500 rpm and the bottom plate containing the liquid waste discarded. Then, filter plate was put on the top of MicroAmp Optical plate (ABI) to recover purified sequence. Finally, 15 µl of sequencing reaction product was added in the middle of the column without touching the Sephadex and centrifuge 2 min at 2000rpm.

3.4.4.5 Running on ABI 3700

MicroAmp Optical plate (ABI) was covered with Thermowell aluminium foil sticker. Then, the plate was placed on ABI 3700. 10X Running Buffer and 3700 POP-6 Polymer were used for ABI 3700. The parameter as follow was used for the sequence running;

Mobility file = DT3700POP6 (BD)v.5mob

Project Name= 3700Project1

Run Module= SeqPop6_deg47

Analysis Module= BC_POP6_SeqOffFtOff.saz

3.4.5 TaqMan Assay for SNP Genotyping

The PCR reaction was carried out in a 5 μl reaction containing 5 μl of PCR solution and 1 μl of DNA 1 ng/1 μl, dried at 37°C before adding PCR solution. There are 2 conditions for TaqMan Assay. Invitrogen PCR Mix or TaqMan Universal PCR Master Mix. If Invitrogen PCR Mix dose not give a good result, TaqMan Universal PCR Master Mix was to be used. PCR reaction consisted of 0.5 μl of 10X PCR buffer, 0.5 μl of 50mM $MgCl_2$, 0.1 μl of Reference Dye, 0.02 μl of Platinum Taq (5U/μl) (Invitrogen), 0.312 μl of dNTPs 8 mM, 0.125 μl of 40X TaqMan probe, which consists of forward primer, reverse primer, and 2 fluorescence labeled probes, and 3.568 μl of H_2O. As Universal PCR Master Mix PCR reaction, 2.5 μl of 2X TaqMan Universal PCR Master Mix, 0.125 μl of primers and probes mix, and 2.375 μl of H_2O. After ABI PRISM 96 well Optical Reaction plate was covered with ABI PRISM Optical Adhesive Covers, it was incubated at 95°C for 10 min, amplification was carried out for 40 cycles with the following temperature cycling parameters; 92°C for 0.15 min and 60°C for 1 min.

Table 3.4 Primers and probes for TaqMan® SNP Genotyping

Assay ID	Name of Primers	Sequence of Primers (5' to 3')	Name of Probes	Sequence of Probes(5' to 3')	Interpretation	Design Strand
G6PDCANTON-GTOT	G6PDCANTON-GTOTF	GCCTCCCAAGCCATACTATGTC	G6PDCANTON-GTOTV2 (VIC)	CCTCA**C**GGAGCTCG	C-VIC: CC (X-axis) Both: CA A-FAM: AA (Y-axis)	Reverse
	G6PDCANTON-GTOTR	GGGCTTCTCCAGCTCAATCTG	G6PDCANTON-GTOTM2 (FAM)	CCTCA**A**GGAGCTCG		
G6PDMAHIDO-1	G6PDMAHIDO-1F	TGATCCTCACTCCCCGAAGAG	G6PDMAHIDO-1V1 (VIC)	CAGCAGA**G**GCTGGAA	G-VIC: GG (X-axis) Both: GA A-FAM: AA (Y-axis)	Forward
	G6PDMAHIDO-1R	AAGGGCTTCTCCACGATGATG	G6PDMAHIDO-1M1 (FAM)	CCAGCAGA**A**GCTGGAA		

Table 3.5 Primers and probes for TaqMan® SNP Genotyping

NCBI SNP Reference	Assay ID	Context Sequence	Gene	SNP Type	Location on HapMap 16	Design Strand	Interpretation
rs5987011	C__26709832_10	TGTGATACTTCAGAGATTAAACCCT**[A/G]**ACAAGATCAACTATACTTATTAAAG	*GAB3*	INTRON	ChX: 152376383	Reverse	A-VIC: AA Both: AG G-FAM: GG
rs5945233	C__29988091_10	TAAGGACTGTCAAAAGGAAGCAACA**[A/T]**ACATAATGGTAACATCATTAGGGAC	*GAB3*	INTRON	ChX: 152406876	Forward	A-VIC: AA Both: AT T-FAM: TT
G6PD Viangchan	C___2228724_10	TGCACCTCTGAGATGCATTTCAACA**[C/T]**CTTGACCTGAGAGAAAGCCAAGGGA	*G6PD*	Exon 9	Chx: 152228888	Reverse	G-VIC: GG Both: GT T-FAM: TT

Table 3.5 Primers and probes for TaqMan® SNP Genotyping (Cont.)

NCBI SNP Reference	Assay ID	Context Sequence	Gene	SNP Type	Location on HapMap 16	Design Strand	Interpretation
rs5970283	C_29615743_10	GGGTTAGACAGATCCTTAAATTGTA**[C/T]**ATAGTCTACTTCTACCCTCATCCTA	*GABRA3*	INTERGENIC/ UNKNOWN	ChX: 150132248	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs10218139	C___357104_10	TTTCAGTCACCTCAGGAATAAAAAA**[A/T]**GGAAGGTGCATTTCAATGACCCCTT	*GABRA3*	INTERGENIC/ UNKNOWN	ChX: 150231600	Reverse	A-VIC: AA Both: AT T-FAM: TT
rs4828596	C__2774818_10	CAACCAACCCACTAGTTGGGAGGGC**[A/G]**GGGGGTGGCAAGAGAGATGTGATTA	-	INTERGENIC/ UNKNOWN	ChX: 150339211	Reverse	A-VIC: AA Both: AG G-FAM: GG
rs5924753	C_29610748_10	GGAGAGATAGATAGGTGATAGATAA**[C/T]**TTATTGACAGATAGATGTATAAATA	*GABRQ*	INTRON	ChX: 150435621	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs2515847	C___329919_10	TAGCATTTTTGTGCCTGCATGAGCT**[C/T]**GCTGTCTCTCTCGCGCGCTCTCTGC	*MAGEA12; CSAG1*	INTRON	ChX: 150525789	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs5970389	C__2594825_10	CACTGTTTAATGGAGTCACCGGGGA**[C/T]**TAGAGCCTGGCCCAGGTGGCAAATA	*NSDHL*	INTRON	ChX: 150638709	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs5925261	C___334132_10	TAAAATCTTTGCCTCTTAGGCAGAT**[G/T]**ACTCAGAAGAAAAACAGACCATTTA	*ZNF185*	INTERGENIC/ UNKNOWN	ChX: 150737182	Forward	G-VIC: GG Both: GT T-FAM: TT
rs4145541	C_26031827_10	TATTAAGAAACTCTTAATTAATGGC**[A/G]**CTCTAACTGAATCTTAGAGTTGGAG	-	INTERGENIC/ UNKNOWN	ChX: 150830182	Forward	A-VIC: AA Both: AG G-FAM: GG
rs5924813	C_29271617_10	TGCATGGTTAAGAGGTCAATATCAC**[C/T]**GAGGACAACTTGCAAAAGTACAAAT	-	INTERGENIC/ UNKNOWN	ChX: 150942649	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs3213466	C__11778692_1	CTCCTTTCCCATGAGCAGGCGTTCC**[C/T]**GTTTTAAATCCTGCCTTGGTCCCTC	*ZNF275*	INTERGENIC/ UNKNOWN	Chx: 151042320	Forward	C-VIC: CC Both: CT T-FAM: TT
rs2285034	C__25473739_10	ACTCGGAAACTGGGGTAAGAAGTCA**[C/T]**TGGGCGCCTTGGTGGCAGTATAAAC	*ATP2B3*	INTRON	ChX: 151282277	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs3761534	C__27478723_10	GTGCAGATTCTGCGCGTAACCGTCC**[A/G]**CCTAGCCCAGGCCCAGCCTAGTTCA	*DUSP9*	INTERGENIC/ UNKNOWN	ChX: 151375455	Forward	A-VIC: AA Both: AG G-FAM: GG
rs4898437	C__27936457_10	GGATGGTCCCAAGGAGCCAATGTCC**[C/T]**GCCTCAGCTTGCACAGGGGCTCTAA	*ABCD1*	INTERGENIC/ UNKNOWN	ChX: 151483857	Reverse	C-VIC: CC Both: CT T-FAM: TT

Table 3.5 Primers and probes for TaqMan® SNP Genotyping (Cont.)

NCBI SNP Reference	Assay ID	Context Sequence	Gene	SNP Type	Location on HapMap 16	Design Strand	Interpretation
rs635	C____341590_10	GAAAAAAATTGATAATTTCCTAACA**[A/G]**AATATAACTTTCTGAAAACCCAACA	-	INTERGENIC/ UNKNOWN	ChX: 151577265	Forward	A-VIC: AA Both: AG G-FAM: GG
rs3027869	C__15765137_10	AGGCTGATGCCCTGGCAGCTGGGGA**[A/G]**GCATCCTCTGTGTGCTCCCACCCAC	*HCFC1; RENBP*	INTERGENIC/ UNKNOWN	ChX: 151679442	Reverse	A-VIC: AA Both: AG G-FAM: GG
rs17435	C___2597094_20	AGCTAGAGTGAGCCTTAGTAAGACA**[A/T]**CCAACACAGTTCTTAGAACAAAGAG	*MECP2*	INTRON	ChX: 151779884	Reverse	A-VIC: AA Both: AT T-FAM: TT
rs1573656	C___7492723_10	GTGGGCCGTGGAGGGGACAGGGCCC**[A/G]**TTGGTTGGAAACTGAGGCGAGGCTA	*OPN1LW*	INTERGENIC/ UNKNOWN	ChX: 151876788	Reverse	A-VIC: AA Both: AG G-FAM: GG
rs2266891	C__16177144_10	CCATTGATGAATTTATCAAGAGTCA**[C/T]**TGGCCGGGAGTGGTGGCTCACGCCT	*TEX28; OPN1MW2*	INTRON	ChX: 151973657	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs2266894	C__16177133_30	GGCAAGAGAGTCTCATCTCTTGCTC**[C/T]**CTAGGAGCTATGAGTTGAGGGCGCC	*TKTL1*	INTRON	ChX: 152016799	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs5945185	C__30138155_10	TGTGGTCTGGCCACACAATGGAGTA**[G/T]**TATGCGGCCATAAAAATAAATGAAG	*FLNA*	INTERGENIC/ UNKNOWN	ChX: 152059378	Forward	G-VIC: GG Both: GT T-FAM: TT
rs2283762	C__15959557_10	ACATTCATAAGGCTGTGCAACTATT**[G/T]**CTACAATCAATTTTAAAACATTGGT	*TAZ;SNORA70;RPL10; DNASE1L1*	INTRON	ChX: 152100111	Forward	G-VIC: GG Both: GT T-FAM: TT
rs7057286	C___2198331_10	AGAGCCAGGTACATGCCAGCTATGA**[C/T]**GATGATGAGCCCAACTGCAACAGGA	*UBL4A; SLC10A3; LAGE3*	UTR3	ChX: 152181688	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs2230037	C__15851848_10	AGAAGACGTCCAGGATGAGGCGCTC**[A/G]**TAGGCGTCAGGGAGCTTCACGTTCT	*G6PD*	INTERGENIC/ UNKNOWN	ChX: 152228205	Reverse	A-VIC: AA Both: AG G-FAM: GG
rs743544	C___2479587_20	ACCCAAAGAAAAGATGAACAAAGCT**[A/G]**GAAATTGGTGTTTGAAAAAGACGAA	*G6PD*	INTERGENIC/ UNKNOWN	ChX: 152232717	Forward	A-VIC: AA Both: AG G-FAM: GG
rs2472393	C__16235463_10	TGAGGCATCAGGCGTGGAAGAAGCC**[C/T]**GGGAGCCGGAGCTGTTCCAGGTGCT	*IKBKG;G6PD*	INTERGENIC/ UNKNOWN	ChX: 152238847	Reverse	C-VIC: CC Both: CT T-FAM: TT
rs4898389	C__27946744_10	CGTTGCCCCAGACCCTATCAGAATC**[A/G]**GTGATCTCTAATTGGTATCTAAGTT	-	INTERGENIC/ UNKNOWN	ChX: 152295188	Reverse	A-VIC: AA Both: AG G-FAM: GG

3.5 Microsatellite Repeat Detection

Three microsatellites $(AC)_n$, $(AT)_n$, and $(CTT)_n$ located within 19 kb downstream of *G6PD* (Figure 3.3), which were genotyped in sub-Saharan African to reconstruct the evolutionary history of G6PD B, A-$^{202A/376G}$, and A^{376G} in African, Southern European, and Middle East, were examined in Thai, Cambodian, Lao, Burmese, and Mon (Tishkoff *et al.*, 2001). Microsatellite repeats were amplified by fluorescence labelled primers followed the previous report (Tishkoff *et al.*, 2001) and analyzed using GENOTYPER by Applied Biosystems.

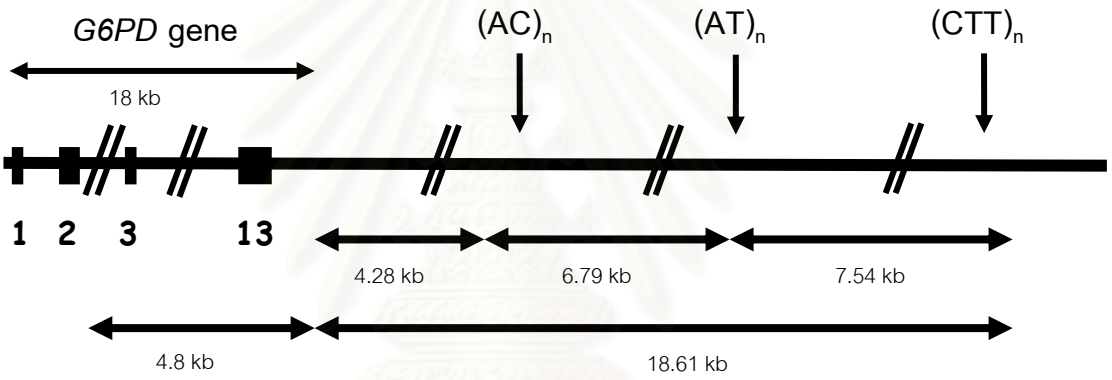


Figure 3.3 Microsatellite repeats AC, AT, CTT locate on downstream of *G6PD*.

Microsatellite haplotypes of Thai, Cambodian, Lao, Burmese, and Mon samples were characterized. The $(AC)_n$ repeat was amplified with primers ACF (AC forward primer), which was labeled with FAM, and ACR (AC reverse primer) to produce fragments from 164 to 188 bp long. The $(AT)_n$ repeat was amplified with primers ATF (AT forward primer), which was labeled with VIC, and ATR (AT reverse primer) to produce fragments from 125 to 179 bp long. The $(CTT)_n$ repeat was amplified with primers CTTF (CTT Forward primers), which was labeled with NED, and CTTR (CTT reverse primer) to produce fragments from 195 to 216 bp long (Tishkoff *et al.*, 2001) (Table 3.6). Amplification was performed with 50 ng of

genomic DNA in a 25 μl total volume reaction mixture. The reaction mixture contained 5 pmol each of fluorescently labeled forward and reverse primer, 200 μM of each dNTP, 50 mM KCl, 10 nM tris-HCl, 1 mM $MgCl_2$, and 0.625 U of *Taq* polymerase. Samples were denatured for 5 min at 94 °C, followed by 35 cycles of 94 °C for 45 sec, 66 °C for 45 sec, and 72 °C for 45 sec, followed by 10 min extension at 72 °C. Amplification products were run on a 6% polyacrylamide gel on an ABI sequencer, and fragment sizes were determined with Genescan software. Amplification of the CTT repeat, located within an *Alu*-rich region, produces some non-specific fragments (predominantly a 154-bp band). Only bands between 195 and 216 bp in size are polymorphic.

Table 3.6 Primers for microsatellite detection

Microsatellite	Primer	Primer Sequence (5' to 3')	Fluorescence	Size (bp)
AC	ACF	TCACTTGGGCCATGATCAC	FAM	164-188
	ACR	TTAATTTGTATCATGGGGTCCTAG	-	
AT	ATF	CATGGTTTCTGTGGAGTCTAGC	VIC	125-179
	ATR	GGTGGGAGGATTGCTTGAAG	-	
CTT	CTTF	GTTCAAGCGATTCTAGTGCCC	NED	195-216
	CTTR	CGGGTAGATTGCTTGAGCC	-	

4. Statistical Analysis

4.1 Analysis of Epidemiological Study

4.1.1 Epidemiological Statistical Analysis

Statistical analyses and model fitting were conducted using the statistical package Genstat 7.1. All individuals in the study protocol were included in the analyses, irrespective of whether their family structure was known. The effect of G6PD status, age and hamlet on the maximum parasite density recorded for each

individual of either *P. falciparum* or *P. vivax* were analysed by fitting a Generalized Linear Mixed Model (GLMM) with a Poisson error structure with date as a factor in the random model to account for the effect of seasonality. Age was factored into eight groups (<1, 1-4, 5-9, 10-14, 15-24, 25-39, 40-59 and 60+ years of age). Age groups were then combined if they were not significantly different from one another in the analysis. Hamlet was removed being non-significant. Because the data were over-dispersed a dispersion parameter was estimated. Wald statistics, which approximate to a χ^2 distribution, were established. The number of malaria cases was similarly analysed. Genders were analysed separately and together, with status defined as normal, hemizygote male, heterozygote female and homozygote female.

4.1.2 Genetic Statistical Analyses

Statistical analyses were carried out as above including gender but excluding G6PD status. The residual variance not explained by these "environmental" factors was generated (Phimpraphi *et al.*, 2008). Because a non-normal error distribution was used, Pearson rather than standardized normal residuals were generated. The residual values were then used in the genetic family-based association analyses to assess the effect of G6PD status on parasite phenotype.

4.1.3 Family Base Association Test (FBAT)

To avoid spurious association due to population structure, family-based association test (FBAT) statistic introduced by Rabinowitz & Laird in 2000 was used using FBAT software package version 2.0.2., which can analyze markers on chromosome X (Rabinowitz and Laird 2000; Schneiter *et al.*, 2007). FBAT built on original transmission disequilibrium test (Spielman *et al.*, 1993), in which alleles transmitted to affected offspring are compared with the expected distribution of alleles among offspring, but can be used with different disease phenotypes including

qualitative, quantitative, censored and multiple measured traits with covariates. In FBAT, the expected genotype distribution is derived using Mendel's law of segregation and conditioning on the observed phenotype under the null hypothesis, which is " no association, no linkage" in our case. Since conditioning eliminates the assumption of distribution of the phenotype, the technique avoids confounding due to model misspecification as well as admixture or population stratification. The test can be applied to multiple phenotypes and to phenotypic data obtained in longitudinal studies without making any distributional assumptions for the phenotypic observations based on generalized estimating equations (Lange *et al.*, 2003).

4.2 Analysis of G6PD Deficiency Phenotype

4.2.1 Prevalence of G6PD Deficiency

Prevalence of G6PD deficiency by ethnic and gender was defined. The prevalence of G6PD deficiency in Thai, Cambodian, Lao, Burmese, and Mon were previously defined in my previous study.

4.2.2 Mean and Standard Deviation of G6PD Activity

Mean and standard deviation (S.D.) of G6PD activity by mutation and gender of Thai, Cambodian, Lao, Burmese, and Mon samples were investigated. While, mean and S.D. of G6PD activity of *G6PD* mutations in Karen could not be defined due to they were screened using qualitative method (FST).

4.3 Analysis of Evolutionary Study

4.3.1 Allele Frequency of Mutations and SNPs

4.3.1.1 Pedcheck

To verify that there was mendelian inheritance within families of the mutations and SNPs genotype in the data set of Karen, Pedcheck was performed. The

inconsistency genotypic data were resolved by re-examination of the raw data and re-genotyping where necessary.

PedCheck is a program for detecting marker typing incompatibilities in pedigree data (O'Connell 1998). There are 4 levels for error detection algorithms;

Level 1: Use the genotypes of each individual as given in the pedigree to check for inconsistencies between parents and offspring. It checks on the nuclear family level and detects the following errors.

a.) Incompatible of alleles of a child and parent.

b.) Half-type of a person.

c.) In a sibling have more than 4 alleles.

d.) In a sibling have more than 3 alleles when there is a homozygous child.

e.) If any is specified, the allele is out of bounds.

f.) Males are scored as homozygous when there are X-linked pedigrees.

Level 2: Use the Lange-Goradia algorithm to do genotype elimination. Level 2 is guaranteed to detect if there is an inconsistency. If there are no Level 2 errors detected, then the pedigree is Mendelian consistent. It will analyze when there was no Level 1 error for that family and will report errors for a nuclear family.

Level 3: Determine the "critical genotypes". They are typed individuals who set to "unknown" then remove the inconsistency in the pedigree.

Level 4: Determines the alternative genotypes that a critical genotype can have, and then computes an odds ratio statistic to assist you in determining the most likely person to be in error.

Input files: PedCheck will look for 'pedfile.txt' and ‘name.txt’ as default inputs. PedCheck used for checking raw data, which any subset of markers, before having to specify allele frequencies. For running X-linked data, the command line option '-x' will be used.

Example: pedcheck –p pedfile.txt –n name.txt –o output.txt -x

Output results: all information is printed to the screen and to a file named ‘output.txt’. The name can be reset by using the option ‘-o’.

4.3.1.2 Hardy-Weinberg Equilibrium (HWE)

Allele frequencies were calculated by gene counting. Deviations from Hardy Weinberg equilibrium of allele frequencies were defined by Arlequin v.3.1 (Excoffier 2005). This program tests the hypothesis that observed diploid genotypes are derived from a random union of gametes. The test is analogous to Fisher’s exact test on a two-by-two contingency table but extended to a contingency table of arbitrary size. The genotypic data is test locus by locus. The test uses a modified version of the Markov-chain random walk algorithm described (Guo 1992). The contingency table is first built. The *kxk* entries of the table are the observed allele frequencies and k is the number of alleles. The probability to observe the table under the null-hypothesis of no association is given by (Levene 1949).

$$L_0 = \frac{n!\prod_{i=1}^{k} n_{i*}!}{(2n)!\prod_{i=1}^{k}\prod_{j=1}^{i} n_{ij}!}\,2^H \qquad \text{-(equation 3.2)}$$

Where *H* is the number of heterozygote individuals.

For alternative contingency tables, new contingency tables from an existing one are created. I select two distinct lines i_1, i_2, and two distinct columns j_1, j_2 at random. The new table is obtained by decreasing the counts of the cells (i_1, j_1) $(i_2,$

j_2) and increasing the counts of the cells (i_1, j_1) (i_2, j_2) by one unit. This leaves the allele counts n_i unchanged. The switch to the new contingency table is accepted with a probability R equal to:

$$1)\ R = \frac{L_{n+1}}{L_n} = \frac{n_{i_1 j_1} n_{i_2 j_2}}{(n_{i_1 j_2}+1)(n_{i_2 j_1}+1)} \frac{(1+\delta_{i_1 j_1})(1+\delta_{i_2 j_2})}{(1+\delta_{i_1 j_2})(1+\delta_{i_2 j_1})}, \text{ if } i_1 \neq j_1 \text{ or } i_2 \neq j_2 \quad \text{-(equation 3.3)}$$

$$2)\ R = \frac{L_{n+1}}{L_n} = \frac{n_{i_1 j_1} n_{i2 j2}}{(n_{i_1 j_2}+1)(n_{i_2 j_1}+2)} \frac{4}{1}, \text{ if } i_1 = j_1 \text{ and } i_2 = j_2 \quad \text{-(equation 3.4)}$$

$$3)\ R = \frac{L_{n+1}}{L_n} = \frac{n_{i_1 j_1} (n_{i_2 j_2}-1)}{(n_{i_1 j_2}+1)(n_{i_2 j_1}+1)} \frac{1}{4}, \text{ if } i_1 = j_2 \text{ and } i_2 = j_1 \quad \text{-(equation 3.5)}$$

δ denotes the Kronecker function. R is the ratio of the probabilities of the two tables. The switch to the new table is accepted if R is larger than 1. The P-value of the test is the proportion of the visited tables having a probability smaller or equal to the observed contingency table. The standard error on the P-value is estimated like in the case of the linkage disequilibrium using a system of batches. Markers which showed excessive homo- or heterozygosity were re-examined.

4.3.2 Haplotype Reconstruction

Haplotype reconstruction was performed from population genotype data using the Bayesian statistical method implemented in PHASE v.2.1.1 (Stephens and Donnelly 2003). For this analysis, I stated that the phase of all males (hemizygotes at the X-linked locus) was known, which should greatly improve the performance of the algorithm. The software can deal with SNP, microsatellite and missing data. Software incorporates a recombination hotspot model and specifies known haplotypes in reconstructing haplotype.

Input files: An input file was supplied by the user to specify how many analyzed individuals, how many typed loci/sites each individual, what sort of loci/sites these are (SNP or microsatellite), and the genotypes for each individual. The file also specifies the relative physical positions of the markers.

The default structure for the input file consists of

1. Number of Individuals: the number of individuals who have been genotyped.

2. Number of Loci: the number of loci or sites at which each individual has been typed.

3. The character 'P' (upper case, without quotation marks).

4. Position (*i*): A number indicating the position of locus *i*, relative to some arbitrary reference point. The unit of base pair is recommended. The loci must be in their physical order along the chromosome (These Positions must be increasing).

5. Locus Type (*i*): A letter indicating the type of locus *i*.

(a) S for a biallelic (SNP) locus, or biallelic site in sequence data.

(b) M for microsatellite, or other multi-allelic locus (eg tri-allelic SNP, or HLA allele).

6. ID (*i*): A string, giving a label for individual *i*.

7. Genotype (*i*): The genotypes for the *i*th individual. This is given on two consecutive rows. At each locus, one allele is entered on the first row, and one on the second row. It does not matter which allele is entered on each row. For biallelic loci, any two characters (e.g. A/C, G/T, 0/1) can be used to represent the two alleles, and they do not need to be separated by a space. Missing alleles at SNP loci should be entered as? For multiallelic loci a positive integer must be used for each allele (representing the number of repeats at microsatellite loci), and data for each locus

should be separated by a space. Missing alleles at multiallelic loci should be represented by -1. For example, the example input file, test.inp.

The first two numbers in the file say that there are 3 individuals typed at 5 loci. The next line indicates their relative positions along the chromosome, and the line MSSSM indicates that the first and last locus are microsatellites/multi-allelic, and the other loci are bi-allelic. The genotype information then follows, with three lines for each individual. The first line gives an ID for the individual, and the second and third lines give the genotypes. The third individual is missing genotype data at the first microsatellite locus, and the first SNP locus. Missing alleles at SNP loci should be entered as ?. Missing alleles at multiallelic loci should be represented by -1.

For an additional option, the specifying known phases: The -k option allows the user to specify that some of the phases are known. Including some known haplotypes in the sample can considerably improve performance. The way to use this option is creating a file which specifies the known phases. This file should contain one line for each individual, with a single character (either a *, 0 or 1) for each locus.

* indicates the phase for that individual at that locus is unknown.

0 indicates the phase for that individual at that locus is as in the input genotypes.

1 indicates that the phase for that individual at that locus is the reverse of the phase in the input genotypes.

The file of genotype data should be saved in test.inp and the known phases for individual was saved as eg.known. The file specifying the known phases must follow the –k without using a space. For example, to use the supplied file eg.known with the data in test.inp, use

PHASE -keg.known test.inp test.out.

Output results: The algorithm starts by dividing the data into segments of consecutive loci. It then computes a list of plausible haplotypes within each segment, and then iteratively combines segments to obtain a list of plausible haplotypes, and a best guess for each pair of haplotypes, across the whole region. The division into segments is random. The program runs a number of \burn-in" and \main" iterations on each segment, and informs its progress in terms of how many \segment operations" it has completed (the total number of segment operations that it will perform is approximately twice the number of segments).

The program produces an output file, contains a summary of the individual haplotype estimates for each individual, with parentheses ( ) at positions where the phase was difficult to infer, and square brackets [ ] around alleles that were difficult to infer and haplotype frequencies.

4.3.3 Heterozygosity of Microsatellite Haplotypes

All microsatellite data were analyzed for their expected and observed haplotype heterozygosity (*H*) using Arlequin v.3.1 (Excoffier 2005). Expected heterozygosity is defined as the probability that randomly chosen haplotypes are different in the sample. Expected heterozygosity and its sampling variance are calculated as

$$\hat{H} = \frac{n}{n-1}(1-\sum_{i=1}^{k} p_i^2) \qquad \text{-(equation 3.6)}$$

$$\mathrm{V}(\hat{H}) = \frac{2}{n(n-1)}\left\{2(n-2)\left[\sum_{i=1}^{k} p_i^3 - (\sum_{i=1}^{k} p_i^2)^2\right] + \sum_{i=1}^{k} p_i^2 - (\sum_{i=1}^{k} p_i^2)^2\right\} \qquad \text{-(equation 3.7)}$$

Observed heterozygosity:

$$\hat{H} = (1 - \sum_{i=1}^{k} p_i^2)$$ -(equation 3.8)

4.3.4 Long Range Haplotype (LRH) Estimation

Sweep program was performed to assess the long range haplotype of G6PD Mahidol^{487A}. This program detects the recent positive selection of allele by identifying 'core haplotype', which has no evidence of a recombination or mutation event that breaks the breakdown haplotype with increasingly distant SNPs (http://www.broad.mit.edu/mpg/sweep/documentation/Sweep_Documentation_Dec05.pdf) (Sabeti *et al.*, 2002). To study the decay LD from core haplotype, extended haplotype homozygosity (EHH), relative extended haplotype homozygosity (REHH), core homozygosity, and marker breakdown are performed.

4.3.4.1 Extend Haplotype Homozygosity (EHH)

Extended haplotype homozygosity (EHH) is the probability of 2 randomly chosen chromosomes, carrying the core haplotype of interest is identical by descent for the entire interval from the region to distance x. EHH is on a scale of 0 (no homozygosity or all extended haplotypes are different) to 1 (complete homozygosity or all extended haplotypes are same). EHH uses to detect the transmission of an extended haplotype without recombination.

$$EHH_t = \frac{\sum_{i=1}^{s} \binom{e_{ti}}{2}}{\binom{c_t}{2}}$$ -(equation 3.9)

Where *c*: number of samples of a particular core haplotype, *s*: number of unique extended haplotypes, *e*: number of samples of a particular extended haplotype

4.3.4.2 Relative Extend Haplotype Homozygosity (REHH)

Relative extended haplotype homozygosity (REHH) is the ratio of the EHH on the tested core haplotype compared with the EHH of the grouped set of core haplotypes at the region not including the core haplotype tested. REHH is on a scale of 0 to infinity.

$$\overline{EHH} = \frac{\sum_{j=1, j\neq t}^{n}\left[\sum_{i=1}^{s}\binom{e_i}{2}\right]}{\sum_{i=1, j\neq t}^{n}\binom{c_i}{2}}$$

-(equation 3.10)

Where *n*: number of different core haplotypes

4.3.4.3 Core Homozygosity

Core homozygosity is a measure of the extent of variation at a core by defining the probability that any 2 randomly chosen core haplotypes from a population will be the same. Core homozygosity uses number & characteristics of SNPs genotyped at the core & historical haplotype structure of the region. Application of core homozygosity is achieved by comparing haplotype blocks with the same number of SNPs and matching the core homozygosity's across regions for analogous data.

$$H_{core} = \frac{\sum_{i=1}^{s}\binom{c_i}{2}}{\binom{n}{2}}$$

-(equation 3.11)

Where *n*: number of Chr., *c*: number of samples of a core haplotype, *s*: number of different core haplotypes.

4.3.4.4 Marker Breakdown

Marker breakdown is the degree to which each added marker at a further distance causes the extended haplotypes to decay for all core haplotypes. Marker breakdown evaluates how much historical recombination (observed recombination) has occurred over a distance from the core.

$$allEHH = \frac{\sum_{j=1}^{n}\left[\sum_{i=1}^{s}\binom{e_i}{2}\right]}{\sum_{i=1}^{n}\binom{c_i}{2}} \quad \text{-(equation 3.12)}$$

The Karen dataset core haplotypes were defined manually. Relative extended haplotype homozygosity (REHH) was calculated for each core haplotype at a 1.3-Mb distance from the core. To test for a significant excess of REHH with respect to allele frequency, our dataset was compared to the empirical distribution of "core haplotype frequency *vs.* REHH" calculated for the entire chromosome X from the HapMap II dataset in Han Chinese (The HapMap International Consortium 2007). SNP density of the HapMap II data was matched to that of our study (i.e. one SNP every 70 kb).

4.3.5 Estimating Age and Selection Intensity of *G6PD* Mutation

To jointly estimate the age of the G6PD Mahidol^{487A} mutation, two different methods were performed: one is a Bayesian method (Slatkin 2008) and the other is a maximum-likelihood method based on the Luria-Delbrück approximation (Austerlitz *et al.*, 2003). Both approaches are based on the frequency and the conservation with distance of the assumed ancestral haplotype on which the mutation

of interest appeared. They both use the recombination rate as a molecular clock, which was retrieved for each pair of genotyped SNP from the HapMap Phase II recombination map (The HapMap International Consortium 2007).

I only considered males in this analysis, to eliminate any uncertainty introduced by haplotype reconstruction. I set the population growth rate at zero, which is a realistic assumption given the demographic history of the population (Besaggio *et al.,* 2007). Ten independent runs of 100,000 replicates each were performed for the Bayesian method. Standard and multipoint estimations gave also similar results for the maximum-likelihood method.

CHAPTER IV

RESULTS

1. Prevalence of G6PD Deficiency

Donor blood samples that are fluorescent negative for FST assay and/or have G6PD activity less than 1.5 I.U./g Hb as determined by standardized method for G6PD assay of hemolysates were categorized as G6PD deficient.

1.1 Prevalence of G6PD Deficiency in Karen Population

Eight hundred and forty six subjects (395 males and 451 females) were screened for G6PD deficiency. Ninety three males (23.5%) and thirty seven females (8.2%) were G6PD deficient. Partial deficiency was more common in female subjects (50 of 451; 11.1%) than in male subjects (26 of 395; 6.6%) as shown in Table 4.1.

Table 4.1 Prevalence of G6PD deficiency in Karen

		G6PD deficiency screening			
	N	Deficiency (%)	Partial deficiency (%)	Normal/ partial deficiency (%)	Normal (%)
Male	395	93 (23.5)	26 (6.6)	12 (3.0)	264 (66.8)
Female	451	37 (8.2)	50 (11.1)	8 (1.8)	356 (78.9)
Total	846	130	76	20	620

1.2 Prevalence of G6PD Deficiency in Thai, Cambodian, Lao, Burmese, and Mon subjects

Prevalence of G6PD deficiency was assessed in previous studies in Thai, Cambodian, Lao, and Burmese (Table 4.2). From 215 Cambodian blood samples, we found G6PD deficiency in 26.1% of Cambodian male (31 of 119) and 3.1% of females (3 of 96). Among Cambodian neonates, 21 of 56 males and two of 51 females were G6PD deficient. Among Cambodian adults, 10 of 63 males and one of

45 females were G6PD deficient (Louicharoen and Nuchprayoon 2005). One hundred and sixty two Lao subjects (84 males and 78 females) were analyzed for G6PD deficiency. Twenty (23.8% of 84) males and three (7.7% of 78) females were G6PD deficient. One hundred and ninety eight Thai males and one hundred thirty five Thai females were screened G6PD activity. Thirty three (16.7% of 198) males and eleven (8.1% of 135) females were defined as G6PD deficient. For Burmese subjects, who had been collected in 2002-2003, one hundred and thirty one subjects (72 males and 59 females) were screened for G6PD activity. Seven (9.7% of 72) males and one (1.7% of 59) females were defined as G6PD deficient.

Table 4.2 Prevalence of G6PD deficiency in Cambodian, Lao, Thai, and Burmese (2002-2003)

Ethnic group	Case (N)	Sex (N)	G6PD deficiency (N)	Prevalence
Cambodian	215	M 119	31	0.26*
		F 96	3	0.03*
Lao	162	M 84	20	0.24
		F 78	6	0.08
Thai	333	M 198	33	0.17
		F 135	11	0.08
Burmese	131	M 72	7	0.10
(2002-2003)		F 59	1	0.02

* (Louicharoen and Nuchprayoon 2005)

G6PD activity in 162 Mon males and 178 Burmese males (year 2004-2005) showed a normal distribution. The average G6PD activity was 6.34 ± 2.89 IU/g Hb in Mon and 6.56 ± 2.69 IU/g Hb in Burmese. Ethnic group, origin of subject, and number of G6PD-deficient subjects are shown in Table 4.3. The results show that 17 Burmese male subjects (9.6%) and 19 Mon male subjects (11.7%) were G6PD deficient (Table 4.4).

Table 4.3 Frequency of G6PD deficiency in male Mon and Burmese among various Place of Birth (Nuchprayoon *et al.*, 2008)

Place of birth	Ethnic group	Case (N)	Deficient (N)	Place of birth	Ethnic group	Case (N)	Deficient (N)
Pha-un	Burmese	89	6	Sangkhla Buri	Mon	5	0
	Mon	96	10	Mae-Sot	Mon	4	0
Yangon	Burmese	45	7	Kokarek	Burmese	3	0
	Mon	26	5	Koei	Mon	3	0
Ye	Burmese	2	0	Pal	Mon	2	0
	Mon	8	0	Mijina	Burmese	2	0
Maewadee	Burmese	4	0	Kawthaung	Burmese	1	0
	Mon	6	2	Irrawaddy	Mon	1	0
Dawei	Burmese	22	2	Marid	Burmese	1	0
	Mon	2	0	Yakai	Burmese	1	1
Lamae	Burmese	5	1	Katai	Burmese	1	0
	Mon	4	1	Jai	Burmese	1	0
Ja-eang	Burmese	1	0	Jadee	Mon	1	0
	Mon	3	1	Aeiou	Mon	1	0

Table 4.4 Prevalence of G6PD deficient Burmese and Mon

Ethnic group	Case (N)	Sex (N)	G6PD deficiency (N)	Prevalence
Burmese (2004-2005)	178	M 178 F ND	17 ND	0.10* ND
Mon	162	M 162 F ND	19 ND	0.12* ND

* (Nuchprayoon *et al.*, 2008), ND represents not be defined.

2. Molecular Genetics of *G6PD*

2.1 Molecular Analysis of *G6PD* Mutations

2.1.1 *G6PD* Mutations in Karen

A total of nine hundred and twenty two subjects (425 males and 497 females) were characterised for their *G6PD* mutations. Using TaqMan SNP genotyping, G6PD Mahidol^{487A} allele was found in 21% of Karen subjects. G6PD Viangchan$^{871A,\ 1311T}$ and G6PD Canton^{1376T} were rarely found in Karen subjects representing 0.9% and 0.6%, respectively (Table 4.5). The allele discrimination of *G6PD* mutation analysis using TaqMan genotyping is shown in Figure 4.1. Five G6PD deficient subjects did not carry any of these three major mutations. However, DNA sequencing of the *G6PD* gene revealed one case each of G6PD Union^{1360T} (Figure 4.2A) and Chinese-5^{1024T} (Figure 4.2B) consistent with have previously been reported in Southeast Asia (Iwai *et al.*, 2001; Nuchprayoon *et al.*, 2002; Laosombat *et al.*, 2005) and the remaining three subjects did not carry any of *G6PD* mutations examined.

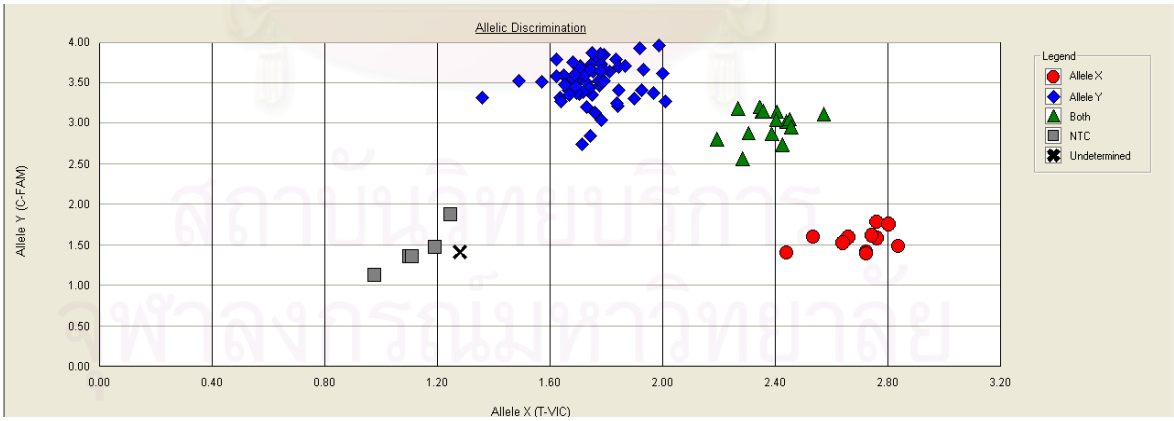


Figure 4.1 Allelic discrimination of TaqMan genotyping

Red circles: homozygote T allele, green triangles: heterozygote C/T allele, dark blue diamonds: homozygote C allele, gray squares: negative control (NTC), black crosses: undetermined sample.

Table 4.5 *G6PD* mutations in Karen

G6PD mutation	Base change	Status	N	G6PD deficinecy screening (N)
G6PD Mahidol	487G→A	Hemizygote	90	deficiency (84), no data (6)
		Heterozygote	162	deficiency (16), partial deficiency (36), partial deficiency/normal (4), normal (92), no data (14)
		Homozygote	21	deficiency (18), normal (2), no data (1)
G6PD Viangchan	871G→A, 1311C→T	Hemizygote	5	deficiency (4), no data (1)
		Heterozygote	6	normal (6)
G6PD Mahidol & G6PD Viangchan	487G→A, 871G→A	Heterozygote	2	deficiency (1), partial deficiency (1)
G6PD Mahidol & G6PD Canton	487G→A, 1376G→T	Heterozygote	2	no data (2)
G6PD Canton	1376G→T	Heterozygote	6	deficiency (1), partial deficiency (3), normal (1), no data (1)
G6PD Union	1360C→T	Hemizygote	1	deficiency (1)
G6PD Chinese-5	1024C→T	Heterozygote	1	deficiency (1)
G6PD Rajanagarindra	477G→C	Hemizygote	1	partial deficiency (1)
		Heterozygote	3	partial deficiency (1), normal (2)
Total			300	

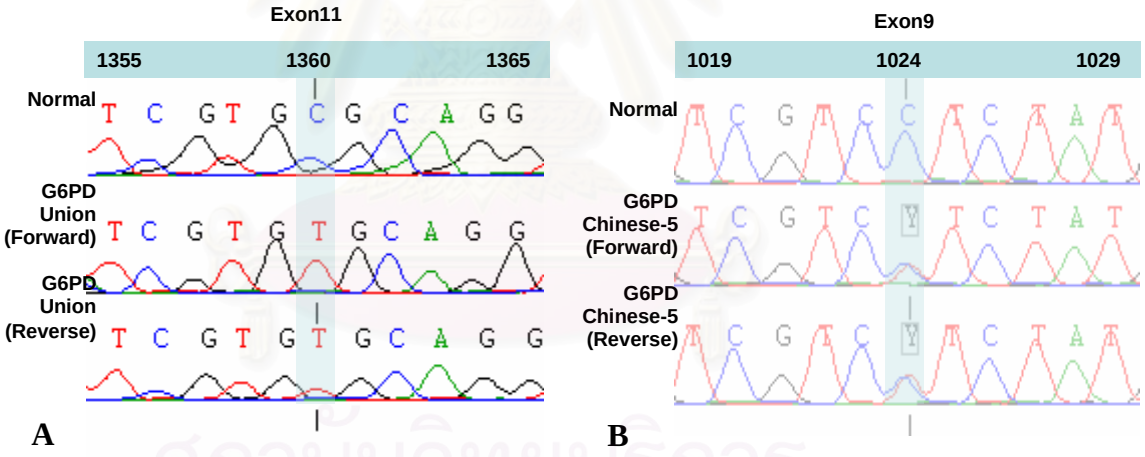


Figure 4.2 A. Chromatogram of G6PD Union^{1360T} B. Chromatogram of G6PD Chinese-5^{1024T} (Y represents C/T.)

Thirty five partial G6PD deficient subjects that did not have any of those three mutations were subsequently sequenced. The results revealed a novel mutation which was a missense mutation in exon 5 nucleotide 477 G→C (Figure 4.3) resulting an amino acid substitution Met159Ilu. This particular mutation found in two related Karens (Figure 4.4A) was designated as G6PD Rajanagarindra^{477C} from the

name of Her Royal Highness Princess Galyani Vadhana KromLuang Naradhiwas Rajanagarinda, honorary chairman of The Tropical Disease Trust Fund provided funding for construction of the RTIC. The mutations of 33 partial deficient subjects remain unidentified. To confirm this novel mutation 477G→C, I performed PCR-RFLP using a primer set (Ex5F and Ex5R) to amplify 320 bp amplicon from exon 5 and using restriction enzyme *Nla*III to digest normal allele resulting three separated bands of 83, 102, and 135 bp, and mutant allele resulting two bands of 135 and 185 bp (Figure 4.5). The PCR-RFLP result showed in each case hemizygous and heterozygous 477G→C, respectively, which confirmed the result obtained by a direct sequencing approach. To further investigate the distribution of this novel mutation in Karen population, PCR-RFLP analysis was performed in all 922 subjects, we found two heterozygous 477G→C; one case was from Family A (Figure 4.4A) and another case was from Family B (Figure 4.4B).

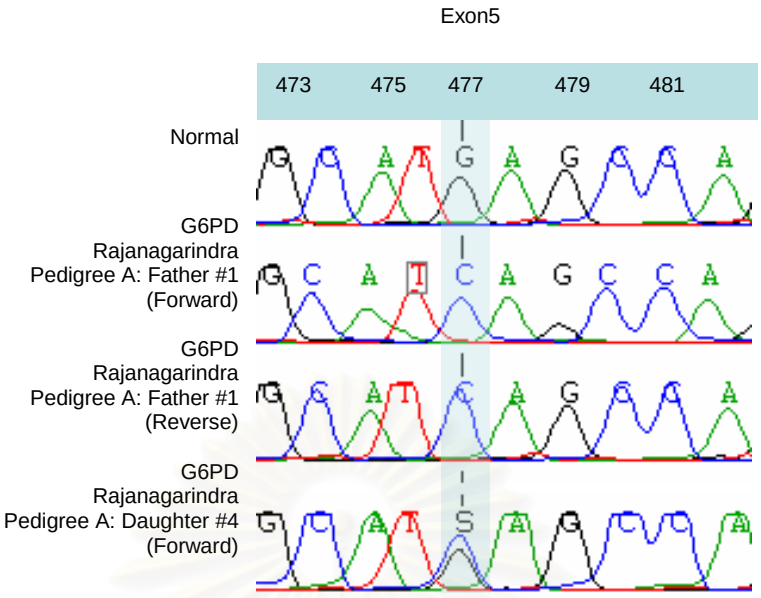


Figure 4.3 Chromatogram of G6PD Rajanagarindra^{477C} (S represents C/G.)

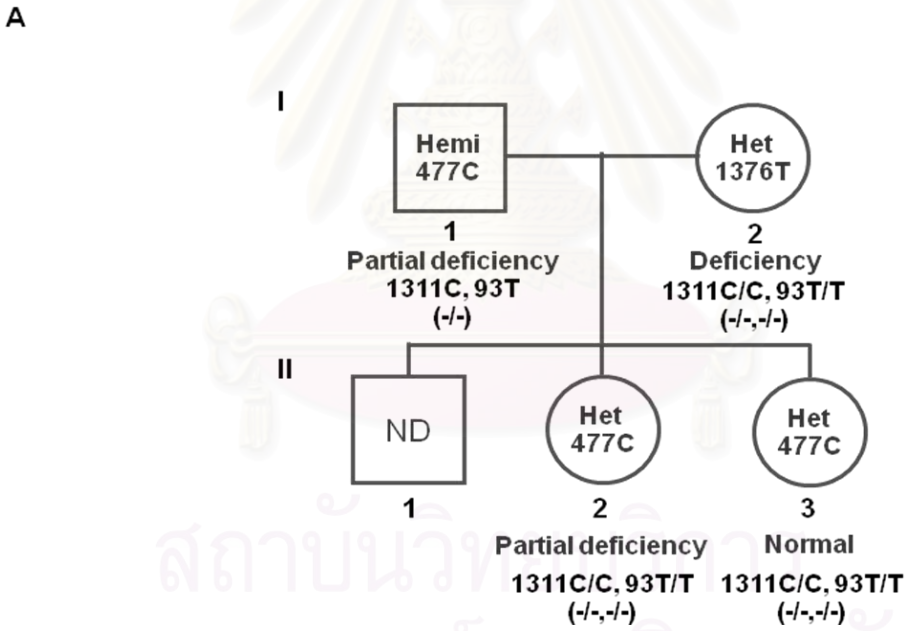


Figure 4.4 A. Pedigree of Family A, B. Pedigree of Family B.

Hemi represents hemizygote; Het represents heterozygote; ND represents no data.

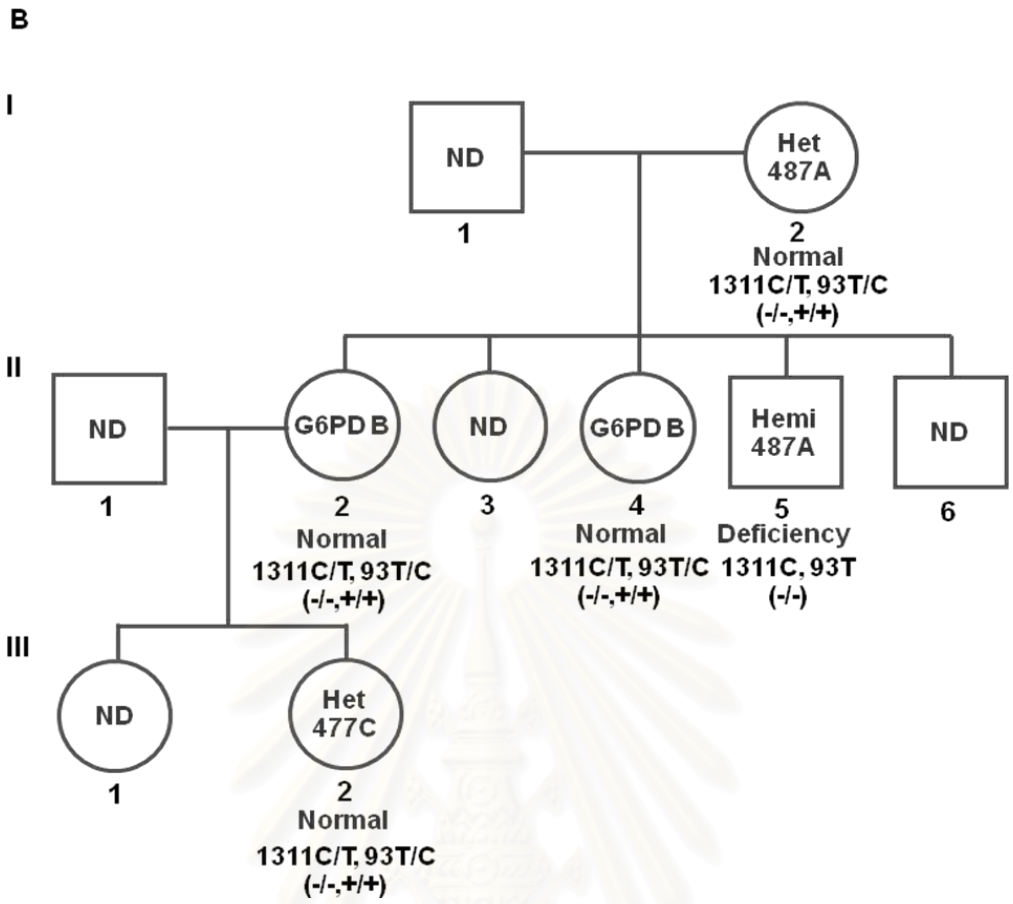


Figure 4.4 A. Pedigree of Family A, B. Pedigree of Family B.

Hemi represents hemizygote; Het represents heterozygote; ND represents no data.

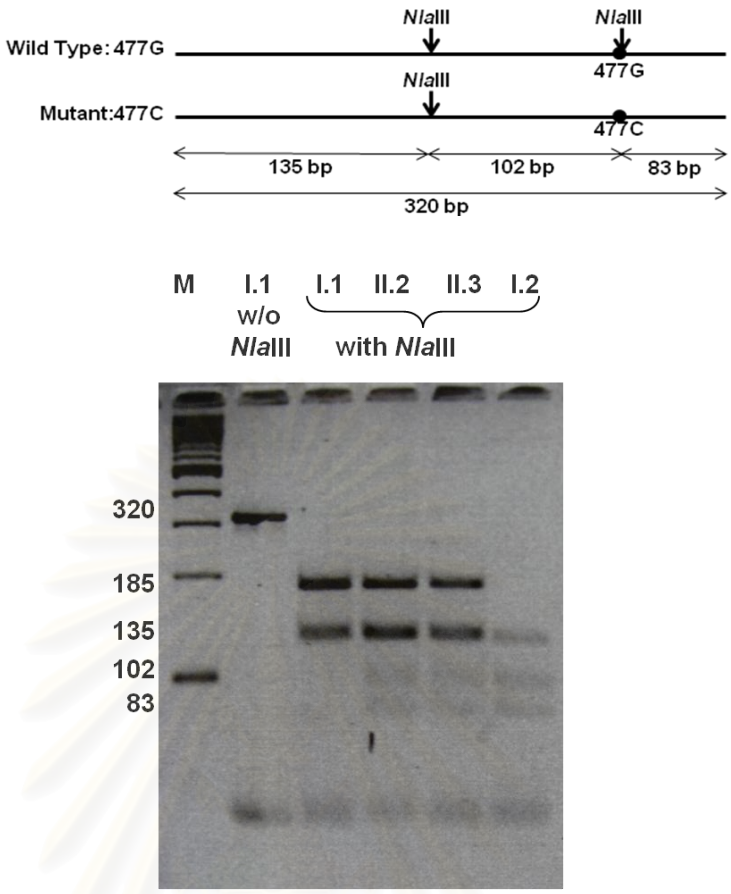


Figure 4.5 PCR-RFLP approach in the detection of G6PD Rajanagarindra^{477C} in all members from Family A. From the left, M in lane 1 represents molecular weight marker. In lane 2, PCR-RFLP with *Nla*III showed a band of an undigested 320 bp (I.1 w/o *Nla*III) and, in lane 3, 135 and 185 bp bands of digested hemizygote 477G→C (I.1 with *Nla*III). Lane 4and 5 showed four digested bands of 83, 102, 135, and 185 bp in heterozygote 477G→C (II.2 and II.3 with *Nla*III) and lane 6 showed three digested bands of 83, 102, and 135 bp in normal control (I.2).

2.1.2 *G6PD* Mutations in Thai, Cambodian, Lao, Burmese, and Mon

G6PD mutations were characterized in Thai, Cambodian, Lao, and Burmese from previous studies (Table 4.6). G6PD Viangchan^{871A} was the most common in Cambodians (82.4% of 34) (Figure 4.6) (Louicharoen and Nuchprayoon 2005), Lao (46.2% of 26), and Thai (47.7% of 44) but was not found in Burmese. G6PD Mahidol^{487A} was a dominant mutation in Burmese (62.5% of 8) but rarely

found in Thai (4.5% of 44) and not found in Cambodian and Lao. G6PD Canton^{1376T} was found in one Burmese (12.5% of 8), Lao (3.8% of 26), and one Thai (2.3% of 44). G6PD Union^{1360T} was found in one each in Cambodian (2.9% of 34) and Lao (3.8% of 26). G6PD Kaiping^{1388A} was found in two Lao (7.7% of 26) and seven Thai (15.9% of 44). G6PD Chinese-5^{1024T} was found in one Thai (2.3% of 44). G6PD Coimbra^{592T} was found in one Cambodian (2.9% of 34). There are 25 G6PD deficient subjects of Burmese, Cambodian, Thai, and Lao ethnic origin that remained of unidentified genotype. The G6PD activity in each variant is shown in Table 4.6.

Figure 4.6 Distribution of *G6PD* mutations in Cambodian. (Louicharoen and Nuchprayoon 2005)**.**

Table 4.6 *G6PD* mutations in each ethnic groups and G6PD variants activity

Mutation	Base Change	Cases (N (%))	Ethnic group (N(%)) Burmese	Cambodian	Lao	Thai	Sex (N)	G6PD level (IU/g Hb)(SD)
Viangchan	871A	61(54.5)	0	28(82.4)*	12(46.2)	21(47.7)	M 49	0.42(0.120)
							F 12	0.51(0.592)
Mahidol	487A	7(6.3)	5(62.5)	0	0	2 (4.5)	M 6	0.62(0.454)
							F 1	1.1
Canton	1376T	6(5.4)	1(12.5)	0	4(3.8)	1(2.3)	M 5	0.56(0.449)
							F 1	0.62
Union	1360T	2(1.8)	0	1(2.9)*	1(3.8)	0	M 2	0.72(0.318)
							F 0	-
Kaiping	1388A	9(8.0)	0	0	2(7.7)	7(15.9)	M 8	0.22(0.314)
							F 1	0
Chinese-5	1024T	1(0.9)	0	0	0	1(2.3)	M 1	0.95
							F 0	-
Coimbra	592T	1(0.9)	0	1(2.9)*	0	0	M 1	0.00
							F 0	-
Unknown		25(22.3)	2(25)	4(11.7)*	7(26.9)	12(27.3)	M 20	0.43(0.454)
							F 6	0.59(0.564)
Total		112(100)	8(100)	34(100)*	26(100)	44(100)	M 91	
							F 23	

* (Louicharoen and Nuchprayoon 2005)

Nineteen G6PD deficient Mon males (12% of 162) were analyzed for mutations. Using PCR-RFLP, 12 (63% of 19) were G6PD Mahidol^{487A}, and one each was G6PD Jammu$^{871A,\ 1311C}$ and G6PD Kaiping^{1388A}. None of these samples carries any of the following mutations: G6PD Canton^{1376T}, G6PD Union^{1360T}, G6PD Chinese-5^{1024T}, G6PD Gaohe^{95G}, G6PD Chinese-4^{392T}, and G6PD Coimbra^{592T}. To specify whether mutation 871G→A is G6PD Viangchan$^{871A,\ 1311T}$ or G6PD Jammu$^{871A,\ 1311C}$, the PCR-RFLP was performed using a restriction enzyme *Bcl*I which showed 1311C in the sample with 871G→A, confirming a case of G6PD Jammu$^{871A,\ 1311C}$ in one Mon subject. DNA sequencing from all coding exons of *G6PD* genes of the remaining five G6PD-deficient Mons revealed one case of G6PD Mediterranean^{563T} (Figure 4.7), and a novel mutation 94(C→G) (Figure 4.8) in a Mon subject from Pha-un province in

Myanmar. It should be noted that the G6PD activity of novel mutation 94G was not detectable (Table 4.7). The types of mutations in three remaining G6PD deficient Mons were unidentified.

Table 4.7 G6PD deficient mutations, activities and associated haplotypes (Nuchprayoon *et al.*, 2008)

Mother's ethnic group	Place of birth	G6PD activity (IU/ g Hb)	*G6PD* mutation	Haplotype	
				1311	93
Burmese 12, Mon 12	Various	0.43 ± 0.48	Mahidol^{487A}	-	-
Mon	Yangon	1.21	Mediterranean^{563T}	-	-
Mon	Pha-un	1.80	Jammu^{871A}	-	-
Mon	Pha-un	1.27	Kaiping^{1388A}	-	-
Mon	Pha-un	0.00	94G	-	-
Burmese	Pha-un	0.64	Valladolid^{406T}	+	+
Burmese	Yangon	0.00	Coimbra^{592T}	-	-
Burmese	Yangon	1.27	Kerala-Kalyan^{949A}	-	-

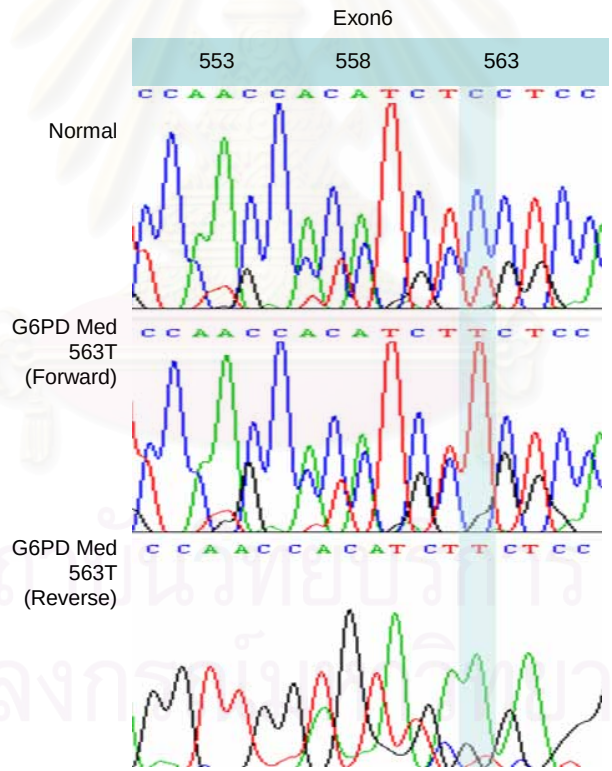


Figure 4.7 Chromatogram of G6PD Mediterranean^{563T}

Seventeen Burmese males (10% of 178) were G6PD deficient. Twelve G6PD deficient Burmese (71%) were G6PD Mahidol^{487A} and one (6%) was G6PD Coimbra^{592T}. DNA sequencing from all coding exons of *G6PD* genes of the four

remaining G6PD-deficient samples were identified as G6PD Kerala-Kalyan^{949A} (Figure 4.9) in one case and G6PD Valladolid^{406T} (Figure 4.10) in another case. Mutations in the remaining two cases were unidentified. Mon and Burmese who have G6PD Mahidol^{487A} came from different birth places in Myanmar (Figure 4.11; Table 4.7). Subjects with G6PD Mahidol^{487A} had variable G6PD activities ranging from undetectable (8 of 24) to some residual activities. The average G6PD activity (± SD) for G6PD Mahidol^{487A} was 0.43 ± 0.48 IU/g Hb (Table 4.7). All *G6PD* mutations in all ethnic groups are summarized in Figure 4.12.

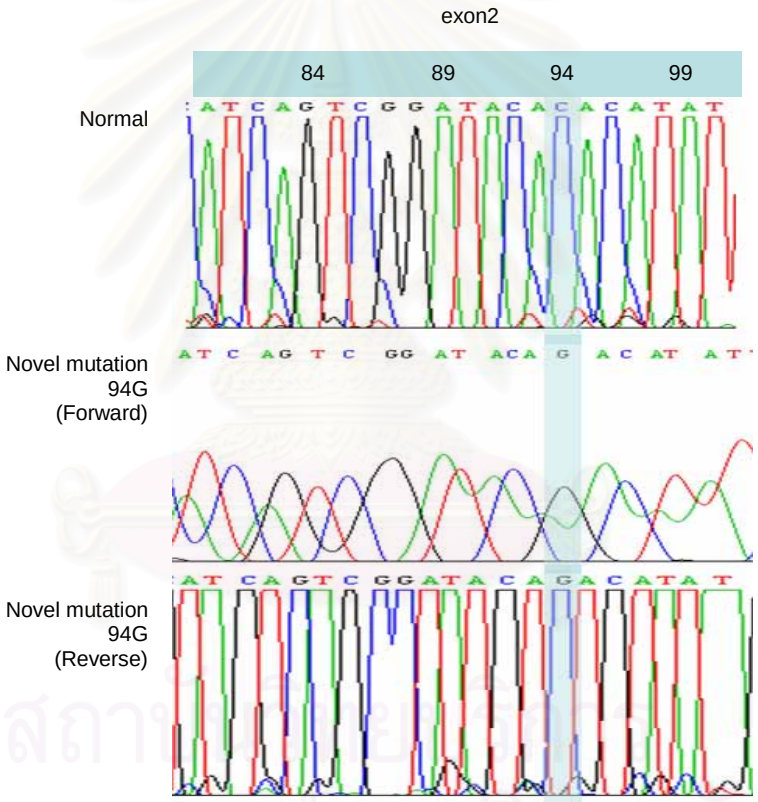


Figure 4.8 Chromatogram of novel mutation 94G

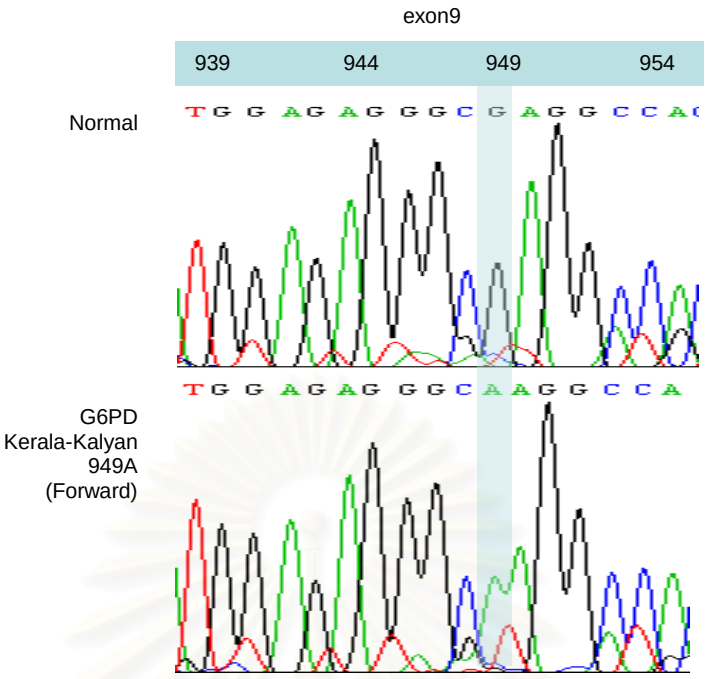


Figure 4.9 Chromatogram of G6PD Kerala-Kalyan^{949A}

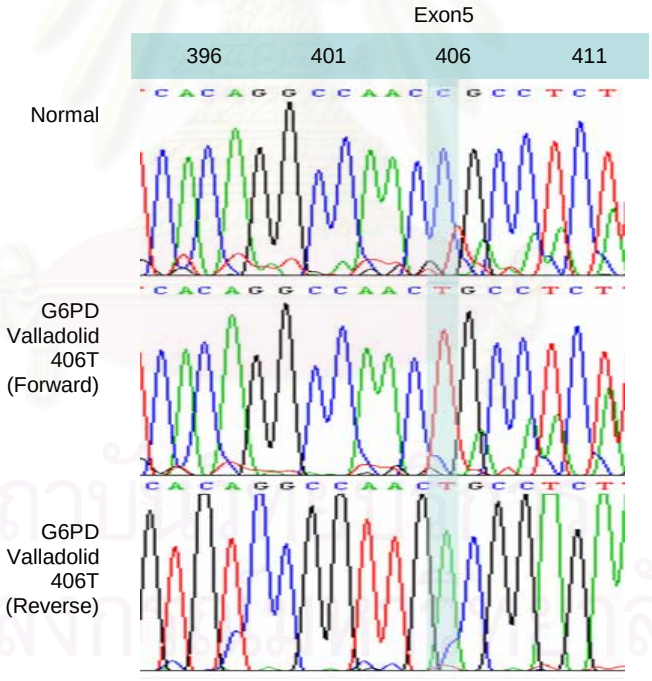


Figure 4.10 Chromatogram of G6PD Valladolid^{406T}

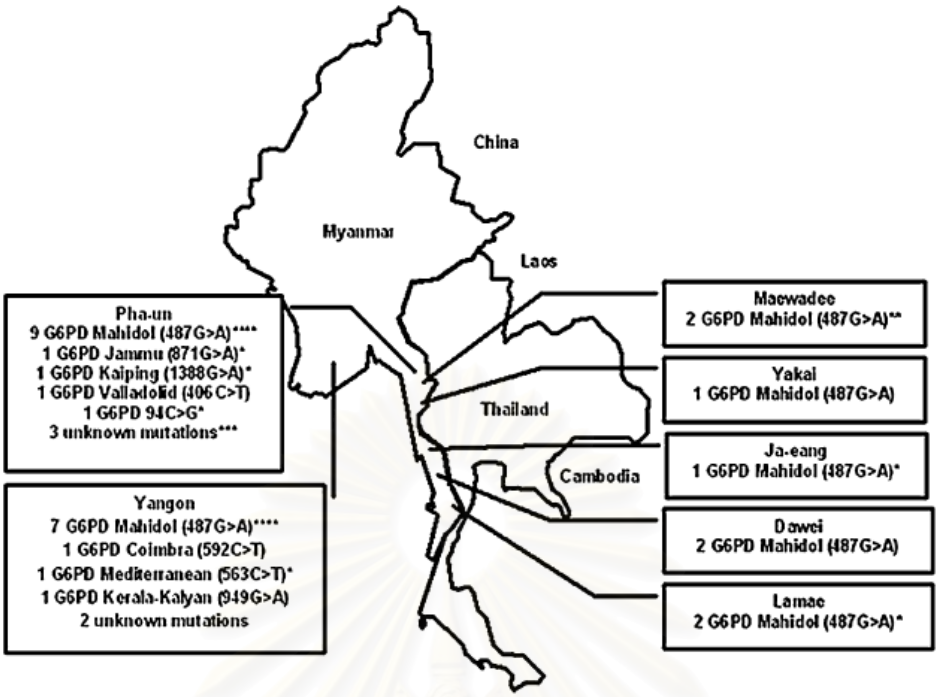


Figure 4.11 Distribution of *G6PD* mutations in Myanmar. Numbers represent number of *G6PD* mutations identified. *one, ** two, * three, **** four cases were Mon** (Nuchprayoon *et al.*, 2008).

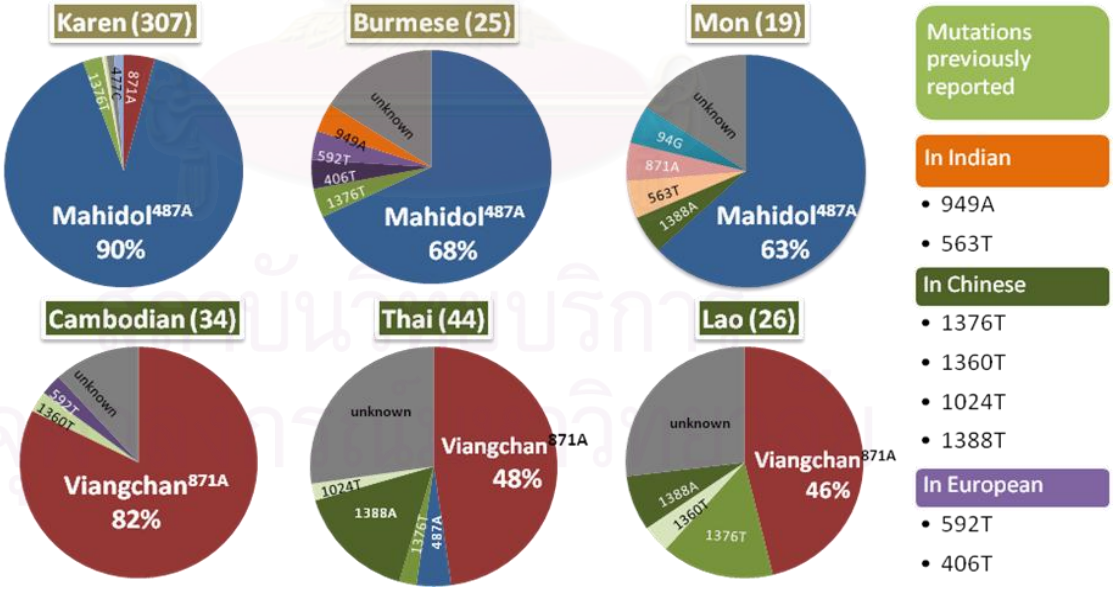


Figure 4.12 All *G6PD* mutations in all ethnic groups.

2.2 Molecular Analysis of SNPs Polymorphisms of *G6PD* Mutations

Six following SNPs consisted of 611G (+), 175T (+), 163T (-), 1116A (-), 1311T (+), and 93C (+) were analyzed in all male G6PD deficient and normal subjects from the previous study population in Cambodia, Laos, Myanmar, and Thailand. Polymorphic haplotypes in *G6PD* mutation are summarized in Table 4.8. 611G (+), 175T (+), 163T (-), and 1116A (-) were not found in our Southeast Asian population. Only 1311T (+) and 93C (+) were found in our samples. Four haplotypes were detected, with haplotype 1311C, 93T (-/-) accounting for 70% of all G6PD B. Haplotype 1311T, 93C (+/+) was found the most frequently in G6PD Viangchan^{871A}. G6PD Mahidol^{487A}, G6PD Canton^{1376T}, G6PD Union^{1360T}, G6PD Kaiping^{1388A}, and G6PD Chinese-5^{1024T} harbored haplotype 1311C, 93T (-/-).

Table 4.8 Haplotypes of the *G6PD* mutations in Southeast Asia population.

Haplotype						*G6PD* mutation (N (%))							
611G	175T	163T	1116G	1311T	93C	G6PD B	871A	487A	1376T	1360T	1388A	1024T	UK*
-	-	+	+	-	-	141 (69.8)	0	6	5	2	8	1	9 (47.4)
-	-	+	+	-	+	13 (6.4)	0	0	0	0	0	0	1 (5.3)
-	-	+	+	+	-	5 (2.5)	3 (6.1)	0	0	0	0	0	0
-	-	+	+	+	+	43 (21.3)	46 (93.9)	0	0	0	0	0	9 (47.4)

* UK represents unknown mutation

In Karens, 1311T, and 93C were genotyped in G6PD-normal (G6PD B) and G6PD-deficient individuals. Haplotype frequencies of *G6PD* mutations are shown in Table 4.9.

Table 4.9 Haplotype frequencies of *G6PD* mutations in Karen

Haplotype	*G6PD* mutation (N(%))							Total
1311/93	G6PD B	487A	871A	1376T	1360T	1024T	477C	
-/-	674 (61.6)	298	0	8	1	1	4	986 (69.5)
-/+	92 (8.4)	0	0	0	0	0	0	92 (6.5)
+/-	104 (9.5)	0	0	0	0	0	0	104 (7.3)
+/+	224 (20.5)	0	13	0	0	0	0	237 (16.7)
Total	1094	298	13	8	1	1	4	1419

In Mon and Burmese samples collected during the year 2004-2005, all G6PD Mahidol^{487A} had haplotype 1311C, 93T (-/-). This haplotype was also the predominant haplotype in all G6PD-deficient Mons and Burmese, except for one case with G6PD Valladolid^{406T} (Table 4.7). Among 31 G6PD-normal (G6PD B) Burmese randomly selected for haplotype analysis, 1311C, 93T (-/-) (n=22, 71.0%) is the most common, followed by 1311T, 93C (+/+) (n=8, 25.8%) and 1311T, 93T (+/-) (n=1, 3.2%), whereas 1311C, 93C (-/+) was not identified. Among 32 G6PD-normal (G6PD B) Mon randomly selected for haplotype analysis, only two haplotypes were found. Similar to the Burmese subjects, haplotype 1311C, 93T (-/-) (n=23, 71.9%) was more common than 1311T, 93C (+/+) (n=9, 28.1%), but the haplotype 1311T, 93T (+/-) was not identified. All haplotypes in G6PD B, G6PD Mahidol^{487A} -/-, and G6PD Viangchan^{871A} +/+ are summarized in Figure 13.

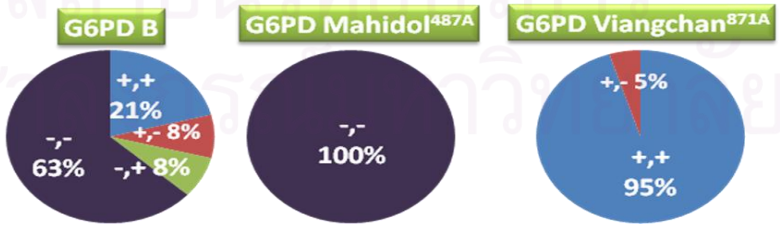


Figure 4.13 All haplotypes in G6PD B, G6PD Mahidol^{487A} -/-, and G6PD Viangchan^{871A} +/+.

3. Positive Selection of *G6PD* Mutations in Southeast Asian Population

3.1 Microsatellite Haplotype of *G6PD* Mutations in Southeast Asian

Thai, Cambodian, Lao, Burmese, and Mon G6PD-deficient and G6PD-normal subjects except Karens were examined for the following three microsatellites: AC, AT, and CTT repeats. Five $(AC)_n$ alleles (from 172 to 180 bp), 19 $(AT)_n$ alleles (from 125 to 169 bp), and 9 $(CTT)_n$ alleles (from 192 to 216 bp) were observed. Microsatellite allele frequencies and heterzygosity values in various populations and in various *G6PD* alleles are summarized in Table 4.10-15.

Haplotype consisting of $(AC)_n$, $(AT)_n$, and $(CTT)_n$ microsatellites and SNPs distinguishing G6PD B alleles (n=241), G6PD Viangchan$^{871A,\ 1311T}$ (n=42), G6PD Jammu$^{871A,\ 1311C}$ (n=1), G6PD Mahidol^{487A} (n=31), G6PD Canton^{1376T} (n=6), G6PD Union^{1360T} (n=2), G6PD Kaiping^{1388A} (n=6), G6PD Chinese-5^{1024T} (n=1), G6PD Coimbra^{592T} (n=1), G6PD Kerala-Kalyan^{949A} (n=1), G6PD Valladolid^{406T} (n=1), G6PD Mediterranean^{563T} (n=1), novel mutation 94G (n=1), and unknown mutation (UK) (n=16) were typed from individuals from ethnically diverse Thai, Cambodian, Lao, Mon, and Burmese. All *G6PD* alleles were further characterized for SNPs according to the presence (+) or absence (-) of the 1311T and 93C. For haplotype reconstruction, I state that the phase of all males (hemizygote at the X-linked locus) was known. This should greatly improve the performance of the algorithm. A total of 92 different AC/AT/CTT haplotypes were identified by ordering size of the AC, then AT, then CTT repeats and are summarized in Table 4.16.

Table 4.10 *G6PD* microsatellite allele frequencies of AC repeat and heterozygosity values in populations. Het. represents heterozygosity

Population	N	172	174	176	178	180	Het.
Thai	107	19(0.18)	53(0.50)	13(0.12)	22(0.21)	0(0.00)	0.6661
Lao	58	17(0.29)	20(0.34)	7(0.12)	14(0.24)	0(0.00)	0.7224
Cambodian	73	13(0.18)	17(0.23)	11(0.15)	32(0.44)	0(0.00)	0.6992
Burmese	64	6(0.09)	42(0.66)	3(0.05)	13(0.20)	0(0.00)	0.5171
Mon	49	12(0.24)	29(0.59)	3(0.06)	4(0.08)	1(0.02)	0.5789
Total	351	67	161	37	85	1	

Table 4.11 *G6PD* microsatellite allele frequencies of AC repeat and heterozygosity values in *G6PD* allele. Het. represents heterozygosity.

Allele	N	172	174	176	178	180	Het.
B-/-	165	56(0.34)	103(0.62)	4(0.02)	2(0.01)	0(0.00)	0.494
B-/+	12	0(0.00)	0(0.00)	2(0.17)	10(0.83)	0(0.00)	0.278
B+/-	7	0(0.00)	1(0.14)	3(0.43)	3(0.43)	0(0.00)	0.612
B+/+	57	3(0.05)	0(0.00)	14(0.25)	40(0.70)	0(0.00)	0.444
871+/+	39	0(0.00)	0(0.00)	11(0.28)	28(0.72)	0(0.00)	0.405
871+/-	3	0(0.00)	1(0.33)	0(0.00)	2(0.67)	0(0.00)	0.444
871-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
487-/-	31	2(0.06)	29(0.94)	0(0.00)	0(0.00)	0(0.00)	0.121
1376-/-	6	2(0.33)	4(0.67)	0(0.00)	0(0.00)	0(0.00)	0.444
1360-/-	2	0(0.00)	2(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
1388-/-	6	2(0.33)	4(0.67)	0(0.00)	0(0.00)	0(0.00)	0.444
1024-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
592-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
949-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
563-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
406+/+	1	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0.000
94-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
UK-/-	11	1(0.09)	9(0.82)	1(0.09)	0(0.00)	0(0.00)	0.314
UK-/+	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	1.000
UK+/+	4	0(0.00)	0(0.00)	1(0.25)	3(0.75)	0(0.00)	0.375
Total	351	66	159	37	88	1	351

Table 4.12 *G6PD* microsatellite allele frequencies of AT repeat and heterozygosity values in populations. Het. represents heterozygosity

Population	N	125	131	133	135	137	139	141	143	145	147	149	151	153	155	157	159	161	167	169	Het.
Thai	107	1(0.01)	2(0.02)	18(0.17)	33(0.31)	11(0.10)	11(0.10)	1(0.01)	3(0.03)	8(0.07)	3(0.03)	2(0.02)	6(0.06)	7(0.07)	0(0.00)	0(0.00)	0(0.00)	1(0.01)	0(0.00)	0(0.00)	0.8399
Lao	58	0(0.00)	3(0.05)	11(0.19)	13(0.22)	2(0.03)	4(0.07)	2(0.03)	1(0.02)	9(0.16)	0(0.00)	0(0.00)	5(0.09)	5(0.09)	1(0.02)	1(0.02)	0(0.00)	1(0.02)	0(0.00)	0(0.00)	0.8639
Cambodian	73	0(0.00)	0(0.00)	9(0.12)	14(0.19)	5(0.07)	3(0.04)	1(0.01)	5(0.07)	3(0.04)	5(0.07)	5(0.07)	15(0.21)	5(0.07)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	3(0.04)	0(0.00)	0.8771
Burmese	64	0(0.00)	1(0.02)	2(0.03)	23(0.36)	16(0.25)	6(0.09)	0(0.00)	4(0.06)	4(0.06)	0(0.00)	3(0.05)	3(0.05)	0(0.00)	0(0.00)	0(0.00)	1(0.02)	0(0.00)	0(0.00)	1(0.02)	0.7856
Mon	49	1(0.02)	2(0.04)	8(0.16)	14(0.29)	14(0.29)	4(0.08)	0(0.00)	1(0.02)	0(0.00)	1(0.02)	3(0.06)	0(0.00)	1(0.02)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.7963
Total	351	2	8	48	97	48	28	4	14	24	9	13	29	18	1	1	1	2	3	1	

Table 4.13 *G6PD* microsatellite allele frequencies of AT repeat and heterozygosity values in *G6PD* allele. Het. represents heterozygosity

Allele	N	125	131	133	135	137	139	141	143	145	147	149	151	153	155	157	159	161	167	169	Het.
B-/-	165	1(0.01)	7(0.04)	37(0.22)	73(0.44)	15(0.09)	19(0.12)	3(0.02)	1(0.01)	6(0.04)	2(0.01)	0(0.00)	1(0.01)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.729
B-/+	12	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.08)	0(0.00)	5(0.42)	3(0.25)	0(0.00)	2(0.17)	1(0.08)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.722
B+/-	7	0(0.00)	0(0.00)	1(0.14)	2(0.29)	0(0.00)	1(0.14)	0(0.00)	0(0.00)	1(0.14)	1(0.14)	1(0.14)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.816
B+/+	57	1(0.02)	1(0.02)	8(0.14)	0(0.00)	2(0.04)	(0.00)	1(0.02)	8(0.14)	11(0.19)	5(0.09)	8(0.14)	4(0.07)	4(0.07)	1(0.02)	1(0.02)	1(0.02)	1(0.02)	0(0.00)	0(0.00)	0.883
871+/+	39	0(0.00)	0(0.00)	0(0.00)	1(0.03)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.03)	0(0.00)	1(0.03)	21(0.54)	14(0.36)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.03)	0(0.00)	0.579
871+/-	3	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.33)	0(0.00)	1(0.33)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.33)	0(0.00)	0.667
871-/-	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
487-/-	31	0(0.00)	0(0.00)	0(0.00)	5(0.16)	26(0.84)	(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.271
1376-/-	6	0(0.00)	0(0.00)	0(0.00)	0(0.00)	3(0.50)	2(0.33)	0(0.00)	0(0.00)	1(0.17)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.611
1360-/-	2	0(0.00)	0(0.00)	0(0.00)	1(0.50)	0(0.00)	1(0.50)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.500
1388-/-	6	0(0.00)	0(0.00)	2(0.33)	4(0.67)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.444
1024-/-	1	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
592-/-	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
949-/-	1	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
563-/-	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
406+/+	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0.000
94-/-	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
UK-/-	11	0(0.00)	0(0.00)	0(0.00)	7(0.64)	1(0.09)	1(0.09)	0(0.00)	0(0.00)	1(0.09)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.09)	0(0.00)	0.562
UK-/+	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
UK+/+	4	0(0.00)	0(0.00)	0(0.00)	1(0.25)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.25)	1(0.25)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.25	0(0.00)	0(0.00)	0.750
Total	351	2	8	48	96	48	29	4	14	24	9	13	29	18	1	1	1	2	3	1	

Table 4.14 *G6PD* microsatellite allele frequencies of CTT repeat and heterozygosity values in population. Het. represents heterozygosity

Population	N	192	195	198	201	204	207	210	213	216	Het.
Thai	107	0(0.00)	25(0.23)	38(0.36)	13(0.12)	1(0.01)	14(0.13)	12(0.11)	3(0.03)	1(0.01)	0.7739
Lao	58	0(0.00)	19(0.33)	14(0.24)	4(0.07)	1(0.02)	11(0.19)	8(0.14)	1(0.02)	0(0.00)	0.7741
Cambodian	73	1(0.01)	8(0.11)	10(0.14)	10(0.14)	3(0.04)	28(0.38)	11(0.15)	1(0.01)	1(0.01)	0.7784
Burmese	64	1(0.02)	17(0.27)	24(0.38)	3(0.05)	4(0.06)	11(0.17)	3(0.05)	1(0.02)	0(0.00)	0.7505
Mon	49	1(0.02)	23(0.47)	16(0.33)	2(0.04)	3(0.06)	3(0.06)	1(0.02)	0(0.00)	0(0.00)	0.6631
Total	351	3	92	102	32	12	67	35	6	2	

Table 4.15 *G6PD* microsatellite allele frequencies of CTT repeat and heterozygosity values in *G6PD* alleles. Het. represents heterozygosity

Allele	N	192	195	198	201	204	207	210	213	216	Het.
B-/-	165	3(0.02)	53(0.32)	63(0.41)	30(0.18)	6(0.04)	4(0.02)	1(0.01)	0(0.00)	0(0.00)	0.692
B-/+	12	0(0.00)	0(0.00)	1(0.08)	0(0.00)	0(0.00)	1(0.08)	6(0.50)	3(0.25)	1(0.08)	0.667
B+/-	7	0(0.00)	0(0.00)	3(0.43)	0(0.00)	2(0.29)	2(0.29)	0(0.00)	0(0.00)	0(0.00)	0.653
B+/+	57	0(0.00)	3(0.05)	6(0.11)	0(0.00)	4(0.07)	25(0.44)	15(0.26)	3(0.05)	1(0.02)	0.717
871+/+	39	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	29(0.74)	10(0.26)	0(0.00)	0(0.00)	0.381
871+/-	3	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	3(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
871-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
487-/-	31	0(0.00)	20(0.65)	11(0.35)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.458
1376-/-	6	0(0.00)	2(0.33)	4(0.67)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.444
1360-/-	2	0(0.00)	1(0.50)	1(0.50)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.500
1388-/-	6	0(0.00)	3(0.50)	3(0.50)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.500
1024-/-	1	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
592-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
949-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
563-/-	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
406+/+	1	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0.000
94-/-	1	0(0.00)	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
UK-/-	11	0(0.00)	5(0.45)	4(0.36)	1(0.09)	0(0.00)	1(0.09)	0(0.00)	0(0.00)	0(0.00)	0.645
UK-/+	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0.000
UK+/+	4	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	2(0.50)	2(0.50)	0(0.00)	0(0.00)	0.500
Total	351	3	92	102	32	12	68	34	6	2	

Table 4.16 Frequencies of haplotypes in *G6PD* mutations of Southeast Asian

No.	Microsatellite haplotype			*G6PD* mutations and SNPs (nt1311, nt93)																				Total
Haplotype	AC	AT	CTT	B-/-	B-/+	B+/-	B+/+	871+/+	871+/-	871-/-	487-/-	1376-/-	1360-/-	1388-/-	1024-/-	592-/-	949-/-	563-/-	406+/+	94-/-	UK-/-	UK-/+	UK+/+	
1	172	131	195	4																				4
2	172	133	192	1																				1
3	172	133	195	9										1										10
4	172	133	198	12		1	1																	14
5	172	133	201	2																				2
6	172	133	204	1																				1
7	172	135	192	1																				1
8	172	135	195	7																	1			8
9	172	135	198	2							2			1										5
10	172	135	201	1																				1
11	172	135	204	1																				1
12	172	137	195	4			1																	5
13	172	137	198	2								1												3
14	172	139	195	1																				1
15	172	139	198	1								1												2
16	172	141	195	1			1																	2
17	172	141	198	1																				1
18	172	145	195	5																				5
19	174	125	195	1																				1
20	174	131	195	1																				1

Table 4.16 Frequencies of haplotypes in *G6PD* mutations of Southeast Asian (cont.)

No.	Microsatellite haplotype			*G6PD* mutations and SNPs (nt1311, nt93)																				Total
Haplotype	AC	AT	CTT	B-/-	B-/+	B+/-	B+/+	871+/+	871+/-	871-/-	487-/-	1376-/-	1360-/-	1388-/-	1024-/-	592-/-	949-/-	563-/-	406+/+	94-/-	UK-/-	UK-/+	UK+/+	
21	174	131	198	2																				2
22	174	133	195	4																				4
23	174	133	198	7										1										8
24	174	133	201	1																				1
25	174	135	195	11							2			2			1				2			18
26	174	135	198	23		2					1		1	1							4			32
27	174	135	201	22											1									23
28	174	135	204	2																				2
29	174	135	207	1																				1
30	174	137	192	1																				1
31	174	137	195								18	1												19
32	174	137	198	5							8	1								1				15
33	174	137	201	3																	1			4
34	174	139	195	5						1		1	1			1		1			1			11
35	174	139	198	11																				11
36	174	139	204	1		1																		2
37	174	141	204	1																				1
38	174	143	198	1																				1
39	174	145	195																		1			1
40	174	145	198									1												1

Table 4.16 Frequencies of haplotypes in *G6PD* mutations of Southeast Asian (cont.)

No.	Microsatellite haplotype			*G6PD* mutations and SNPs (nt1311, nt93)																			Total
Haplotype	AC	AT	CTT	B-/-	B-/+	B+/-	B+/+	871+/+	871+/-	871-/-	487-/-	1376-/-	1360-/-	1388-/-	1024-/-	592-/-	949-/-	563-/-	406+/+	94-/-	UK-/-	UK-/+	UK+/+	
41	174	167	207						1														1
42	176	131	207				1																1
43	176	133	198				1																1
44	176	133	207				1																1
45	176	135	198	1																			1
46	176	137	207				1																1
47	176	143	195				1																1
48	176	143	207		1		3																4
49	176	145	204			1																	1
50	176	145	207	1			2																3
51	176	147	207	2		1	1																4
52	176	147	213				1																1
53	176	149	207			1	1																2
54	176	149	213				1																1
55	176	151	207					8														1	9
56	176	151	210		1			1															2
57	176	153	207					1															1
58	176	153	210					1															1
59	176	167	207																		1		1
60	176	169	207																1				1

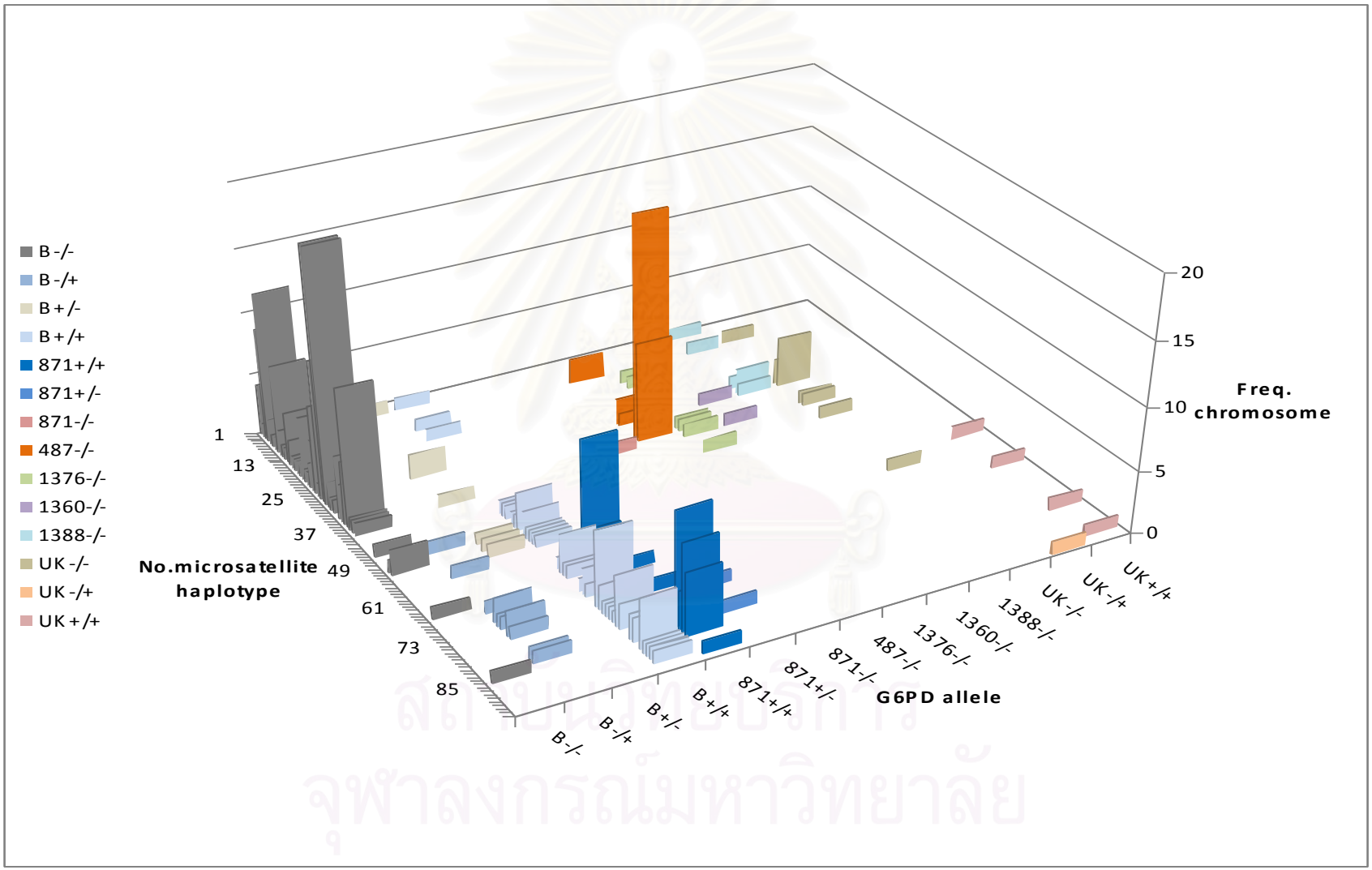
Table 4.16 Frequencies of haplotypes in *G6PD* mutations of Southeast Asian (cont.)

No.	Microsatellite haplotype			*G6PD* mutations and SNPs (nt1311, nt93)																				Total
Haplotype	AC	AT	CTT	B-/-	B-/+	B+/-	B+/+	871+/+	871+/-	871-/-	487-/-	1376-/-	1360-/-	1388-/-	1024-/-	592-/-	949-/-	563-/-	406+/+	94-/-	UK-/-	UK-/+	UK+/+	
61	178	125	207				1																	1
62	178	133	198				3																	3
63	178	133	207				1																	1
64	178	133	210				1																	1
65	178	135	201	1																				1
66	178	135	207					1															1	2
67	178	139	198		1																			1
68	178	143	198				1																	1
69	178	143	207				2																	2
70	178	143	210		2		1																	3
71	178	143	213		1																			1
72	178	143	216		1																			1
73	178	145	207				6	1																7
74	178	145	210		2		2																	4
75	178	145	213		1		1																	2
76	178	147	207				1		1															2
77	178	147	210				1																	1
78	178	147	216				1																	1
79	178	149	204				4																	4
80	178	149	207				2	1																3

Table 4.16 Frequencies of haplotypes in *G6PD* mutations of Southeast Asian (cont.)

No.	Microsatellite haplotype			*G6PD* mutations and SNPs (nt1311, nt93)																			Total
Haplotype	AC	AT	CTT	B-/-	B-/+	B+/-	B+/+	871+/+	871+/-	871-/-	487-/-	1376-/-	1360-/-	1388-/-	1024-/-	592-/-	949-/-	563-/-	406+/+	94-/-	UK-/-	UK-/+	UK+/+	
81	178	149	210		1																	1	2
82	178	149	213		1																		1
83	178	151	207				2	9	1														12
84	178	151	210	1			2	3															6
85	178	153	207					7															7
86	178	153	210				4	5															9
87	178	155	210				1																1
88	178	157	210				1																1
89	178	159	210				1																1
90	178	161	210				1															1	2
91	178	167	207					1															1
92	180	139	195																			1	1
Total				165	12	7	57	39	3	1	31	6	2	6	1	1	1	1	1	1	11	1	4	351

Figure 4.14 Relative frequencies of AC/AT/CTT microsatellite haplotypes on G6PD B and *G6PD* mutation chromosomes. The presence (+) or absence (-) of 1311T/ 93C is defined.



The highest haplotype diversity was found on G6PD B (+/+), G6PD B (-/+), and G6PD B (-/-) (H = 0.98 ± 0.01, 0.97 ± 0.04, and 0.94 ± 0.01). A moderate level of haplotype diversity was found on G6PD Viangchan^{871A} (871+/+) (H = 0.87 ± 0.03) and the most restricted variability was on G6PD Mahidol^{487A} (487-/-) (H = 0.61 ± 0.08) (Table 4.17).

Table 4.17 Heterozygosity value of *G6PD* alleles

Allele	N	Heterozygosity (SD.)
B+/+	57	0.98 (0.01)
B-/+	12	0.97 (0.04)
B+/-	7	0.95 (0.10)
B-/-	165	0.94 (0.01)
871+/+	39	0.87 (0.03)
871+/-	3	1.00 (0.27)
487-/-	31	0.61 (0.08)

Pattern of microsatellite haplotype variability and scatter plot of LD associated with the different *G6PD* alleles are shown in Figure 4.12-15. The microsatellite haplotypes of G6PD B (-/-) alleles and G6PD B (+/+) alleles were clearly different. G6PD B (-/-) alleles were associated with either a 172- or 174-bp AC alleles, while G6PD B (+/+) alleles were associated with either a 176- or 178-bp AC alleles. There were wider ranges of AT alleles on G6PD B (+/+) alleles. Moreover, G6PD B (+/+) alleles always associated with only large-sized CTT alleles ranging from 198- to 210-bp. In contrast, G6PD B (-/-) alleles had primarily small CTT alleles (range from 192- to 204-bp). Microsatellite haplotypes on G6PD B (-/+) alleles were those found in a group of G6PD B (+/+), which was opposite to haplotypes on G6PD B (+/-) alleles that were a subset of G6PD B (-/-) group. G6PD Viangchan^{871A} (871+/+), (871+/-) alleles appeared to have microsatellite haplotype variability presenting in G6PD B (+/+) group with four common microsatellite

haplotypes containing 176- to 178-bp AC alleles, 151- to 153-bp AT alleles, and 207- to 210-bp alleles. Microsatellite haplotype diversity of G6PD Mahidol^{487A} (487-/-) alleles were those in a group of G6PD B(-/-) alleles with only two major microsatellite haplotypes containing a 174-bp AC allele, a 137-bp AT allele, and 195- to 198-bp CTT alleles. The pattern of haplotype diversity on G6PD Mahidol^{487A} was identical in all populations, indicating a single common ancestor of the G6PD Mahidol^{487A} in Thai, Burmese, and Mon.

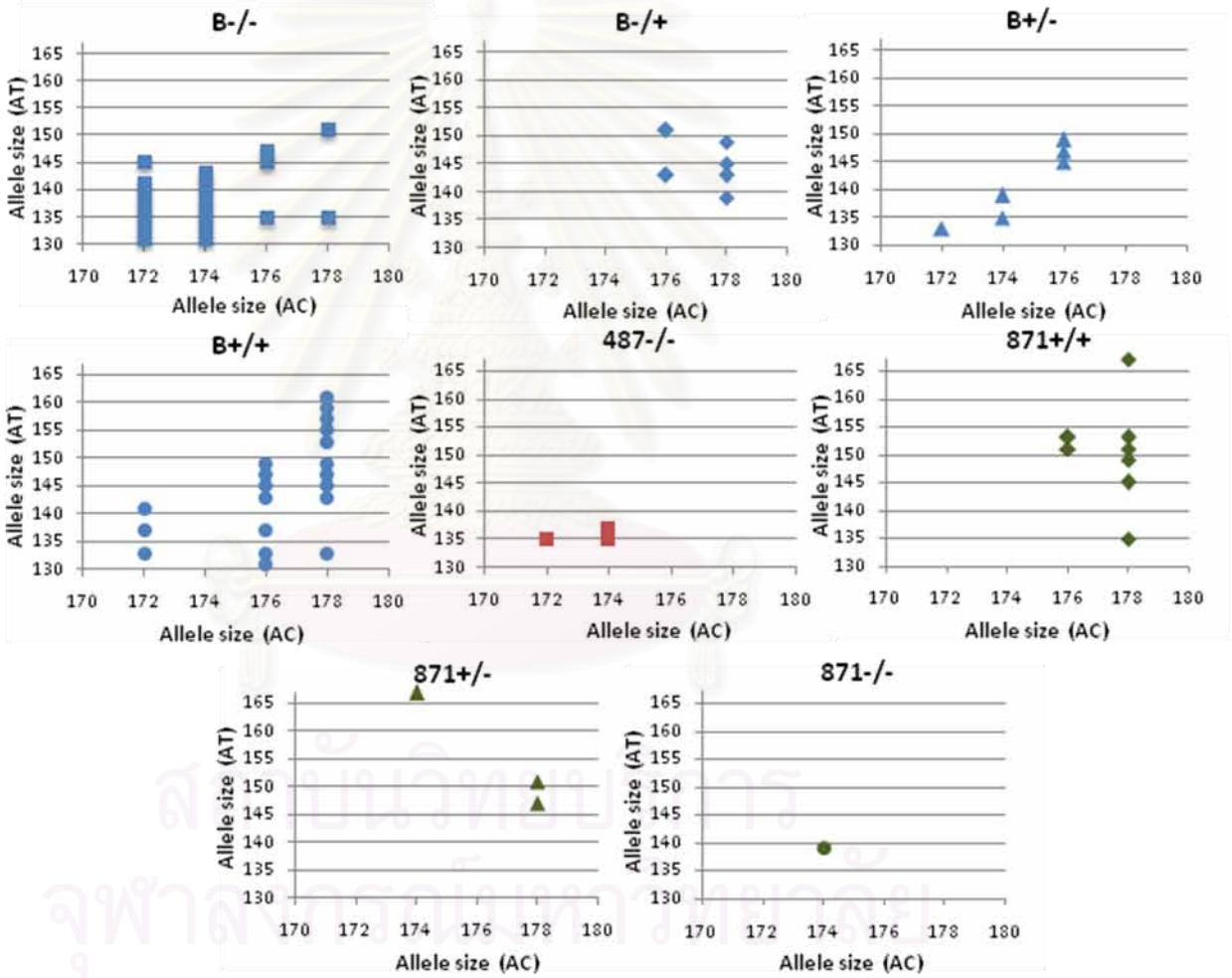


Figure 4.15 Scatter plot of distribution of AC and AT allele on various *G6PD* alleles. The clustering of points represents LD between microsatellite and *G6PD* alleles. 871+/+: green diamonds; 871+/-: green triangles; 871-/-: green circles; 487-/-: red squares; B-/-: blue squares; B-/+: blue diamonds; B+/-: blue triangles; B+/+: blue circles.

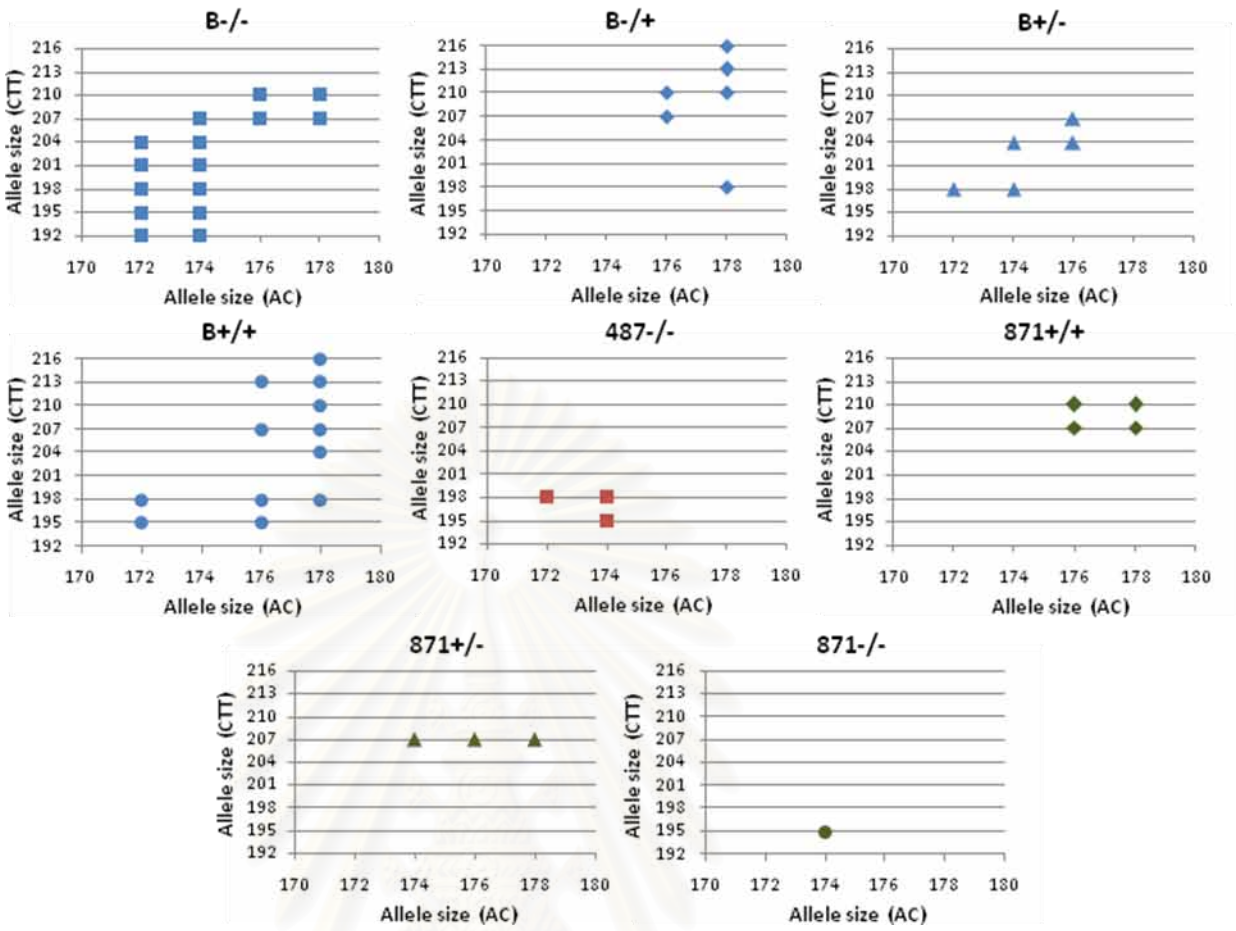


Figure 4.16 Scatter plot of distribution of AC and CTT allele on various *G6PD* alleles. The clustering of points represents LD between microsatellite and *G6PD* alleles. 871+/+: green diamonds; 871+/-: green triangles; 871-/-: green circles; 487-/-: red squares; B-/-: blue squares; B-/+: blue diamonds; B+/-: blue triangles; B+/+: blue circles.

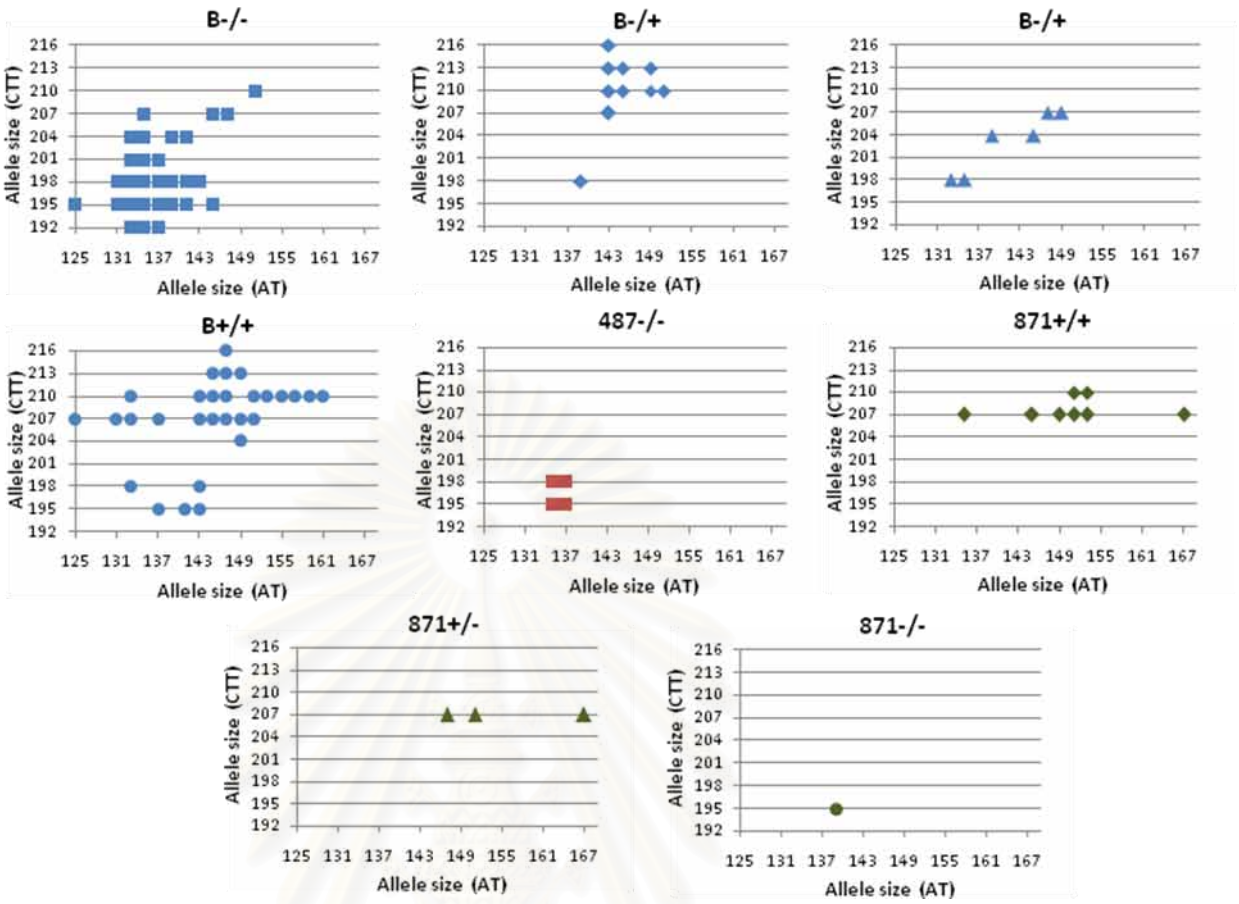


Figure 4.17 Scatter plot of distribution of AT and CTT allele on various *G6PD* alleles. The clustering of points represents LD between microsatellite and *G6PD* alleles. 871+/+: green diamonds; 871+/-: green triangles; 871-/-: green circles; 487-/-: red squares; B-/-: blue squares; B-/+: blue diamonds; B+/-: blue triangles; B+/+: blue circles.

The low allelic microsatellite diversity and high frequency of microsatellite haplotypes on G6PD Mahidol^{487A} (-/-) indicated the recent or strong positive selection theory of G6PD Mahidol^{487A} (-/-) in Southeast Asian population. G6PD Viangchan^{871A} (+/+) presented high allelic microsatellite diversity and low frequency of microsatellite haplotype supporting the hypothesis that it is an ancient allele in Southeast Asian.

3.2 Long Range Haplotype (LRH) of G6PD Mahidol^{487A} and G6PD Viangchan^{871A}

LRH test was performed to identify mutations/ haplotypes under recent positive selection. Three hundred and eighty four Karen randomly selected from unrelated individuals were genotyped using 30 SNPs –including the Mahidol mutation dispersed along a 2.4-Mb region encompassing the *G6PD* gene. The PCR-RFLP result of rs3752409 genotyping is shown in Figure 4.16.

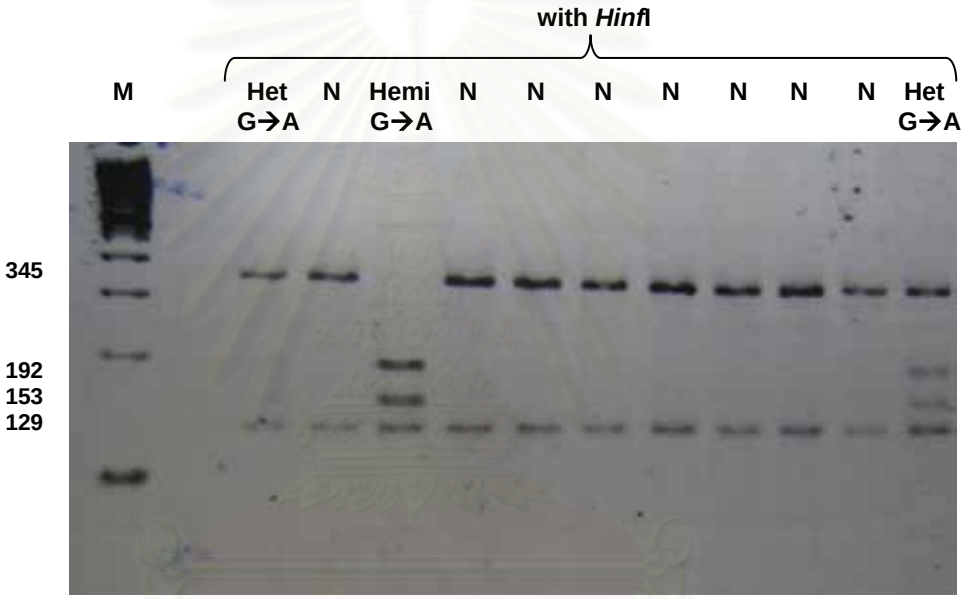


Figure 4.18 PCR-RFLP approach in detection rs3752409. M represents molecular weight marker. PCR-RFLP results showed a single band of 474 bp in undigested sample with *Hinf*I, three bands of 129, 153, and 192 bp in digested hemizygote G→A (Hemi G→A with *Hinf*I), four bands of 129, 153, 192, and 345 bp in digested heterozygote G→A (Het G→A with *Hinf*I), and two bands of 129 and 345 bp in digested normal control (N).

The phase of extended haplotypes was reconstructed using the PHASE program, assuming that male haplotypes were known haplotype. Then, LRH test was performed to define the recent positive selection by comparing the frequency of G6PD Mahidol^{487A} with the breakdown of LD of SNPs dataset around it (Sabeti *et al.*, 2002). This test detects an excess of homozygosity at SNPs associated with a given mutation and is thus a proxy for the age of the mutation. When the homozygosity around an allele is unexpectedly high with respect to the frequency of the mutation, the allele has arisen to high frequency too rapidly for its estimated age and is a sign of recent positive selection (Sabeti *et al.*, 2002). In this population, the G6PD Mahidol^{487A} mutation showed extreme levels of extended homozygosity: 63% of the Mahidol-bearing haplotypes showed complete haplotype conservation over the entire 2.4-Mb region (Figure 4.17-19), with no evidence of recombination or mutation events.

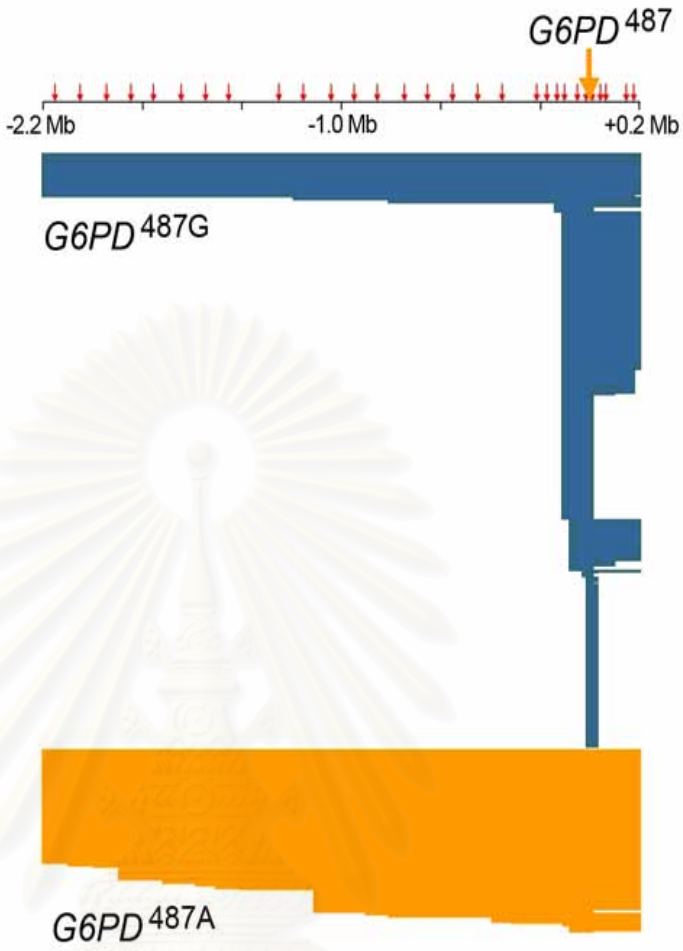


Figure 4.19 Comparison of extents of homozygozity flanking the G6PD Mahidol^{487A} allele and the non G6PD Mahidol^{487G} allele over a 2.4-Mb region. Positions of the 30 genotyped SNPs are tagged by red arrows.

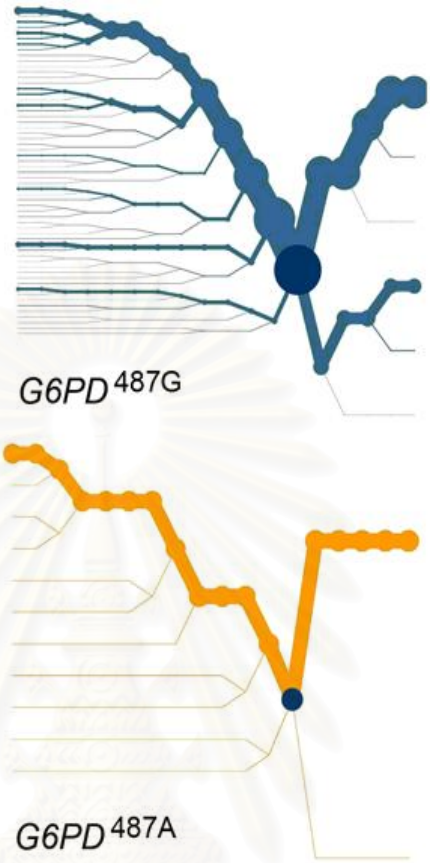


Figure 4.20 Haplotype bifurcation plots for the two G6PD 487 alleles. The thickness of lines represents the number of haplotypes in our Thai sample. Each successive bifurcation corresponds to evidence for recombination at an increasing distance from the core G6PD 487 alleles (dark blue dots).

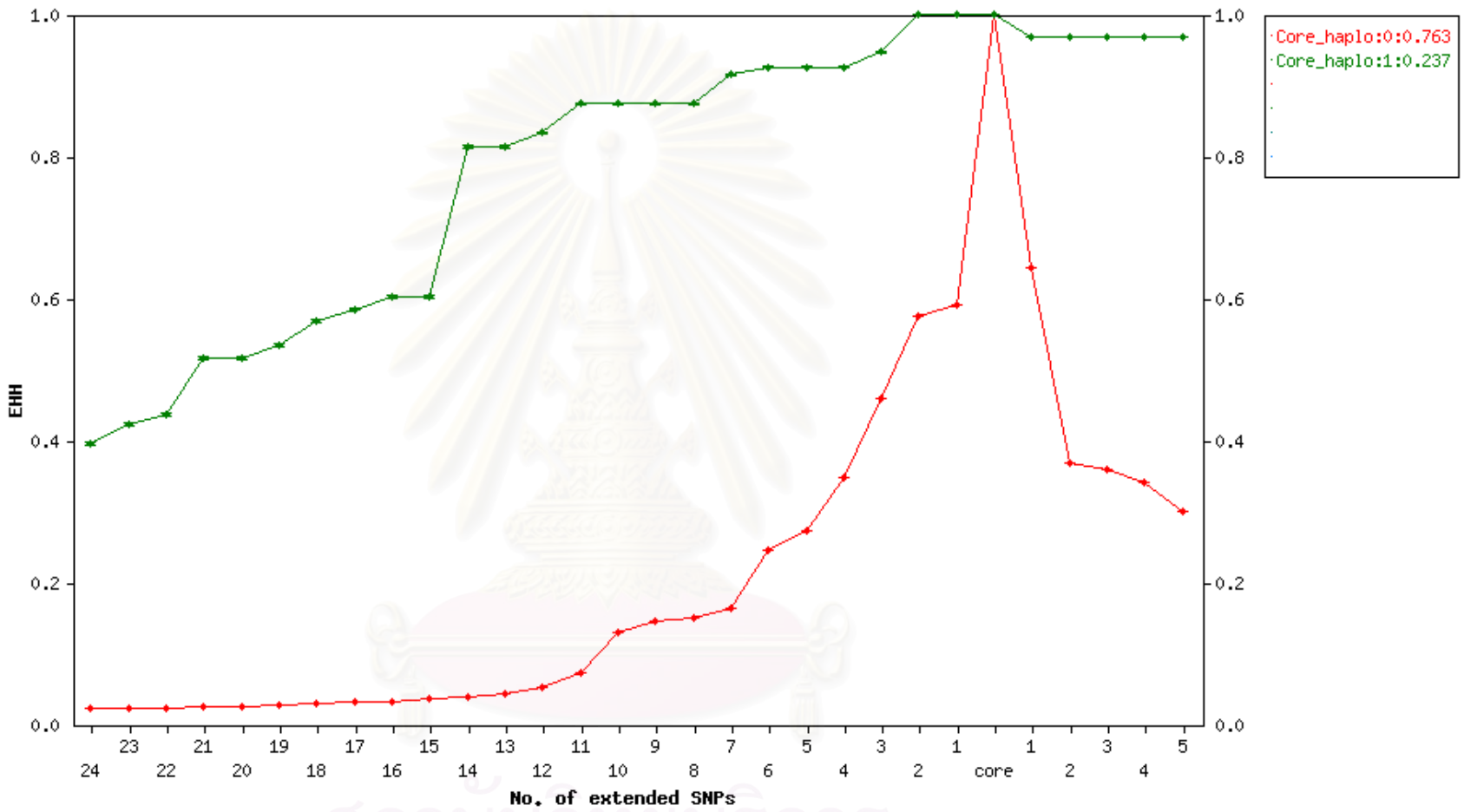


Figure 4.21 Extended haplotype homozygosity (EHH) at varying extended SNPs from G6PD Mahidol region (core) on G6PD Mahidol^{487A} haplotype (core_haplo:1: green line) and non G6PD Mahidol^{487G} haplotype (core_haplo:0: red line).

A comparison of this observation with the empirical distribution of allelic homozygosity *vs*. frequency for the X chromosome retrieved from HapMap Phase II data in Han Chinese was performed after matching for SNP density (Figure 4.20). The excess of homozygosity around the Mahidol mutation was highly significant given its frequency ($P<6.7x10^{-4}$). Thus, the analysis clearly shows that the Mahidol mutation is under strong and recent positive selection in the population, pointing to a strong selective advantage conferred by this mutation on human survival.

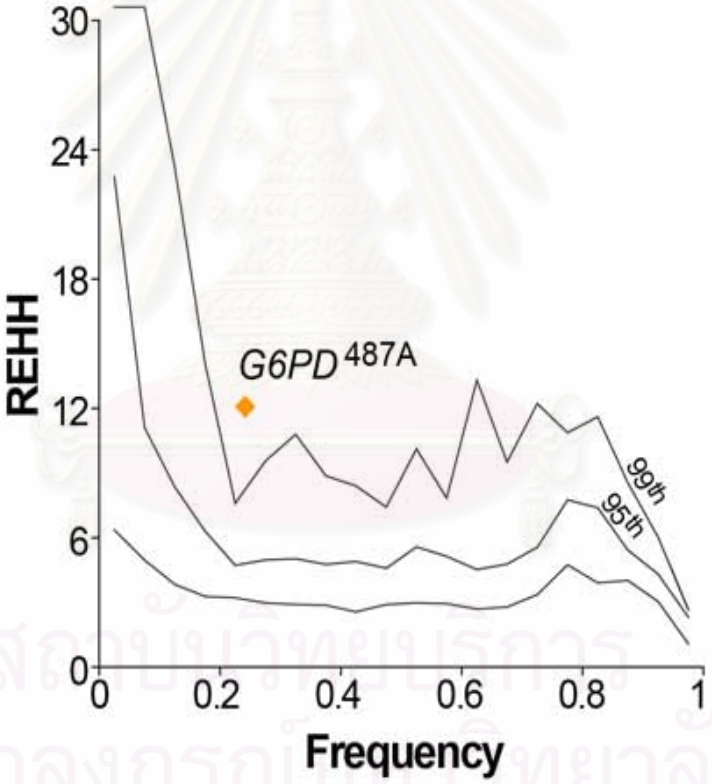


Figure 4.22 Relative Extended Haplotype Homozygosity (REHH) of the Mahidol allele (orange diamond) against an empirical distribution of REHH retrieved from HapMap II dataset of the X chromosome of Han Chinese, matched for allele frequency and SNP density. The 95th and 99th percentiles of the empirical distribution were reported.

Estimating the age and the selection coefficient necessary to explain such a signal of strong positive selection was next performed. Using different methods (Austerlitz *et al.,* 2003; Slatkin 2008), I obtained comparable estimates: the mutation started to grow in frequency ~1,500 years before present (YBP) with a selection intensity of ~0.23 (Table 4.18).

Table 4.18 Age and intensity of positive selection targeting the G6PD Mahidol^{487A} in Karen populations. ML stands for Maximum-Likelihood and CI for Confidence Interval.

Method	Age (generations)	95% CI	Selection coefficient	95% CI
Bayesian method (Slatkin 2008)	64.4	38-94	0.228	0.16-0.40
ML deterministic method (Austerlitz *et al.,* 2003)	60.8	53.7-73.6	0.235	0.20-0.30

4. Epidemiological Study

To determine the nature of the selective advantage conferred by the Mahidol^{487A} mutation, I examined the effect of this mutation on the outcome of infection by either *P. falciparum* or *P. vivax*. To this end, I analyzed a community-based longitudinal cohort study in the Suan Phung district of Thailand. Suan Phung has a total population of 5,368 living in seven hamlets, the majority of whom are Karen. Among these villagers 3,484 of all ages, participated in the study. Between 1998 and 2005 there were 19,162 independent clinical presentations were reported for 2,545 individuals. Of these 2,430 cases from 1,120 individuals were positive for *P. falciparum*. *P. vivax* was found in 1,280 cases, from 636 individuals. I obtained reliable parasite (trophozoite) density data for 1,795 slides from 949 individuals for *P. falciparum*, and for 975 slides from 517 individuals for *P. vivax*, 39 slides with mixed parasite species were excluded. We obtained genotypes at G6PD Mahidol^{487A} position

(i.e. G6PD B/G6PD B, 487A/G6PD and 487A/487A for females; G6PD B/Y and 487A/Y for males) for 515 individuals infected by either of the two parasite species (Table 4.19). This included 800 observations on *P. falciparum* parasite density in 390 individuals and 222 individuals with 411 observations on *P. vivax* parasite density.

Table 4.19 Number of people with each G6PD Mahidol^{487A} status group

Specie	G6PD B/Y Male	G6PD B/G6PD B Female	487A/Y Male	487A/G6PD B Female	487A/487A Female
P. falciparum	173	98	47	65	7
P. vivax	108	54	24	33	3

We first performed a statistical analysis of the effect of G6PD Mahidol^{487A} on the maximum parasite density experienced by each individual taking into account age and environmental covariates. Both age and Mahidol^{487} genotype had a significant effect on *P. vivax* parasite density (Fig. 4.21A). Parasite density decreased with age (χ^2_2=20.95, P<0.001), indicative of the acquisition of anti-parasite immunity. Accounting for age, G6PD Mahidol^{487A} carriers had significantly lower *P. vivax* parasite density than non-carriers (χ^2_1=8.39, P=0.004). There was no significant difference in *P. vivax* parasite density between 487A/Y, G6PD B/487A and 487A/487A individuals (Fig. 4.21B). However, analysing sexes separately revealed a more significant protective effect for hemizygous 487A/Y males than heterozygous 487A/G6PD B females, with respect to their *G6PD* normal counterparts (Males χ^2_1=6.46, P=0.011, females χ^2_1=2.42 P=0.12). 487A/487A females could not be analysed independently because only three 487A/487A females infected by *P. vivax* were observed in our study. The presence of G6PD Mahidol^{487A} accounted for 3.1% of the observed variation in *P. vivax* parasite density. By contrast, although increasing age was again associated with decreasing *P. falciparum* parasite density (χ^2_2=52.02, P<0.001), G6PD Mahidol^{487A} was associated with a significant increase in *P.*

falciparum parasite density (χ^2_1=4.35, *P*=0.037, Fig. 4.21C). However, this significant association was explained by only seven 487A/487A females found to be infected by *P. falciparum* (χ^2_1= 22.38 *P*<0.001) (Fig. 4.21D). By contrast to the effect of this mutation on parasite density phenotypes, G6PD Mahidol^{487A} had no effect on the number of cases of clinical malaria due to either *P. vivax* or *P. falciparum*, reported for each individual during the seven-year observation period.

To avoid spurious associations that may result from population admixture or stratification, we then carried out a family-based association study using FBAT method. FBAT compares the expected genotype of an offspring, based on parental genotypes and the null hypothesis of no association, with the observed genotype(s) in each family. I studied 35 nuclear families with at least one parent who was heterozygote or hemizygote for G6PD Mahidol^{487A} and at least one offspring who presented with *P. vivax* infection. I found that G6PD Mahidol^{487A} was significant associated with lower maximum *P. vivax* parasite density (*P*=0.018). A permutation test with 10,000 iterations confirmed that these results were significant (*P*=0.019). We included 44 families with sufficient data to investigate association with *P. falciparum* infection. G6PD Mahidol^{487A} was significantly associated with higher maximum *P. falciparum* density (*P*=0.016).

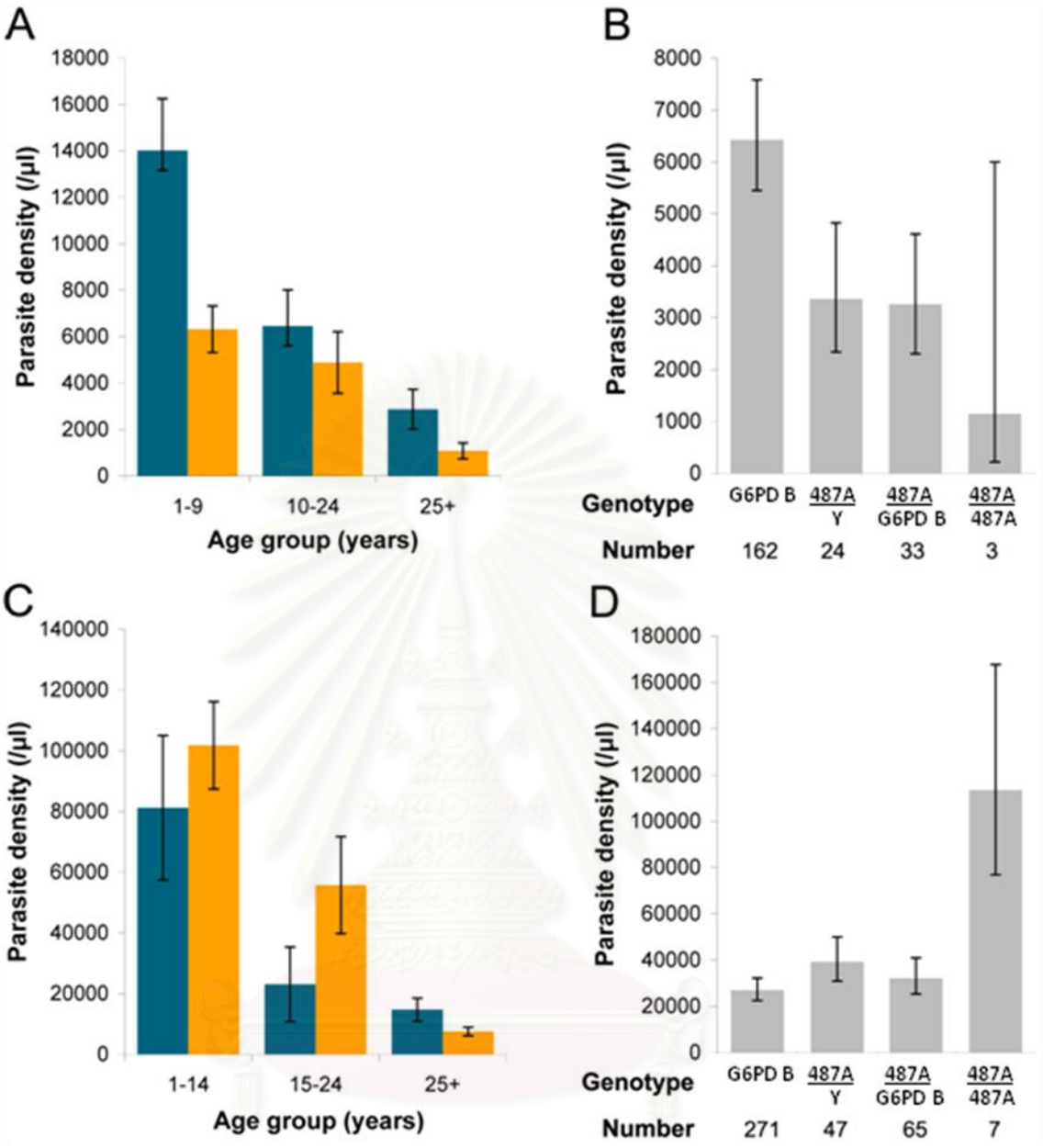


Figure 4.23 Effect of G6PD status on parasite densities

(**A**) *P. vivax* and (**C**) *P. falciparum* maximum parasite densities (mean ±SE) experienced by each individual according to age group and G6PD Mahidol^{487A} status (blue – G6PD Mahidol^{487A} negative [wildtype], yellow – G6PD Mahidol^{487A} positive [mutant]). (**B**) *P. vivax* and (**D**) *P. falciparum* maximum densities (mean±SE) according to G6PD Mahidol^{487A} genotype status (Normal and 3 deficient classes: Heterozygote mutant females, Homozygote mutant females and Hemizygote mutant males) taking into account age.

CHAPTER V

CONCLUSION AND DISCUSSION

Since G6PD deficiency is highly prevalent in Southeast Asian, its molecular heterogeneity has been widely investigated in various ethnic groups. However, there are minority tribes; Mon and Karen, who inhabit the Burma-Thailand border, and who have never been characterized with respect to their G6PD deficiency prevalence and molecular mutations. Therefore, moreover, it has been proposed that the high overall population frequency of deficiency alleles result from their otherwise protective effect against malaria: the global distribution of G6PD deficiency overlaps with that of malaria (Allison 1960). Analyses of the naturally-occurring variation at the *G6PD* locus support the occurrence of local and recent positive selection targeting G6PD-deficient alleles (G6PD A-$^{202A/376G}$) in Africa (Tishkoff *et al.,* 2001; Sabeti *et al.,* 2002). Here, the prevalence of G6PD deficiency, *G6PD* mutations in Southeast Asian and its positive selection force in protective against malaria infection were investigated.

1. Prevalence of G6PD Deficiency in Southeast Asian

In the study of Karen population the prevalence of G6PD deficiency was found to be high in Karen males (23.5%) and females (8.2%). The higher prevalence of G6PD deficiency in males is largely a consequence of X-linked location of *G6PD* gene, with thus complete penetrance of any significant mutation in hemizygous males as opposed to variable expression in heterozygous females;

random inactivation of one of the pair of X chromosomes in heterozygous females leads to variable effects on G6PD activity.

In Mon and Burmese, the results from my previous study indicated the prevalence of G6PD deficiency in Mon and Burmese males were 12% and 10%, respectively (Nuchprayoon *et al.,* 2008), comparable with results from other studies reporting that G6PD deficiency in Burmese was 7.3-11% (Iwai *et al.,* 2001; Matsuoka *et al.,* 2004). However, with a Mon population size approximately 3.6 times larger than that of reported by Iwai's group (3 of 42; 6.7%) (Iwai *et al.,* 2001), my results may more accurately represent the prevalence of G6PD deficiency in Mon.

In Cambodians, I found that G6PD deficiency was 26.1% in Cambodian males (Louicharoen and Nuchprayoon 2005), which is comparable to that of northeastern Thai (21.7%) (Kittiwatanasarn *et al.,* 2003). Concurrently, additional studies reported 13.4% and 12.6% of G6PD deficiency in Cambodian boys in central Cambodia and in Cambodian villagers in Battambang, Kampot, and Rattanakiri provinces, respectively (Monchy *et al.,* 2004; Matsuoka *et al.,* 2005).

The overall high level of G6PD deficiency in Karen, Cambodian, Burmese, and Mon are similar to other ethnic groups in Thailand and neighbouring countries in Southeast Asia, including 10.8% in Shans (Iwai *et al.,* 2001), 6.3 and 7.1% in Kachin and Danu ethnic groups in Myanmar (Iwai *et al.,* 2001), 7.2% in Lao (Iwai *et al.,* 2001), and 9.8% in southern Phuket islanders (Ninokata *et al.,* 2006). In this study, a high prevalence of G6PD deficiency among Southeast Asians was postulated to be due to selection pressure from malaria, which was hyperendemic in Southeast Asia (Flatz G 1963). The mechanism of resistance to malaria infection is still controversial, but it is probable that G6PD deficiency could reduce NADPH in oxidative stress, thus affecting growth of *Plasmodium* (Beutler 1994).

2. Molecular Heterogeneity of *G6PD* Mutations in Southeast Asia and Their Evolutionary History

The distribution of G6PD-deficient molecular mutations in Southeast Asia varies with ethnic groups. From molecular analysis, there are two most common *G6PD* mutations in Southeast Asia; G6PD Viangchan^{871A} and G6PD Mahidol^{487A}. Taking all regional evidence together, the *G6PD* mutations in the Southeast Asian peninsular can be described as follows. Burmese, Karen, and Mon in the west of the peninsular are quite homogenous for G6PD Mahidol^{487A} (Iwai *et al.,* 2001). Lao and Cambodian in the eastern part of the peninsular are relatively homogeneous for G6PD Viangchan^{871A}. On the other hand, Thai in the central peninsular are heterogeneous.

G6PD Viangchan^{871A}, a dominant mutation among Cambodian (82-98%) (Louicharoen and Nuchprayoon 2005; Matsuoka *et al.,* 2005), Thai (31-54%) (Nuchprayoon *et al.,* 2002; Laosombat *et al.,* 2005), Lao (46-100%) (Hsia *et al.,* 1993; Iwai *et al.,* 2001), and the ethnic groups in Vietnam (Kinh and K'Ho) (32% and 100%) (Matsuoka *et al.,* 2007), was infrequently found in Karen (4%). Indeed, G6PD Viangchan^{871A} has not been found in Burmese or Mon (Matsuoka *et al.,* 2004; Nuchprayoon *et al.,* 2008). Population admixture of Karen with Thai that carried G6PD Viangchan^{871A} is the likely cause of G6PD Viangchan^{871A} in Karen. From haplotype analysis, all G6PD Viangchan^{871A} in Karen, Lao, Thai, and 94% of G6PD Viangchan^{871A} in Cambodian carried haplotype 1311T, 93C (+/+), which have been previously reported in all G6PD Viangchan^{871A} in Cambodian, Malay, Bajo (Indonesian), and Kinh (Yusoff *et al.,* 2002; Matsuoka *et al.,* 2005; Kawamoto *et al.,* 2006; Matsuoka *et al.,* 2007). Haplotype 1311T, 93C (+/+) was highly prevalent in G6PD-normal Cambodian, Lao, and Thai. My result supports the hypothesis that G6PD Viangchan^{871A} (+/+) in Cambodian, Lao, Malays, Kinh, Bajo, Karen, and Thai

have a common ancestry, likely derived from G6PD-normal (+/+); however 6% of G6PD Viangchan^{871A} (+/-) in Cambodian probably divided from a minority group of G6PD-normal (+/-) (2.5-7.3%). The finding that G6PD Viangchan^{871A} is highly frequent in Cambodian, Lao, and Thai suggests a common ancestry of these populations. Thai (93.5%) and Lao (71%) speak Daic or Thai (Tai) language, whereas Cambodian (94.3%) speaks Khmer, a distinctive Austro-Asiatic language (http://www.ethnologue.com). However, there are many Cambodian refugees in Thailand, including migrant laborers and those married with Thai in border provinces.

G6PD Mahidol^{487A} was found to be the dominant mutation (93%) in Karen as found in other ethnic groups in Myanmar: Burmese (71%) (Iwai *et al.*, 2001; Matsuoka *et al.*, 2004; Nuchprayoon *et al.*, 2008) and Mon (63%) (Nuchprayoon *et al.*, 2008). It was, however, rarely found in Thai (4.5%). Indeed, G6PD Mahidol^{487A} has not been found in Lao and Cambodian (Louicharoen and Nuchprayoon 2005). Population admixture of Thai with other ethnic groups in Southeast Asia that carried G6PD Mahidol^{487A} (-/-) is the likely cause of G6PD Mahidol^{487A} in Thai. All G6PD Mahidol^{487A} in Karen, Burmese, Mon (Nuchprayoon *et al.*, 2008), and Thai were inherited as the haplotype 1311C, 93T (-/-). Moreover, this haplotype was also predominant in G6PD-normal Southeast Asian population (Nuchprayoon *et al.*, 2008). My results support the hypothesis that G6PD Mahidol^{487A} (-/-) in Karen, Burmese, Mon, and Thai have a common ancestry, likely derived from G6PD-normal (-/-). In Myanmar, the eight largest groups are Burmese (Burman), Karen, Rakhine, Kachin, Kayin, Kayah, Chin, and Mon. Mon people speak Monic (Mon), an Austro-Asiatic, Mon-Khmer language (Gordon 2005). Historically, Mon were among the earliest people to settle in present-day southern Myanmar. Burmese people migrated to Myanmar around 800 AD and became the largest ethnic group in

Myanmar. A Mon kingdom existed until 1757, after which it was repressed by the Burmese (Church 2003). There is nothing known about the origin of the Karen. However, Karen characteristics suggest that Tibet is a possible origin (Schliesinger 2000). From there, they migrated southwards and gradually entered Myanmar around the sixth-seventh century AD, and eventually into Thailand. Burmese natives speak Burmese (Bamar), a Tibeto-Burman language. In spite of distinctive cultural backgrounds and languages, G6PD Mahidol^{487A} was the predominant mutation in Burmese, Karen, and Mon indicating that they share a common origin that differs from the ancestry of Cambodians, Lao, and Thai.

The *G6PD* mutations in Chinese such as G6PD Canton^{1376T}, G6PD Union^{1360T}, G6PD Chinese-5^{1024T}, and G6PD Kaiping^{1388A} were infrequently found in Southeast Asian population. G6PD Canton^{1376T}, found in Karen (2.5%), Burmese (12.5%), Lao (3.8%) and Thai (2.3-10%) (Nuchprayoon *et al.,* 2002), was the most prevalent in Chinese from southern China (43%) (Chiu *et al.,* 1991; Chen *et al.,* 1998; Du *et al.,* 1999; Yang *et al.,* 2001), Malaysian Chinese (42.3-50%) (Ainoon *et al.,* 1999; Ainoon *et al.,* 2004), and Singapore Chinese (24%) (Saha *et al.,* 1994). G6PD Union^{1376T}, found in Karen (0.3%), Cambodian (2.9%), and Lao (3.8%) and G6PD Chinese-5^{1024T} found in Karen (0.3%) and Thai (2.3%), were both found in Malaysian Chinese (0.8% and 1.5%) (Ainoon *et al.,* 2004) and Thai (equally 2.6%) (Nuchprayoon *et al.,* 2002). G6PD Kaiping^{1388A} is found in Chinese (32%) (Yan *et al.,* 2006), in southern Thailand (20.1%) (Laosombat *et al.,* 2005), Phuket Islanders in southern Thailand (3%) (Ninokata *et al.,* 2006), and Malaysian Malay (2.3%) (Ainoon *et al.,* 2003), was found in Mon (5%), Lao (7.7%), and Thai (15.9%). The infrequent occurrence of *G6PD* mutations of Chinese in Karen, Mon, Burmese, Lao, and Cambodians support low level of admixture with Chinese. On the other hand, *G6PD*

mutations of Chinese could be attributed to assimilated Chinese immigrants to Thailand in the recent decades.

G6PD Coimbra^{592T} is widely distributed across Europe and Asia at low frequencies (Corcoran *et al.,* 1992). G6PD Coimbra^{592T} was previously reported in two G6PD-deficient Burmese (Matsuoka *et al.,* 2004); one was from Yangon, which is similar to my case. G6PD Coimbra^{592T} was also found in 3.5% of G6PD-deficient Malaysian Malay (Iwai *et al.,* 2001; Ainoon *et al.,* 2003), in Flores Island, Indonesia (Kawamoto *et al.,* 2006) and, in my cases, Cambodians (2.9%). The haplotype 1311T, 93C (+/+) has never been reported in G6PD Canton^{1376T}, G6PD Union^{1360T}, G6PD Coimbra^{592T}, and G6PD Chinese-5^{1024T}. I found that all G6PD Canton^{1376T}, G6PD Union^{1360T}, G6PD Coimbra^{592T}, G6PD Kaiping^{1388A}, and G6PD Chinese-5^{1024T} in all of my samples carry haplotype 1311C, 93T (-/-), which has previously been reported in G6PD Kaiping^{1388A} in Chinese (Yan *et al.,* 2006).

G6PD Valladolid^{406T} has been previously identified in Spaniards (Zarza *et al.,* 1997) and Mexicans (Vaca *et al.,* 2003). This mutation occurs in exon 5 and is predicted to code for cysteine at residue 135 instead of arginine resulting in a reduction of G6PD activity (0.64 IU/g Hb). How this mutation occurs in a G6PD-deficient Burmese subject is unclear. The mutation could have arisen independently, as there is no known strong historical linkage between Spain and Myanmar. However, a distinct haplotype 1311T, 93C (+/+) seen in this case was different than in Burmese at large, suggesting a different ethnic origin of this individual, possibly resulting from British occupation of Myanmar.

A novel mutation, G6PD Rajanagarindra^{477C}, has been identified in my study. This mutation is a missense mutation at exon 5 nt477 G→C, resulting in an amino acid substitution of methionine to isoleucine at residue 159. Although the

mutated residue of G6PD Rajanagarindra^{477C} is located in the N-terminal dinucleotide binding domain (residues 27-200), it is not predicted to have a functional effect on human G6PD tertiary structure (Naylor *et al.,* 1996). G6PD Rajanagarindra^{477C} was found in partial G6PD deficient and G6PD normal subjects, supporting its class IV classification (3.87 IU./g Hb). Most of mutations located in exon 5 have been classified in the non severe classes or class II, III, and IV (Cappellini and Fiorelli 2008); these include G6PD A^{376A} (Asn126Asp) (Yoshida *et al.,* 1967), G6PD Ilesha^{466G} (Glu156Lys) (Vulliamy *et al.,* 1988), and G6PD Quing Yuan^{392T} (Gly131Val) (Chiu *et al.,* 1993). G6PD Rajanagarindra^{477C} was found in 4 Karen from 2 unrelated families.

I also identified a few other G6PD-deficient mutations and a novel mutation: a 94 (C→G) in a Mon individual from Pha-un province in Myanmar. The point mutation 94 (C→G), which occurs in exon 2, is predicted to change amino acid from histidine to aspartic acid at residue 32 resulting in an absence of G6PD activity in this subject. Thus, this mutation belongs to a class II variant, which is closely related to G6PD Gaohe^{95G} by changing the same amino acid residue from histidine to arginine.

Few mutations were suggestive of an Indian contribution to the Mon and Burmese G6PD-deficient gene pool: G6PD Jammu$^{871A,\ 1311C}$, G6PD Mediterranean^{563T}, and G6PD Kerala-Kalyan^{949A}. G6PD Jammu$^{871A,\ 1311C}$ was previously found in an Indian (Beutler et al. 1991). G6PD Mediterranean^{563T} is widely distributed in different populations in the Mediterranean regions of southern Europe, the Middle East, and India. The haplotype of my G6PD Mediterranean^{563T} case was 1311C, 93T (-/-), which is similar to those with the Indian, rather than that of the Middle Eastern haplotype (Beutler and Kuhl 1990). The prevalence of G6PD Kerala-

Kalyan^{949A} was reported to be 24.9% in India (Sukumar et al. 2004). None of these mutations were found in Thai, Lao, Cambodian, and Karen, whereas G6PD Mediterranean^{563T} was found in approximately 30% of G6PD-deficient Indonesians from central Java (Soemantri et al. 1995), 27% of G6PD-deficient Malaysian Malays (Ainoon et al. 2002) and occasionally in Thai of the southern province of Thailand (Laosombat et al. 2005). Furthermore, G6PD Kerala-Kalyan^{949A} was also identified in Urak-Lawoi, a sea Gypsy population of the Andaman Sea who inhabited Phuket Island in southern Thailand (Ninokata et al. 2006). Both polymorphisms C and T are present at 1311 in Indians with G6PD Kerala-Kalyan^{949A} (Sukumar et al. 2004). My finding of G6PD Kerala-Kalyan^{949A} in a Burmese from Yangon suggests the flow of this gene from India southward.

3. Positive Selection of *G6PD* Mutation in Southeast Asian Population

Reduced microsatellite haplotype diversity and increased microsatellite haplotype frequency were observed in G6PD Mahidol^{487A}, opposite to the high microsatellite haplotype diversity and restricted haplotype frequency in G6PD Viangchan^{871A}. This finding is consistent with the hypothesis of the recent positive selection of G6PD Mahidol^{487A} possibly because of selection by malaria and the ancient origin of G6PD Viangchan^{871A} in Southeast Asia peninsula.

The recent positive selection of G6PD Mahidol^{487A} was confirmed using LRH test, which monitors the breakdown of LD of SNPs dataset around G6PD Mahidol^{487A} and its frequency. The extended LD of G6PD Mahidol^{487A} was around 2.4 Mb with no recombination or mutation events, which was larger than LD range of G6PD A-$^{202A/376G}$ in African (15 kb) (Sabeti *et al.*, 2002), and other important genes indicated in protection against malaria -HbE in Thai (100 kb) (Ohashi *et al.*, 2004),

and HbC in African (1 kb) (Wood *et al.*, 2005). The unexpectedly high homozygosity (63%) around G6PD Mahidol^{487A} allele is highly significant (P<6.7x10^{-4}) after comparing with the empirical distribution of homozygosity *vs*. frequency for the X chromosome retrieved from HapMap Phase II data in Han Chinese. G6PD Mahidol^{487A} allele has arisen to high frequency too rapidly for its estimated age and is thus a sign of recent positive selection.

G6PD Mahidol^{487A} is estimated to have expanded 1,500 YBP with a selection intensity of ~0.23. The estimate of the selection intensity of the G6PD Mahidol^{487A} is among the strongest detected in the human genome, including malaria-protective G6PD A-$^{202A/376G}$ (~0.2) (Saunders *et al.*, 2005; Slatkin 2008) and *β*-globin mutations (~0.08-0.26) (Wood *et al.*, 2005), as well as lactase persistence (~0.1) (Enattah *et al.*, 2008). A general link between *G6PD* alleles and their protection against malaria together with the recurrent hypothesis stating that malaria has imposed a very strong selective pressure on the human species strongly suggest that G6PD Mahidol^{487A} does protect against malarial infection, thereby increasing population fitness.

The nature of the selective advantage conferred by the Mahidol^{487A} mutation is considered a protective effect against *P. vivax* parasite density, which is most notable in hemizygous males. There are too few homozygote deficient females infected with *P. vivax* for independent analyses, but the same trend is observed in both homozygote and heterozygote mutant females. Here the protective effect of the Mahidol^{487A} mutation against *P. vivax* can be observed in the youngest age groups that have little or no immunity. As anti-parasite immunity develops with age, the impact of the mutation becomes less important. By contrast, G6PD Mahidol^{487A} has no effect on the number of clinical malaria cases due to *P. vivax* infection. This

observation suggests that Mahidol^{487A} does not prevent infection but rather controls parasite proliferation and hence the degree of harm inflicted by the parasites. However, the low force of infection in this site would likely generate considerable heterogeneity in exposure to infection thereby increasing the importance of environmental *vs.* genetic effects on the number of *P. vivax* infections. Although G6PD Mahidol^{487A} protects against *P. vivax* parasite density, this mutation is associated with an increasing *P. falciparum* parasite density. This observation is consistent with several previous reports (Bienzle *et al.*, 1979), suggesting that G6PD-deficiency may increase *P. falciparum* parasite proliferation but certainly does not confer protection.

The family-based replication study excluded population admixture/stratification from being the cause of the significant association between Mahidol^{487A} and *P. vivax* parasite density. Therefore, these results indicate that Mahidol^{487A} exerts a genuine protective effect against *P. vivax*. Although the precise mechanism underlying this protective effect remains to be determined, it is likely to be linked to the effects of G6PD deficiency on red cell physiology, in particular an increased oxidative stress. Young red cells (i.e. reticulocytes) have more anti-oxidant enzymes than mature red cell populations (Prchal and Gregg 2005). The preference of *P. vivax* for reticulocytes suggests that *P vivax* is more sensitive to oxidative stress than *P. falciparum*, which has no red-cell preference. In normal red blood cells, the store of G6PD is sufficient to maintain the redox balance for their 120 day lifespan. *G6PD* mutations affect catalytic efficiency or the number of fully active molecules. The latter can result from altered RNA splicing, defective protein folding, reduced protein stability or a failure to dimerize, which lead to increased rates of protein degradation. The mutant enzyme half-life is considerably reduced such that activity

may be affected even in reticulocytes, a situation that would be exacerbated by host fever response to infection. G6PD Mahidol^{487A} results in impaired protein folding likely due to steric hindrance that considerably reduces enzyme activity and markedly increases protein thermo-instability (Panich *et al.*, 1972). Suitable red cell availability was previously invoked as the mechanism by which G6PD deficiency protects against malaria: parasite rate was 2-80 times higher in normal than in deficient erythrocytes from the same individual. Under these conditions, reduced G6PD activity in reticulocytes will thus have a relatively greater impact upon *P. vivax* because it preferentially invades reticulocytes with respect to *P. falciparum* that has no red cell preference.

The expansion of malaria, in particular that due to *P. falciparum* and *P. vivax*, has been classically linked to the agricultural revolution. Indeed, the emergence of agriculture both generated a breeding ground for mosquitoes and led to a high human population density (Hume *et al.*, 2003), thereby increasing human-mosquito contact and the conditions for stable malaria transmission. In East Asia, the introduction of farming is mainly associated with the development of the rice culture in China at around 8,000 YBP, which spread to Southeast Asia over the following 4,000 years. Although there is evidence of wet rice cultivation as early as 4,200 YBP in the Southeast Asian peninsula, rice cultivation has developed rapidly mainly over the last 2,000 years (Cavalli-Sforza 1994). The Karen people belong to the Sino-Tibetan language group. They are thought to be of Tibetan origin and to have entered Myanmar by ~1,500 YBP (Besaggio *et al.*, 2007). Interestingly, the estimated age of Mahidol^{487A} at 1,500 YBP coincides with the proposed arrival of the Karen people into the region and with the time at which rice started to be extensively cultured. This

supports a link between the onset of agriculture in East Asia and an increased incidence of mosquito-borne malaria.

In conclusion, G6PD Mahidol^{487A} mutation has been under strong and recent positive selection for the last 1,500 years. The mutation reduces *P. vivax* parasite density in the host and provides evidence that *P. vivax* is likely to be the agent responsible for the strong selective advantage conferred by this mutation. These findings support the notion that *P. vivax* historically had a considerable impact on human health, at least in Southeast Asia (Carter and Mendis 2002). The virulent nature of *P. vivax* infection has been seriously understated and there are increasing reports of its association with severe morbidity and mortality (Tjitra *et al.,* 2008). Antimalarial drugs –Primaquine- remains the most effective drug against *P. vivax* liver stage infections and yet can cause oxidant-induced hemolytic anemia, particularly among individuals with G6PD deficiency. Discovery of the protective effect of G6PD Mahidol^{487A} against *P.vivax,* which is sensitive to oxidative stress, may be a strategy in an administration of antimalarial drugs both in non G6PD deficient and G6PD deficient individuals.

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APPENDIX

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ORIGINAL ARTICLE

Chalisa Louicharoen · Issarang Nuchprayoon

G6PD Viangchan (871G>A) is the most common G6PD-deficient variant in the Cambodian population

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Abstract Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common hereditary enzymopathy among Southeast Asians. We studied *G6PD* mutations in 108 migrant Cambodian laborers in Chanthaburi province and cord blood samples from 107 Cambodian newborns at Buriram Hospital. Thirty-one (26.1%) of 119 Cambodian males and three of 96 (3.1%) females were G6PD deficient and were assayed for *G6PD* mutations. *G6PD Viangchan* (871G > A) was identified in most G6PD-deficient Cambodians (28 of 34; 82.4%); *G6PD Union* (1360C > T) and *G6PD Coimbra* (592C > T) was found in one case each. We concluded that *G6PD Viangchan* (871G > A) was the most common mutation among Cambodians. This finding is similar to G6PD-deficient Thais and Laotians, suggesting a common ancestry of people from these three countries.

Keywords Glucose-6-phosphate dehydrogenase deficiency · *G6PD Viangchan* · Cambodian · Thai · Laotian · Myanmar population

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common hereditary enzymopathy. The prevalence of G6PD deficiency is high in the Southeast Asian population, which correlated with malaria endemicity (Nuchprayoon et al. 2002; Iwai et al. 2001). G6PD (MIM# 305900) is a housekeeping enzyme that provides NADPH in catalysis of the pentose phosphate pathway (PPP) (Poggi et al. 1990). Inherited deficiency of this enzyme can cause acute or chronic hemolytic anemia, neonatal hyperbilirubinemia, and favism (Beutler 1994). The *G6PD* gene consists of 13 exons distributed over approximate 18 kb on the distal long arm of the X chromosome (Xq28) (Martini et al. 1986). At least 442 *G6PD* variants have been described by the biochemical characterization (Xu et al. 1995). To date, more than 130 different mutations in the *G6PD* gene, most of which are nucleotide substitution, have been described (Hamel et al. 2002).

Certain *G6PD* mutations are associated with specific ethnic groups in tropical Asia (Iwai et al. 2001). Epidemiological and molecular studies had previously shown that G6PD deficiency in Southeast Asia is heterogeneous. *G6PD Viangchan* (871G > A; Val291Met) seems to be the most common variant in Thais, Laotians, and Malaysian Malays (Nuchprayoon et al. 2002; Iwai et al. 2001; Ainoon et al. 2003) while *G6PD Mahidol* (487G > A; Gly163Ser) is the most variant in the Myanmar population (Matsuoka et al. 2004). *G6PD* mutations in Cambodians, however, were not known. In this study, we report the prevalence of G6PD deficiency and have identified the G6PD-deficient mutations among Cambodians.

Materials and methods

Sample collections

One hundred and eight peripheral blood samples were collected from consenting migrant Cambodian laborers in Chanthaburi province, Thailand, as a part of a health screening program for work permit in Thailand between March and April 2002. The immigrants' Cambodian provinces of residence were recorded. Cord blood samples of 107 newborns of consenting Khmer-speaking mothers were collected from the delivery room of Buriram Hospital, Buriram Province, Thailand, between April and May 2003. From each subject, 3 ml ACD blood samples were collected for G6PD activity assay, and 2 ml EDTA blood samples collected for DNA analysis. Blood samples were stored at 4°C until used.

C. Louicharoen · I. Nuchprayoon (✉)
Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Rama IV Rd., Bangkok, 10330, Thailand
E-mail: fmedinp@md2.md.chula.ac.th
Tel.: +66-2-2564949
Fax: +66-2-2564949

G6PD activity assay

G6PD activity assays were performed according to the WHO standard (Betke et al. 1967) within 7 days of collection. In our laboratory, G6PD activity was 7.39 ± 2.57 IU/g Hb (mean ± SD) in normal males and 6.94 ± 2.51 IU/g Hb in normal females. G6PD activity < 1.5 IU/g Hb is defined as G6PD deficiency (Betke et al. 1967).

DNA extraction

Genomic DNA was extracted from G6PD-deficient EDTA blood samples using QIAamp DNA Blood Minikit (Qiagen, Germany) according to manufacturer's instruction.

Mutation analysis

To identify *G6PD* mutations, we first screened all G6PD-deficient samples for two mutations, *G6PD Viangchan* (871G > A) and *G6PD Mahidol* (487G > A), which were previously reported to be the most common in the Southeast Asian population (Nuchprayoon et al. 2002; Iwai et al. 2001; Ainoon et al. 2003). For G6PD-deficient samples in which mutation remained unknown, they were assayed for eight common Chinese mutations: *G6PD Canton* (1376G > T), *G6PD Union* (1360C > T), *G6PD Kaiping* (1388G > A), *G6PD Chinese-5* (1024C > T), *G6PD Gaohe* (95A > G), *G6PD Chinese-3* (493A > G), *G6PD Chinese-4* (392G > T), and *G6PD Coimbra* (592C > T) (Nuchprayoon et al. 2002; Ainoon et al. 2003; Tang et al. 1992; Ainoon et al. 1999; Huang et al. 1996; Saha et al. 1994).

For *G6PD* mutation assay, the target gene was amplified using a PCR-based technique with primers that were previously designed to create restriction sites (Nuchprayoon et al. 2002; Huang et al. 1996). The typical PCR reaction was carried out in a 50-μl reaction containing 1× PCR buffer, 1 U of Taq polymerase (Fermentas), 50 ng of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, and approximate 300 ng DNA template. After incubation at 94°C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and final extension for 15 min at 72°C. Ten microliters of PCR product were digested with 5 U of an appropriate restriction enzyme digestion set (Huang et al. 1996) according to manufacturer's protocols (New England Biolabs). The digestion was incubated at 37°C 2–4 h, subjected to electrophoresis on 6% acrylamide gel, and then stained with ethidium bromide.

For nt 1311C > T polymorphism, two primers previously reported to create a restriction site of *Bcl*I (Vulliamy et al. 1991) were used in a PCR reaction similar to the *G6PD* mutation assays. The PCR amplification was performed on the DNA thermal cycler for 1 cycle of 94°C for 5 min, then 35 cycles of 1 min at 94°C, 1 min at 63°C, 1 min at 72°C, and a final extension at 72°C for 15 min. Ten microliters of PCR product were digested with *Bcl*I following the technique described above.

Results

Prevalence of G6PD deficiency

From the 215 Cambodian blood samples, we found G6PD deficiency in 26.1% of Cambodian male (31 of 119) and 3.1% of females (3 of 96). Among Cambodian neonates, 21 of 56 males and two of 51 females were G6PD deficient. Among Cambodian adults, 10 of 63 males and one of 45 females were G6PD deficient (Table 1).

Prevalence of *G6PD* mutations

G6PD genotype was examined in both G6PD-deficient neonates and adults. The results are shown in Table 1. Only three *G6PD* mutations were identified in 34 G6PD-deficient Cambodians. *G6PD Viangchan* (871G > A) was found in 28 cases (82.4%, Fig. 1), *G6PD Union* (1360C > T) and *G6PD Coimbra* (592C > T) in one case each. We also screened for *G6PD Mahidol* (487G > A), *G6PD Canton* (1376G > T), *G6PD Kaiping* (1388G > A), *G6PD Chinese-5* (1024C > T), *G6PD Gaohe* (95A > G), *G6PD Chinese-3* (493A > G), and *G6PD Chinese-4* (392G > T) but did not identify any of these mutations in G6PD-deficient Cambodians. In four G6PD-deficient samples, the mutation remained unidentified.

Because *G6PD* mutation 871G > A can be *G6PD Viangchan* (871G > A, nt 1311C > T) or *G6PD Jammu* (871G > A, wild type nt 1311), we assayed for the nt 1311C > T by a PCR-restriction enzyme method. We found that all samples with 871G > A had nt 1311C > T,

Table 1 G6PD mutations in G6PD-deficient Cambodian adults and neonates

Age	Gender	Total	Deficient	Mutation			
				Viangchan	Union	*Coimbra*	Unknown
Adult	Male	63	10	8	–	–	2
	Female	45	1	1	–	–	–
Neonate	Male	56	21	19	1	1	–
	Female	51	2	–	–	–	2

Fig. 1 Distribution of glucose-6-phosphate dehydrogenase (*G6PD*) variants in Cambodians. Numbers represent number of *G6PD* variant/population tested by province. Only provinces in which *G6PD* variants were found were reported

consistent with *G6PD Viangchan* (Fig. 2). For three G6PD-deficient females, all samples were heterozygote for *G6PD Viangchan* (Fig. 3).

Discussion

There were few reports on genetic markers in Cambodians because the country was not accessible to international communities until recently. Cambodians speak Khmer, a distinctive Austro-Asiatic language (http://www.ethnologue.com). Due to poor living conditions in Cambodia, many Cambodians migrated into Thailand as refugees, migrant laborers, and through marriages with Thais in border provinces. Because of their distinctive language and culture, it has been controversial

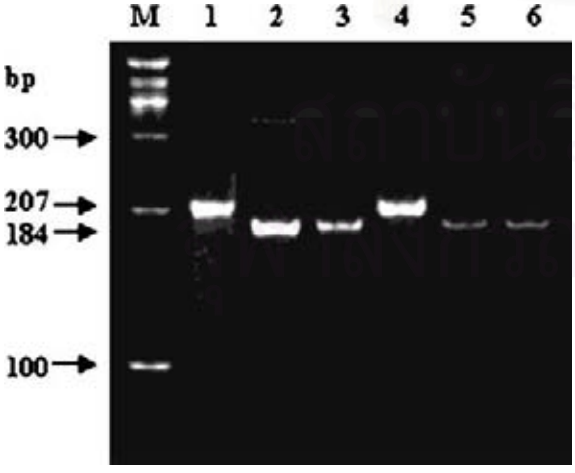

Fig. 2 PCR-RFLP for nt 1311C > T. *Lane M* 100-bp ladder, *lane 1* undigested nt 1311C > T showed a 207 band, *lanes 2–3, 5–6* digested nt 1311C > T showed 184 bp, *lane 4* digested nt 1311C showed 207 bp

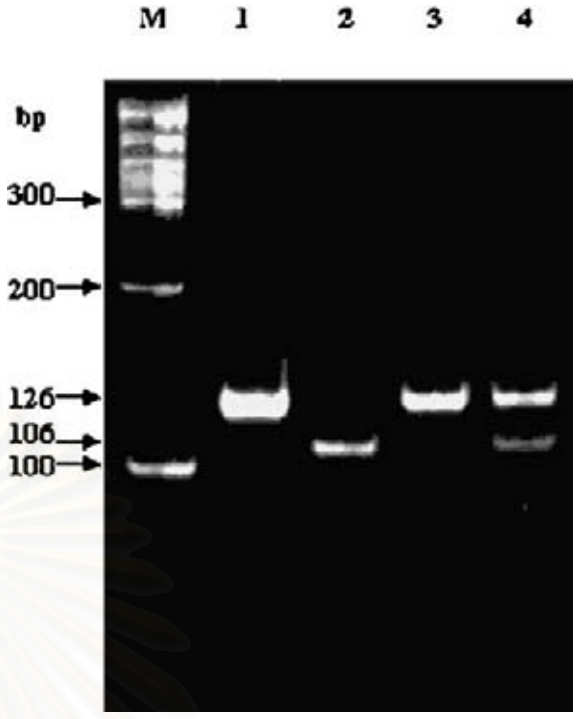

Fig. 3 PCR-RFLP for *G6PD Viangchan*. *Lane M* 100 bp ladder, *lane 1* undigested *G6PD Viangchan* showed a 126 bp band, *lane 2* digested *G6PD Viangchan* that reduced to 106 bp, *lane 3* digested normal, *lane 4* digested female heterozygote

whether today's Cambodians share a common ancestry with people of neighboring countries.

G6PD deficiency is highly prevalent in Cambodians. A recent study found G6PD deficiency in 13.4% of school boys in central Cambodia (Monchy et al. 2004). In this study, we found a prevalence of 26.1% in Cambodian males, which is comparable to Laotians and northeastern Thais (21.7%, Kittiwatanasarn et al. 2003). The high prevalence of G6PD deficiency among Southeast Asians was postulated to be due to selection pressure from malaria, which was hyperendemic in Southeast Asia (Flatz et al. 1963). The mechanism of resistance to malaria infection is still controversial, but it is probable that G6PD deficiency reduces NADPH in oxidative stress that affects growth of *Plasmodium* (Beutler 1994).

The objective of our study was to identify as many G6PD-deficient cases as possilble to characterize their mutations. For this reason, females were included in the study. We used quantitative G6PD activity testing in all samples without using G6PD screening methods. We also have previously established a histogram of cord blood G6PD levels in males and females in our population (Sanpavat et al. 2001). In that study, we found that the cord blood of female babies whose G6PD activities are in deficient range are heterozygotes for G6PD mutation.

We found that *G6PD Viangchan* was the most common variant in Cambodians, with calculated allele frequency of 0.23. This variant is also the most common

among Thais (Nuchprayoon et al. 2002) and Laotians (Iwai et al. 2001). Our finding is in favor of the theory that proposed a common ancestry of Thais, Laotians, and Cambodians (Church et al. 2003). In contrast, *G6PD Mahidol*, the most common *G6PD* variant in the Myanmar population (Matsuoka et al. 2004), was not found in Cambodians. We screened for seven common Chinese mutations and found only one case each of *G6PD Union* (1360G > T) and *G6PD Coimbra* in Cambodians. These two *G6PD* variants could be attributed to assimilated Chinese immigrants to Cambodia in the recent decades.

Taking all regional evidence together, the *G6PD* mutations of people in the Southeast Asian peninsular can be described. Myanmese in the west of the peninsular are quite homogenous for *G6PD Mahidol* (Iwai et al. 2001) while Laotians and Cambodians in the eastern part of the peninsular are relatively homogeneous for *G6PD Viangchan*. For Thais in the central part of the peninsular, *G6PD* mutations are more heterogeneous but still predominated by *G6PD Viangchan* (Nuchprayoon et al. 2002). *G6PD* mutations become even more heterogeneous in Thais in the south of Thailand (Laosombat et al. 2005) and Malays in Malaysia (Ainoon et al. 2003). Overseas, Chinese immigrants in the recent century contributed Chinese *G6PD* variants to the Southeast Asian gene pools.

In addition to *G6PD Viangchan*, hemoglobin E (HbE) is known to be highly prevalent in Thais, particularly in the Northeastern region (Wasi et al. 1967). A recent study also suggests that HbE is prevalent in Khmer-speaking people in border provinces of Thailand (Fuchareon et al. 2002). This and our finding favors the theory that the Thais, Cambodians, and Laotians were people of the same ancestors living in the Southeast Asia peninsula for some time during the past millennium. Malarial selection pressure may be at work in these indigenous people during the age of agriculture (Tishkoff et al. 2001). Haplotype analysis of the *G6PD* locus could provide further evidence to support this hypothesis.

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ORIGINAL ARTICLE

Glucose-6-phosphate dehydrogenase mutations in Mon and Burmese of southern Myanmar

Issarang Nuchprayoon · Chalisa Louicharoen · Warisa Charoenvej

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Abstract Glucose-6-phosphate dehydrogenase (G6PD) deficiency is highly prevalent in Southeast Asians. *G6PD* mutations are associated with specific ethnic groups in Southeast Asia. Mon is a minority ethnic group in Myanmar, which speaks Monic, a distinct language of Mon-Khmer classification. We studied *G6PD* mutations in Mon and Burmese males of southern Myanmar who migrated to Thailand in Samutsakhon province. G6PD deficiency was identified in 19 (12%) of 162 Mon males and 17 (10%) of 178 Burmese males, and then assayed for *G6PD* mutations. Among 19 G6PD-deficient Mons, 12 were G6PD Mahidol; one case each was G6PD Jammu (871G > A; nt 1311C), G6PD Kaiping (1388G > A), G6PD Mediterranean (563C > T), a novel mutation 94(C > G); and three remain unidentified. Among 17 G6PD-deficient Burmese, 12 were G6PD Mahidol; one each was G6PD Coimbra (592C > T), G6PD Kerala-Kalyan (949G > A), and G6PD Valladolid (406C > T); and two remain unidentified. G6PD Mahidol (487G > A) is the most common mutation among Mons and Burmese. All G6PD deficient Mon and Burmese, except for a person with G6PD Valladolid, shared the same haplotype nt93T, nt1311C. Despite a similar language root with Cambodian's Khmer language, our study suggests that Mon people share a common ancestry with Burmese rather than Cambodians.

Keywords Glucose-6-phosphate dehydrogenase deficiency · G6PD Mahidol · G6PD Jammu · Mon · Burmese

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) (MIM# 305900) is an X-linked enzyme that catalyzes the production of nicotinamide adenine dinucleotide phosphate (NADPH) in the pentose phosphate pathway (PPP) of the red cell (Poggi et al. 1990). G6PD deficiency is the most common enzyme disorder in humans (WHO 1989). This disorder causes many clinical manifestations, including neonatal jaundice, acute or chronic hemolytic anemia, neonatal hyperbilirubinemia, and favism (Beutler 1994). The prevalence of G6PD deficiency is high in the Southeast Asian population where malaria has historically been endemic (Nuchprayoon et al. 2002; Iwai et al. 2001).

The *G6PD* gene consists of 13 exons distributed over approximately 18 kb on the distal long arm of the X chromosome (Xq28) (Martini et al. 1986). To date, at least 442 *G6PD* variants have been described by biochemical characterization (Xu et al. 1995), and 130 *G6PD* mutations by molecular technique (Hamel et al. 2002). *G6PD* mutations in Southeast Asians are specific to certain ethnic groups. G6PD Viangchan (871G > A; Val291Met) is the most common mutation among Thais (Nuchprayoon et al. 2002), Laotians (Iwai et al. 2001), and Cambodians (Louicharoen et al. 2005; Matsuoka et al. 2005), whereas G6PD Mahidol (487G > A; Gly163Ser) is the most

I. Nuchprayoon (✉)
Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Rama IV Rd, Bangkok 10330, Thailand
e-mail: issarangn@yahoo.com

I. Nuchprayoon · C. Louicharoen
Chula Medical Research Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

W. Charoenvej
Department of Clinical Pathology, Samutsakhon Hospital, Samutsakhon, Thailand

common mutation among Burmese in Myanmar (Matsuoka et al. 2004).

Mon is a minority tribe in southern Myanmar. Mon people speak Monic, an Austro-Asiatic language similar to Thais and Cambodians, whereas Burmese speak Burmese (Bamar), a Tibeto-Burman language. We report here a study of G6PD deficiency and mutations in Mon and Burmese in southern Myanmar and demonstrate that their G6PD-deficient mutations are similar despite their distinct language and cultural roots.

Materials and methods

Subjects

Blood samples were collected from 162 Mon and 178 Burmese male migrant foreign laborers in Samutsakhon province, Thailand, as part of a health screening program before receiving a work permit in Thailand. Interviews of participants included their self-reported ethnic classification and place of birth. Blood samples were divided into two parts, 3 ml in acid citrate dextrose (ACD) for G6PD activity assay and 2 ml in ethylenediaminetetraacetate (EDTA) for molecular characterization, and were stored at 4°C until used. Samples that were found to be G6PD deficient were tested further for mutation analysis and haplotype analysis. Samples that were G6PD normal were randomly selected for haplotype analysis.

G6PD activity assay

G6PD activity assay was performed within 7 days according to our previous study, which was based on the World Health Organization (WHO) recommended standard test (Betke et al. 1967).

G6PD mutations analysis

Genomic DNA was extracted using a DNA blood mini kit (QIAGEN, Germany) according to manufacturer's instruction. To identify *G6PD* mutations, we initially screened for G6PD Mahidol (487G > A), which was previously found to be the most common mutation in the Burmese population (Iwai et al. 2001; Matsuoka et al. 2004). G6PD-deficient DNA samples whose mutation remained unknown were assayed for eight common mutations that were previously reported from Southeast Asians: G6PD Viangchan (871G > A), G6PD Canton (1376G > T), G6PD Union (1360C > T), G6PD Kaiping (1388G > A), G6PD Chinese-5 (1024C > T), G6PD Gaohe (95A > G), G6PD Chinese-4 (392G > T), and G6PD Coimbra (592C > T) (Nuchprayoon et al. 2002; Tang et al. 1992; Saha et al. 1994; Huang et al. 1996; Ainoon et al. 1999, 2002). For *G6PD* mutation assays, the target gene was amplified using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique with an appropriate primer set (Table 1) (Nuchprayoon et al. 2002; Huang et al. 1996). The typical PCR reaction was carried out in a 10-μl reaction containing 1× PCR buffer, 0.5 U of Taq polymerase (Fermentas), 20 ng of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, and approximately 50 ng DNA template. After incubation at 94°C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 0.45 min, 56°C for 0.45 min, 72°C for 0.45 min, and final extension for 7 min at 72°C. Five microliters of PCR product was digested with 1 U of an appropriate restriction enzyme digestion set (Huang et al. 1996) according to manufacturer's protocols (New England Biolabs). The digestion was incubated for 2–4 h, subjected to electrophoresis on 6% acrylamide gel, and then stained with ethidium bromide.

For all unknown mutation samples, PCR direct sequencing was performed for each exon with primers in Table 1 (Nuchprayoon et al. 2002; Huang et al. 1996; Tang et al. 1992; Ninokata et al. 2006). For haplotype analysis, PCR-RFLP technique was performed for detecting nt1311C > T polymorphism in exon 11 and nt93T > C polymorphism in intron 11 (Vulliamy et al. 1991; Beutler et al. 1991). Cases with G6PD Vianchan (871G > A, 1311T) were distinguished from G6PD Jammu (871G > A, 1311C) by the presence of nt1311C > T.

Results

G6PD activity in 162 healthy Mon males and 178 Burmese males showed a normal distribution. The average G6PD activity was 6.34 ± 2.89 IU/g Hb [mean ± standard deviation (SD)] in Mon and 6.56 ± 2.69 IU/g Hb in Burmese. G6PD activity less than 1.5 IU/g Hb was classified as G6PD deficiency. Ethnic group, origin of subject, and number of G6PD-deficient subjects are shown in Table 2.

Nineteen (12% of 162) Mon males were G6PD deficient and assayed for mutation. Using PCR-RFLP assays, 12 (63% of 19) were G6PD Mahidol, and one each was G6PD Jammu (871G > A; nt 1311C) and G6PD Kaiping (1388G > A). We also screened for G6PD Canton (1376G > T), G6PD Union (1360C > T), G6PD Chinese-5 (1024C > T), G6PD Gaohe (95A > G), G6PD Chinese-4 (392G > T), and G6PD Coimbra (592C > T) but could not identify any of these mutations in G6PD-deficient Mons. To identify whether mutation 871G > A was G6PD

Table 1 Nucleotide sequences of primers and the exons of the glucose-6-phosphate dehydrogenase (*G6PD*) gene amplified

Primers	Sequence
871[a]	5′-TGGCTTTCTCTCAGGTCTAG-3′
	5′-GTCGTCCAGGTACCCTTTGGGG-3′
487[b]	5′-GCGTCTGAATGATGCAGCTCTGAT-3′
	5′-CTCCACGATGATGCGGTTCAAGC-3′
1360 [b]	5′-ACGTGAAGCTCCCTGACGC-3′
	5′-GTGAAAATACGCCAGGCCTTA-3′
1376 [b]	The same as for primer 1360
1388 [b]	The same as for primer 1360
	5′-GTGCAGCAGTGGGGTGAACATA-3′
1024[b]	5′-GTCAAGGTGTTGAAATGCATC-3′
	5′-CATCCCACCTCTCATTCTC-C3′
592[b]	5′-GAGGAGGTTCTGGCCTCTACTC-3′
	5′-TTGCCCAGGTAGTGGTCGCTGC-3′
95[b]	5′-CTCTAGAAAGGGGCTAACTTCTCA-3′
	5′-GATGCACCCATGATGATGAATACG-3′
392[b]	5′-GGACTCAAAGAGAGGGGCTG-3′
	5′-GAAGAGGCGGTTGGCCGGTGAC-3′
Exon2[c]	5′-CTCTAGAAAGGGGCTAACTTCTCAA-3′
	5′-GGAATTCCTGGCTTTTAAGATTGGG-3′
Exon3–4[c]	5′-AGGATGATGTAGTAGGTCG-3′
	5′-CCGAAGTTGGCCATGCTGGG-3′
Exon5	5′-GTGTGTCTGTCTGTCCGTGTC-3′
	5′-CACGCTCATAGAGTGGTGGG-3′
Exon6[d]	5′-GGGAGGGCGTCTGAATGA-3′
	5′-ACCTTGGGCCTCTGTGGTG-3′
Exon7	5′-TCCACCTTGCCCCTCCCTGC-3′
	5′-CCAGCCTCCCAGGAGAGAGG-3′
Exon8[d]	5′-CATGCCCTTGAACCAGGTGA-3′
	5′-GCATGCACACCCCAGCTC-3′
Exon9–10	5′-TTCTCTCCCTTGGCTTTCTC-3′
	5′-TACAGAGAAGGAGCAGTGTG-3′
Exon11[b,c]	5′-GAAGCCGGGCATGTTCTTCAAC-3′
	5′-GTGAAAATACGCCAGGCCTTA-3′
Exon12[b,c]	5′-ACGTGAAGCTCCCTGACGC-3′
	5′-CCAGGGCTCAGAGCTTGTG-3′
Exon13[d]	5′-TGCCTCTCCTCCACCCGTCA-3′
	5′-GTCAATGGTCCCGGAGTC-3′
nt1311[e]	5′-TGTTCTTCAACCCCGAGGAGT-3′
	5′-AAGACGTCCAGGATGAGGTGATC-3′

[a] Nuchprayoon et al. (2002), [b]Huang et al. (1996), [c]Tang et al. (1992), [d]Ninokata et al. (2006), [e]Vulliamy et al. (1991)

Viangchan (1311C > T) or G6PD Jammu (1311C), we used a PCR-RFLP technique with restriction enzyme *Bcl*I and identified nt 1311C in the sample with 871G > A, establishing a case of G6PD Jammu in a Mon subject.

DNA sequencing from all coding exons of G6PD genes of the five remaining G6PD-deficient Mons revealed a case of G6PD Mediterranean (563C > T), and a novel mutation 94(C > G) in a Mon individual from Pha-un province in Myanmar (Fig. 1). The G6PD activity of 94(C > G) was not detectable (Table 3). The mutations of three G6PD-deficient Mons remained unidentified.

Seventeen (10% of 178) Burmese males were G6PD deficient. Twelve (71%) G6PD-deficient Burmese were G6PD Mahidol (487G > A) and one (6%) was G6PD Coimbra (592C > T). DNA sequencing from all coding exons of G6PD genes of the four remaining G6PD-deficient samples identified G6PD Kerala-Kalyan (949G > A) in one case and G6PD Valladolid (406C > T) in another case, whereas two remained unidentified.

Mon and Burmese who had G6PD Mahidol came from various places of birth in Myanmar (Fig. 2; Table 2). Subjects with G6PD Mahidol had variable G6PD activities ranging from no detectable activity (8 of 24) to some residual activities. The average G6PD activity (± SD) for G6PD Mahidol was 0.43 ± 0.48 IU/g Hb (Table 3). All cases of G6PD Mahidol had haplotype 93T, 1311C. This haplotype was also the predominant haplotype in all G6PD-deficient Mons and Burmese, except for one case with G6PD Valladolid (406C > T).

Among 31 G6PD-normal Burmese randomly selected for haplotype analysis, 93T, 1311C (n = 22, 71.0%) was the most common, followed by 93C, 1311T (n = 8, 25.8%) and 93T, 1311T (n = 1, 3.2%), whereas 93C, 1311C was not identified. Among 32 G6PD-normal Mon randomly selected for haplotype analysis, only two haplotypes were found. Similar to the Burmese subjects, haplotype 93T, 1311C (n = 23, 71.9%) was more common than 93C, 1311T (n = 9, 28.1%), but the haplotype 93T, 1311T was not identified.

Discussion

Through a study for G6PD deficiency, we found the prevalence of G6PD deficiency to be high in Mon (12%) and Burmese (10%) males. Our result in Mons was higher than the previous report (6.7%; 3 of 42) (Iwai et al. 2001). The sample of Mons in our study was 3.6 times larger than that study and thus more accurate. The high prevalence of G6PD deficiency in Southeast Asians may be due to natural selection by malaria, which is endemic in Southeast Asia (Flatz et al. 1963).

There are many distinct ethnic groups in Myanmar. The eight largest groups are Burmese (Burman), Karen, Rakhine, Kachin, Kayin, Kayah, Chin, and Mon. Because of their distinctive language and cultural background and closed society until very recently, we could rely only on the self-reported ethnic classifications of the study participants.

Table 2 Frequency of glucose-6-phosphate dehydrogenase (G6PD) deficiency in male Mons and Burmeses among various places of birth

Place of birth	Ethnic group	Number	Deficient (cases)	Place of birth	Ethnic group	Number	Deficient (cases)
Pha-un	Burmese	89	6	Sangkhla Buri	Mon	5	0
	Mon	96	10	Mae-Sot	Mon	4	0
Yangon	Burmese	45	7	Kokarek	Burmese	3	0
	Mon	26	5	Koei	Mon	3	0
Ye	Burmese	2	0	Pal	Mon	2	0
	Mon	8	0	Mijina	Burmese	2	0
Maewadee	Burmese	4	0	Kawthaung	Burmese	1	0
	Mon	6	2	Irrawaddy	Mon	1	0
Dawei	Burmese	22	2	Marid	Burmese	1	0
	Mon	2	0	Yakai	Burmese	1	1
Lamae	Burmese	5	1	Katai	Burmese	1	0
	Mon	4	1	Jai	Burmese	1	0
Ja-eang	Burmese	1	0	Jadee	Mon	1	0
	Mon	3	1	Aeiou	Mon	1	0

Mon people speak Monic (Mon), which is an Austro-Asiatic, Mon-Khmer language, spoken by Mons and Cambodians (Gordon 2005). Historically, Mons were among the earliest people to settle in present-day southern Myanmar. Burmese people migrated to Myanmar around 800 AD and became the largest ethnic group in Myanmar. A Mon kingdom existed until 1757, after which it was repressed by the Burmese (Church 2003). Until now, there were few reports on genetic markers in Mon.

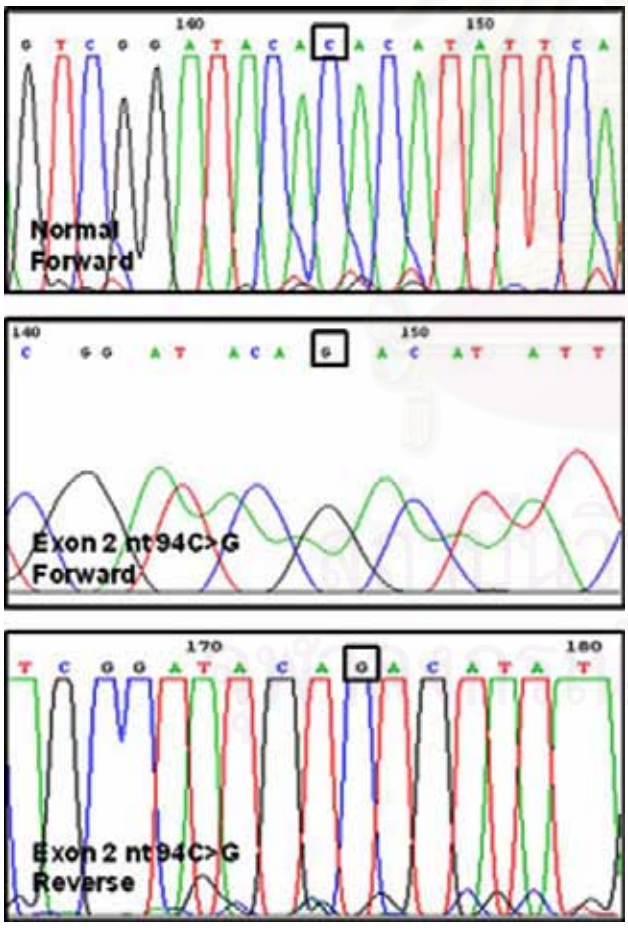

Fig. 1 Chromatogram of glucose-6-phosphate dehydrogenase (G6PD) 94C > G

Burmese natives speak Burmese (Bamar), a Tibeto-Burman language. Because of their distinctive language, it has been controversial whether today's Mon share a common ancestry with Burmese or Thai-Cambodian. We found that G6PD Mahidol was the most common mutation in Mon and Burmese males, with an allele frequency of 0.08 and 0.07, respectively. This finding confirms the results of previous reports that G6PD Mahidol is the predominant mutation in Burmese and Mon people in Myanmar (Iwai et al. 2001; Matsuoka et al. 2004). Our findings in Mon are in sharp contrast with two previous studies of Cambodians in which G6PD Viangchan was found to be the most common G6PD deficient mutation: 28 of 34 (82.4%) in one study (Louicharoen and Nuchprayoon 2005) and 46 of 47 (96.5%) in the other (Matsuoka et al. 2005) and G6PD Mahidol was not present in any of the G6PD-deficient Cambodians.

We also found that all subjects with G6PD Mahidol and almost all other Mon and Burmese mutations had haplotype 1311C and 93T. This haplotype is present in the majority of G6PD-normal Mon as well as Burmese. This is different from G6PD Viangchan in Cambodians and other populations, which have haplotype 1311T by definition, and 93T (Yan et al. 2006). Although haplotypes of Cambodians have not been reported in the literature, this evidence supports a common ancestry between Mon and Burmese rather than Cambodians.

In addition to G6PD Mahidol, we also identified a few other G6PD-deficient mutations and a novel mutation: a 94

Table 3 Glucose-6-phosphate dehydrogenase (G6PD)-deficient mutations, activities and associated haplotypes

Mother's ethnic group	Place of birth	G6PD activity (IU/ g Hb)	*G6PD* mutation	Haplotype	
				nt1311	nt93
Burmese 12, Mon 12	Various	0.43 ± 0.48	Mahidol (487G > A)	C	T
Mon	Yangon	1.21	Mediterranean (563C > T)	C	T
Mon	Pha-un	1.80	Jammu (871G > A)	C	T
Mon	Pha-un	1.27	Kaiping (1388G > A)	C	T
Mon	Pha-un	0.00	94C > G	C	T
Burmese	Pha-un	0.64	Valladolid (406C > T)	T	C
Burmese	Yangon	0.00	Coimbra (592C > T)	C	T
Burmese	Yangon	1.27	Kerala-Kalyan (949G > A)	C	T

(C > G) in a Mon individual from Pha-un province in Myanmar. The point mutation 94 (C > G), which occurs in exon 3, is predicted to change amino acid from histidine to aspartic acid at residue 32 and result in no G6PD activity in the subject, thus is a class II variant. This mutation is close to G6PD Gaohe (95A > G), which changes the same amino acid residue from histidine to arginine.

A few mutations were suggestive of an Indian contribution to the Mon and Burmese G6PD-deficient gene pool: G6PD Jammu (871G > A; 1311C), G6PD Mediterranean, and G6PD Kerala-Kalyan (949G > A). G6PD Jammu was previously found in an Indian (Beutler et al. 1991). G6PD Mediterranean is widely distributed in different populations in the Mediterranean regions of southern Europe, the Middle East, and India. The haplotype of our G6PD Mediterranean case was 1311C and 93T, which is similar to those with the Indian, rather than the Middle Eastern, type (Beutler and Kuhl 1990). G6PD Kerala-Kalyan (949G > A) was reported to be prevalent (24.9%) in India (Sukumar et al. 2004). None of these mutations were found in Thai, Laotians, or Cambodians, whereas G6PD Mediterranean was found in approximately 30% of G6PD-deficient Indonesians from central Java (Soemantri et al. 1995), 27% of G6PD-deficient Malaysian Malays (Ainoon et al. 2002), occasionally in Thais of the southern province of Thailand (Laosombat et al. 2005), and G6PD Kerala-Kalyan was also identified in Urak-Lawoi, a sea Gypsy population of the Andaman Sea who inhabited Phuket Island in southern Thailand (Ninokata et al. 2006). Both polymorphisms C and T are present at nt 1311 in Indians

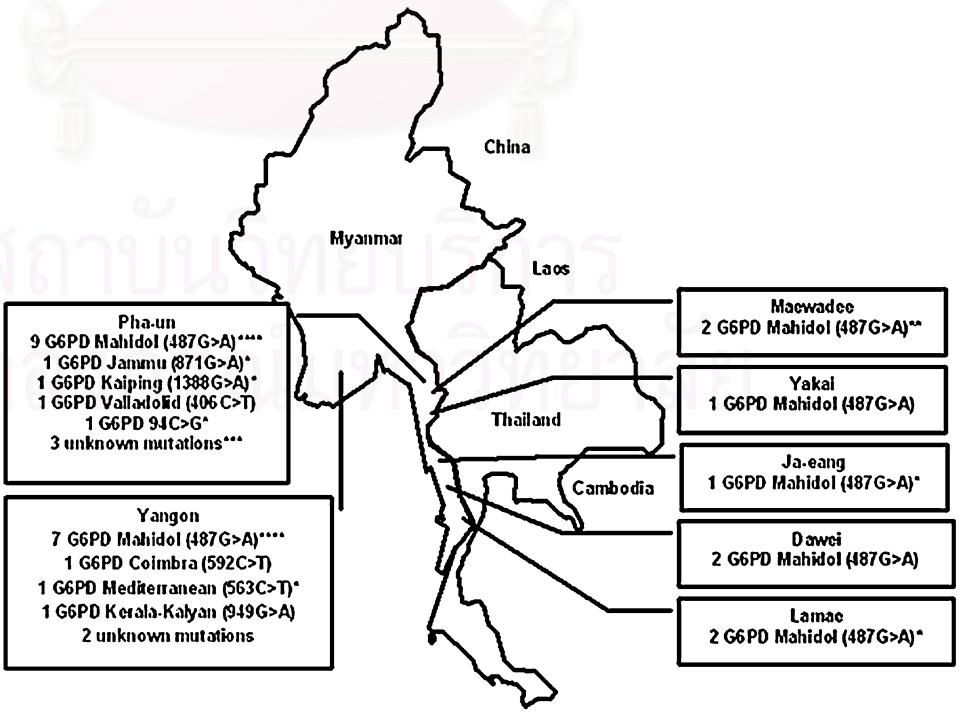

Fig. 2 Distribution of glucose-6-phosphate dehydrogenase (*G6PD*) mutations in Myanmar. *Numbers* represent number of *G6PD* mutations identified. *one, ** two, *** three, **** four cases were Mon

with G6PD Kerala-Kalyan (Sukumar et al. 2004). Our finding of G6PD Kerala-Kalyan in a Burmese from Yangon suggests the flow of this gene from India southward.

G6PD Kaiping is a predominant mutation among Chinese (Yan et al. 2006). This mutation is also the second most common mutation in southern Thailand (Laosombat et al. 2005). It is found in a few cases of Phuket Islanders in southern Thailand (Ninokata et al. 2006) and a few cases of Malaysian Malay (Ainoon et al. 2002). The haplotype of Chinese with G6PD Kaiping is also uniformly 1311C and 93T (Yan et al. 2006), similar to our Mon case in this study.

G6PD Coimbra (592C > T) is widely distributed across Europe and Asia but at low frequencies (Corcoran et al. 1992). G6PD Coimbra was previously reported in two G6PD-deficient Burmese (Matsuoka et al. 2004): one was from Yangon, which is similar to our case. G6PD Coimbra was also found in 3.5% of G6PD-deficient Malaysian Malay (Ainoon et al. 2002; Iwai et al. 2001) and in Flores Island, Indonesia (Kawamoto et al. 2006).

G6PD Valladolid (406C > T) has been previously identified in Spaniards (Vives-Corrons et al. 1997; Zarza et al. 1997) and Mexicans (Vaca et al. 2003). This mutation occurs in exon 5 and is predicted to code for cysteine at residue 135 instead of arginine and has reduced G6PD activity (0.64 IU/g Hb). How this mutation occurs in a G6PD-deficient Burmese subject is unclear. The mutation could have arisen independently, as there is no known strong historical linkage between Spain and Myanmar. However, a distinct haplotype seen in this case was different than in Burmese at large, suggesting a different ethnic origin of this individual, possibly during British occupation of Myanmar.

In summary, the G6PD mutations in Burmese are more heterogenous than previously suggested (Iwai et al. 2001; Matsuoka et al. 2004). Despite this heterogeneity, G6PD Mahidol is by far the most common G6PD-deficient mutation among Burmese and Mon populations. Its uniform and identical haplotype in both Mon and Burmese also confirm the common origin of these ethnic groups.

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เอกสารชี้แจงข้อมูล / คำแนะนำสำหรับผู้เข้าร่วมโครงการ

1. ชื่อโครงการวิจัย บทบาทของ G6PD Mahidol ต่อการป้องกันมาลาเรียในเอเชียตะวันออกเฉียงใต้ (A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA)

2. แพทย์ผู้ทำวิจัย

ชื่อ รศ. นพ. อิศรางค์ นุชประยูร

ที่อยู่ ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กทม. 10330

เบอร์โทรศัพท์ 02-256-4949

ผู้ช่วยวิจัย

ชื่อ นางสาวชาลิสา หลุยเจริญ

ที่อยู่ ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กทม. 10330

เบอร์โทรศัพท์ 02-256-4949

3. สถานที่วิจัย หน่วยตรวจสุขภาพแรงงานต่างด้าว โรงพยาบาลสมุทรสาคร, โรงพยาบาลจันทบุรี และโรงพยาบาลบุรีรัมย์

4. ผู้สนับสนุนการวิจัย ทุนโครงการปริญญาเอกกาญจนาภิเษก (คปก) สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

เรียน ผู้เข้าร่วมโครงการวิจัยทุกท่าน

เอกสารนี้เป็นเอกสารที่แสดงข้อมูลเพื่อใช้ประกอบการตัดสินใจของท่านในการเข้าร่วมการศึกษาวิจัย อย่างไรก็ตามก่อนที่ท่านตกลงเข้าร่วมการศึกษาดังกล่าว ขอให้ท่านอ่านเอกสารฉบับนี้อย่างละเอียดเพื่อให้ท่านได้ทราบถึงเหตุผลและรายละเอียดของการศึกษาวิจัยในครั้งนี้ หากท่านมีข้อสงสัยใดๆ เพิ่มเติม กรุณาซักถามจากผู้ทำวิจัยในโครงการซึ่งจะเป็นผู้สามารถให้ความกระจ่างแก่ท่านได้

ท่านสามารถขอคำแนะนำในการเข้าร่วมโครงการวิจัยนี้จากครอบครัว และเพื่อนได้ ถ้าท่านตัดสินใจแล้วว่าจะเข้าร่วมโครงการวิจัยนี้ ขอให้ท่านเซ็นชื่อยินยอมในเอกสารฉบับนี้

5. ความเป็นมาของโครงการ ที่ทำให้ต้องศึกษาเรื่องนี้

ภาวะพร่องเอ็นไซม์กลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนส (Glucose-6-phosphate dehydrogenase, G-6-PD deficiency) เป็นความผิดปกติที่มีการถ่ายทอดทางพันธุกรรมจากแม่สู่ลูกชาย โดยความผิดปกติที่เกิดขึ้นมีผลกระทบต่อเม็ดเลือดแดงทำให้เกิดภาวะฮีโมลัยซิส (hemolysis)ภาวะพร่อง G-6-PD เป็นปัญหาทางสาธารณสุขที่สำคัญของเอเชียตะวันออกเฉียงใต้

ภาวะพร่องเอ็นไซม์ G-6-PD เป็นความผิดปกติในระดับยีน *G6PD* โดยความผิดปกติที่พบบ่อยคือการกลายพันธุ์ (mutation) ของยีน *G6PD* ซึ่งส่งผลต่อเอ็นไซม์ G-6-PD ที่ผิดปกติ ในปัจจุบันการศึกษาทางพันธุศาสตร์ (Molecular technique) พบการกลายพันธุ์ของยีน *G6PD* ทั้งหมด 130 ชนิด โดยการกลายพันธุ์ของยีน *G6PD* แต่ละชนิดมีชื่อเรียกแตกต่างกันไป และจะแตกต่างกันไปในแต่ละกลุ่มประชากร โดยการกลายพันธุ์ชนิดที่พบบ่อยในคนไทย และลาว คือ G6PD Viangchan ส่วนในคนพม่า *G6PD* mutation ที่พบได้บ่อย คือ G6PD Mahidol การศึกษาด้านการกลายพันธุ์ของยีน *G6PD* สามารถนำมาเป็นข้อมูลในการอธิบายวิวัฒนาการของประชากรได้

ด้วยข้อมูลดังกล่าวทำให้มีความสนใจที่ทำการศึกษา *G6PD* mutations ในประชากรเอเชียตะวันออกเฉียงใต้ เพื่อนำมาเป็นข้อมูลในการอธิบายวิวัฒนาการ ประวัติศาสตร์ความสัมพันธ์ของประชากรในภูมิภาคเอเชียตะวันออกเฉียงใต้

6. วัตถุประสงค์ของการศึกษา

เพื่อศึกษาหาภาวะพร่องเอ็น ไซม์ G-6-PD และตรวจหาข้อมูลทางพันธุกรรมของยีนนี้เพื่อเปรียบเทียบกับประชากรชาวไทย ลาว พม่า มอญ และกัมพูชา เพื่อนำข้อมูลไปอธิบายวิวัฒนาการของยีนนี้ในประชากรเอเชียตะวันออกเฉียงใต้

7. วิธีการที่เกี่ยวข้องกับการวิจัย

หากท่านมีคุณสมบัติที่เหมาะสมและยินยอมที่จะเข้าร่วมในโครงการวิจัยนี้

- ผู้วิจัยจะขอให้ท่านกรอกข้อมูลประวัติส่วนตัว ครอบครัวลงในแบบฟอร์มที่ได้แนบมานี้ และขอเก็บตัวอย่างเลือดของท่านเพิ่มขึ้นอีก 5 มิลลิลิตร (1 ช้อนชา)เพื่อทำการวิจัย นอกเหนือจากที่ท่านเจาะเลือดเพื่อตรวจร่ายกายแล้ว
- ท่านไม่ต้องเสียค่าใช้จ่ายใดๆในการเข้าร่วมการศึกษาวิจัยครั้งนี้
- ท่านไม่ต้องมาพบผู้วิจัยอีกภายหลังจากผู้วิจัยขอเก็บตัวอย่างเลือดแล้ว

8. ประโยชน์

1. ทำให้ทราบลักษณะพันธุศาสตร์ประชากรของชาวกัมพูชา ลาว พม่า มอญ และ กระเหรี่ยง
2. ทำให้สร้างองค์ความรู้ใหม่ในการอธิบายวิวัฒนาการของประชากรเอเชียตะวันออกเฉียงใต้

9. ความเสี่ยงที่ได้รับจากการเจาะเลือด

ท่านมีโอกาสที่จะเกิดอาการเจ็บ เลือดออก ช้ำจากการเจาะเลือด อาการบวมบริเวณที่เจาะเลือดและโอกาสที่จะเกิดการติดเชื้อบริเวณที่เจาะเลือดพบได้น้อยมาก

หากท่านมีข้อสงสัยใดๆ เกี่ยวกับความเสี่ยงที่อาจได้รับจากการเข้าร่วมในโครงการวิจัย ท่านสามารถสอบถามจากแพทย์ผู้ทำวิจัยได้ตลอดเวลา

10. การเข้าร่วมและการสิ้นสุดการเข้าร่วมโครงการวิจัย

การเข้าร่วมในโครงการวิจัยครั้งนี้เป็นไปโดยความสมัครใจ หากท่านไม่สมัครใจจะเข้าร่วมการศึกษาแล้ว ท่านสามารถถอนตัวได้ตลอดเวลา และการถอนตัวจากโครงการวิจัยจะไม่มีผลต่อการตรวจร่างกายของท่านแต่อย่างใด

สิทธิ์ของผู้เข้าร่วมโครงการวิจัย

ในฐานะที่ท่านเป็นผู้เข้าร่วมในโครงการวิจัย ท่านมีสิทธิ์ดังต่อไปนี้

1. ท่านจะได้รับทราบถึงลักษณะและวัตถุประสงค์ของการวิจัยในครั้งนี้
2. ท่านจะได้รับการอธิบายเกี่ยวกับระเบียบวิธีการวิจัยทางการแพทย์
3. ท่านจะได้รับการอธิบายถึงความเสี่ยงและความไม่สบายที่จะได้รับจากการวิจัย
4. ท่านจะมีโอกาสได้ซักถามเกี่ยวกับงานวิจัยหรือขั้นตอนที่เกี่ยวข้องกับงานวิจัย
5. ท่านจะได้รับทราบว่าการยินยอมเข้าร่วมโครงการวิจัยนี้ ท่านสามารถขอถอนตัวจากโครงการเมื่อไรก็ได้ โดยผู้เข้าร่วมในโครงการวิจัยสามารถขอถอนตัวจากโครงการ โดยไม่ได้รับผลเสียใดๆทั้งสิ้น
6. ท่านจะได้รับสำเนาเอกสารใบยินยอมที่มีลายเซ็นและวันที่
7. ท่านจะได้รับโอกาสในการตัดสินใจว่าจะเข้าร่วมในโครงการวิจัยหรือไม่ก็ได้ โดยปราศจากการใช้อิทธิพลบังคับ ข่มขู่ หรือการหลอกลวง

11. ค่าตอบแทน

โครงการวิจัยนี้ไม่ได้จ่ายค่าตอบแทนให้

12. การปกป้องรักษาข้อมูลของอาสาสมัคร

ข้อมูลที่อาจนำไปสู่การเปิดเผยตัวของท่าน จะได้รับการปกปิดและจะไม่เปิดเผยแก่สาธารณชน ในกรณีที่ผลการวิจัยได้รับการตีพิมพ์ ชื่อและที่อยู่ของท่านจะต้องได้รับการปกปิดอยู่เสมอ โดยจะใช้เฉพาะรหัสประจำโครงการวิจัยของท่าน

13. เจ้าหน้าที่หรือแพทย์ผู้ที่ท่านสามารถติดต่อได้

ถ้าท่านมีข้อสงสัยประการใด โปรดสอบถามแพทย์ผู้รับผิดชอบโครงการ ได้แก่ นายแพทย์อิศรางค์ นุชประยูร โทร . 0-2256-4000 ต่อ 4949 ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย หรือผู้ช่วยวิจัย นางสาว ชาลิสา หลุยเจริญ โทร. 0-2256-4000 ต่อ 3567

ขอขอบคุณในการร่วมมือของท่านมา ณ ที่นี้

หนังสือให้ความยินยอมเข้าร่วมโครงการวิจัย

วันที่……

ข้าพเจ้า……..……..……..อายุ…….ปี ขอทำหนังสือเล่มนี้ให้ไว้ต่อหัวหน้าโครงการวิจัยเพื่อเป็นหลักฐานแสดงว่า

ข้อ 1. ข้าพเจ้าได้รับทราบโครงการวิจัยของ รศ. นพ. อิศรางค์ นุชประยูร และคณะ เรื่อง บทบาทของ G6PD Mahidol ต่อการป้องกันมาลาเรียในเอเชียตะวันออกเฉียงใต้ (A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA)

ข้อ 2. ข้าพเจ้ายินยอมเข้าร่วมโครงการวิจัยนี้ด้วยความสมัครใจ โดยมิได้มีการบังคับหลอกลวงแต่ประการใด และพร้อมจะให้ความร่วมมือในการวิจัย

ข้อ 3. ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยเกี่ยวกับวัตถุประสงค์ของการวิจัย วิธีการวิจัย ประสิทธิภาพ ความปลอดภัย อาการ หรืออันตรายที่อาจเกิดขึ้น รวมทั้งประโยชน์ที่จะได้รับจากการวิจัยโดยละเอียดแล้วจากเอกสารการวิจัยที่แนบท้ายหนังสือให้ความยินยอมนี้ ข้าพเจ้าได้รับสำเนาใบยินยอมเข้าร่วมโครงการวิจัยที่ข้าพเจ้าได้ลงนาม และวันที่

ข้อ 4. ข้าพเจ้าได้รับการรับรองจากผู้วิจัยว่า จะเก็บข้อมูลส่วนตัวของข้าพเจ้าเป็นความลับ จะเปิดเผยเฉพาะผลสรุปการวิจัยเท่านั้น และจะเปิดเผยได้เฉพาะเมื่อได้รับการยินยอมจากข้าพเจ้าเท่านั้น บุคคลอื่นในนามคณะกรรมการพิจารณาจริยธรรมการวิจัยหรือผู้ได้รับอำนาจมอบหมายให้เข้าตรวจและประมวลข้อมูลของผู้เข้าร่วมวิจัย ทั้งนี้จะต้องกระทำไปเพื่อวัตถุประสงค์เพื่อตรวจสอบความถูกต้องของข้อมูลเท่านั้น โดยการตกลงที่เข้าร่วมการศึกษานี้ข้าพเจ้าได้ให้คำยินยอมที่จะให้มีการตรวจสอบข้อมูลประวัติทางการแพทย์ของผู้เข้าร่วมวิจัยได้

ข้อ 5. ข้าพเจ้าได้รับทราบจากผู้วิจัยแล้วว่าหากมีอันตรายใดๆ ในระหว่างการวิจัยหรือภายหลังการวิจัยอันพิสูจน์ได้จากผู้เชี่ยวชาญของสถาบันที่ควบคุมวิชาชีพนั้นๆ ได้ว่าเกิดขึ้นจากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการดูแลและค่าใช้จ่ายในการรักษาพยาบาลจากผู้วิจัยและ / หรือผู้สนับสนุนการวิจัย และจะได้รับค่าชดเชยรายได้ที่สูญเสียไปในระหว่างการรักษาพยาบาลดังกล่าวตามมาตรฐานค่าแรงขั้นต่ำตามกฎหมาย ตลอดจนมีสิทธิได้รับค่าทดแทนความพิการที่อาจเกิดขึ้นจากการวิจัยตามมาตรฐานค่าแรงขั้นต่ำตามกฎหมายและในกรณีที่ข้าพเจ้าได้รับอันตรายจากการวิจัยถึงแก่ความตาย ทายาทของข้าพเจ้ามีสิทธิได้รับค่าชดเชยและค่าทดแทนดังกล่าวจากผู้วิจัยและ / หรือผู้สนับสนุนการวิจัยแทนตัวข้าพเจ้า

ข้อ 6.ข้าพเจ้าได้รับทราบว่า ข้าพเจ้ามีสิทธิจะบอกเลิกการร่วมโครงการวิจัยนี้เมื่อใดก็ได้ และการบอกเลิกการร่วมโครงการวิจัยจะไม่มีผลกระทบต่อการได้รับบรรดาค่าใช้จ่าย ค่าชดเชยและค่าทดแทนตามข้อ 5 ทุกประการ

ข้อ 7. หัวหน้าผู้วิจัยได้อธิบายเกี่ยวกับรายละเอียดต่างๆของโครงการ ตลอดจนประโยชน์ของการวิจัย รวมทั้งความเสี่ยงและอันตรายต่างๆ ที่อาจจะเกิดขึ้นในการเข้าโครงการนี้ให้ข้าพเจ้าได้ทราบ และตกลงรับผิดชอบตามคำรับรองในข้อ 5 ทุกประการ

ข้าพเจ้าเข้าใจว่า ข้าพเจ้ามีสิทธิ์ที่จะตรวจสอบหรือแก้ไขข้อมูลส่วนตัวของข้าพเจ้าและสามารถเลิกการให้สิทธิในการใช้ข้อมูลส่วนตัวของข้าพเจ้าได้ โดยต้องแจ้งให้ผู้วิจัยรับทราบ ข้าพเจ้าได้ตระหนักว่าข้อมูลในการวิจัยรวมถึงข้อมูลทางการแพทย์ที่ไม่มีการเปิดเผยชื่อ จะผ่านกระบวนการต่างๆ เช่น การเก็บข้อมูล การบันทึกข้อมูลในคอมพิวเตอร์ การตรวจสอบ การวิเคราะห์และการรายงานเพื่อวัตถุประสงค์ทางวิทยาศาสตร์ รวมทั้งการใช้ข้อมูลทางการแพทย์ในอนาคตเท่านั้น

ข้าพเจ้าได้อ่านและเข้าใจข้อความตามหนังสือนี้โดยตลอดแล้ว เห็นว่าถูกต้องตามเจตนาของข้าพเจ้า จึงได้ลงรายมือชื่อไว้เป็นสำคัญ พร้อมกับหัวหน้าผู้วิจัยและต่อหน้าพยาน

ลงชื่อ……..……ผู้ยินยอม
(…….…..…)
ลงชื่อ……...หัวหน้าผู้วิจัย
(……..…..)
ลงชื่อ……...…พยาน
(……)
ลงชื่อ……...…พยาน
(……)

หมายเหตุ 1) กรณีผู้ยินยอมตนให้ทำวิจัย ไม่สามารถอ่านหนังสือได้ ให้ผู้วิจัยอ่านข้อความในหนังสือให้ความยินยอมนี้ ให้แก่ผู้ยินยอมให้ทำวิจัยฟังจนเข้าใจดีแล้ว และให้ผู้ยินยอมตนให้ทำวิจัยลงนาม หรือพิมพ์ลายนิ้วหัวแม่มือรับทราบในการให้ความยินยอมดังกล่าวด้วย

2) ในกรณีผู้ให้ความยินยอมมีอายุไม่ครบ 20 ปีบริบูรณ์ จะต้องมีผู้ปกครองตามกฎหมายเป็นผู้ให้ความยินยอมด้วย

<u>เลขที่ .....</u>

<u>แบบบันทึกข้อมูลแรงงานต่างด้าว</u>

เชื้อชาติ :☐ พม่า ☐ ฉาน ☐ กะเหรี่ยง ☐ ยะไข่ ☐ จีน ☐ มอญ ☐ อินเดีย ☐ ดานู ☐ คาชิน ☐ ลิซู ☐ อคา

☐ กัมพูชา

☐ ลาว

☐ อื่นๆ.....

สถานที่เกิด (จังหวัด).....

เชื้อชาติของ :

มารดา:☐ พม่า ☐ ฉาน ☐ กะเหรี่ยง ☐ ยะไข่ ☐ จีน ☐ มอญ ☐ อินเดีย ☐ ดานู ☐ คาชิน ☐ ลิซู ☐ อคา

☐ กัมพูชา

☐ ลาว

☐ อื่นๆ.....

ตา :☐ พม่า ☐ ฉาน ☐ กะเหรี่ยง ☐ ยะไข่ ☐ จีน ☐ มอญ ☐ อินเดีย ☐ ดานู ☐ คาชิน ☐ ลิซู ☐ อคา

☐ กัมพูชา

☐ ลาว

☐ อื่นๆ.....

ยาย : ☐ พม่า ☐ ฉาน ☐ กะเหรี่ยง ☐ ยะไข่ ☐ จีน ☐ มอญ ☐ อินเดีย ☐ ดานู ☐ คาชิน ☐ ลิซู ☐ อคา

☐ กัมพูชา

☐ ลาว

☐ อื่นๆ.....

Volunteer Information Sheet

1. Research title A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA

2. Researcher Assoc. Prof. Dr. Issarang Nuchprayoon
Miss Chalisa Louicharoen

3. Research place Tarksin Hospital, Samutsakorn Hospital, Buriram Hospital

4. Research Foundation The Royal Golden Jubilee Ph.D. Program (RGJ), The Thailand research Found (TRF)

You are invited to participate in research project that study population genetic of the *G6PD* gene in blood cell. Then we will describe the evolution of this gene in Southeast Asian population from the genetic information. Before you decide to participate this study, we would like you to know the reasons and details in this study.

5. Background of research project

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is the most common disorder in humans that is frequently found in Southeast Asian. Inherited deficiency of this enzyme cause hemolytic anemia, which means that red blood cell die before aging. People with G6PD deficiency are often healthy except when they take some medication or expose to certain chemical agents, they can become pale and has dark urine. Mutation of *G6PD* gene affects the G-6-PD deficiency. Certain *G6PD* mutations are associated with specific ethnic groups in Southeast Asian. The objective of this study is to compare the data of *G6PD* among patients in many ethnic groups and healthy persons to describe the evolution of this gene in Southeast Asian.

6. Objectives

1. To study the deficiency and *G6PD* mutations in Thai, Cambodian, Laotian, Karen, Mon and Myanmese
2. To describe the evolution of *G6PD* mutations in Southeast Asian population

7. Process of research participation

We will draw 7 ml. (2 teaspoon) of blood from your vein once. The amount of blood is divided in to 2 parts: health screening program for work permit in Thailand and doing the research project. We will perform a test to see whether you are G6PD deficient at no cost to you.

8. Application

1. Know the population genetic of Southeast Asian.

2. Reconstruct the evolution of Southeast Asian from the population genetic database.

9. The effect of being volunteer in research project

The amount of blood is not harmful to your health. Because you are getting blood for other purpose only extra blood will be collected.

10. Rightness in participation of research project

You can refuse to be volunteer if you do not agree to participate. The decline does not affect your access in health care service.

11. Remuneration

The volunteer will not be paid.

12. Your data is confidential

The result of this study is only in the objective of research. Your individual information will be kept secret and be presented only in the form of research conclusion.

13. Counselor in research project

If you have problem to be volunteer, you can connect to Dr. Issarang Nuchprayoon Tel. 01-641-4797.

Consent form

Date……

Name……Age……

This document was constructed to be evidence

1. I confirm that I have read and understand the information of the research project "A ROLE FOR G6PD MAHIDOL IN PROTECTION AGAINST MALARIA IN SOUTHEAST ASIA" and that all my questions have been answered satisfactory.

2. I agree to take part in the above study.

3. The researcher has informed me about objectives, methodology, safety, benefit and risk of this research.

4. The researcher promised to keep my personal information secret and will present only in the form of research conclusion.

5. I understand that responsible individuals managing the registry and regulatory authorities. (i.e. institutional review board) may have access to my records.

6. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care being affected.

Signature……..……volunteer

(……)

Signature……..…leader of researcher

(……)

Signature……witness

(……)

Signature……witness

(……)

Questionnaire

No…….

Nationality: □ Burma (Aka) □ Chan □ Karen □ Yakai □ Chinese □ Mon □ Indian

□ Danu □ Kachin □ Lisu

□ Cambodian

□ Laotian

□ Other.....….....

Place of birth…….

Nationality of

Mother: □ Burma (Aka) □ Chan □ Karen □ Yakai □ Chinese □ Mon □ Indian

□ Danu □ Kachin □ Lisu

□ Cambodian

□ Laotian

□ Other.....….....

Grandfather: □ Burma (Aka) □ Chan □ Karen □ Yakai □ Chinese □ Mon □ Indian

□ Danu □ Kachin □ Lisu

□ Cambodian

□ Laotian

□ Other.....….....

Grandmother: □ Burma (Aka) □ Chan □ Karen □ Yakai □ Chinese □ Mon □ Indian

□ Danu □ Kachin □ Lisu

□ Cambodian

□ Laotian

□ Other.....….....

បញ្ជីព័ត៌មានអ្នកស្ម័គ្រចិត្ត

១. ចំណងជើងនៃការស្រាវជ្រាវ: ប្រភេទសេនេទិចនៃសែន G6PD របស់ប្រជាជនក្នុងតំបន់អាស៊ីអាគ្នេយ៍

២. អ្នកស្រាវជ្រាវ: បណ្ឌិតសាស្ត្រាចារ្យ Issarang Nuchprayoon

កញ្ញា Chalisa Louicharoen

៣. ទីកន្លែងស្រាវជ្រាវ: មន្ទីរពេទ្យ Tarksin

៤. ប្រូលនិធិនៃការស្រាវជ្រាវ: កម្មវិធីសំរាប់ថ្នាក់បណ្ឌិតរបស់ The Royal Golden Jubilee (RGJ), ការបង្កើតគំរោងស្រាវជ្រាវរបស់ប្រទេសថៃ

អ្នកត្រូវបានគេអញ្ជើញអោយចូលរួមក្នុងគំរោងស្រាវជ្រាវមួយដែលសិក្សាពីសែន G6PD នៃប្រភេទសេនេទិចក្នុងកោសិកាឈាម។ បន្ទាប់មកយើងនឹងពិពណ៌នាអំពីការវិវត្តនៃសែននេះនៃប្រជាជនក្នុងតំបន់អាស៊ីអាគ្នេយ៍ពីព័ត៌មានសេនេទិច។ យើងចង់អោយអ្នកដឹងពីហេតុផល និងព័ត៌មានពិស្តារមួយចំនួន មុនសំរេចចិត្តចូលរួមក្នុងការសិក្សាស្រាវជ្រាវនេះ។

៥. ប្រវ្តិនៃគំរោងស្រាវជ្រាវ

កង្វះអង់ស៊ីម Glucose-6-phosphate dehydrogenase គឺជាភាពគ្មានសណ្តាប់ធ្នាប់ដ៏សាមញ្ញបំផុតក្នុងសារពាង្គកាយមនុស្សដែលគេរកឃើញជារឿយៗនៅតំបន់អាស៊ីអាគ្នេយ៍។ កង្វះអង់ស៊ីមនេះអាចបណ្តាលអោយកើតមានជំងឺខ្វះឈាមក្រហម មានន័យថាកោសិកាឈាមក្រហមងាប់មុនអាយុ។ មនុស្សដែលខ្វះ G6PD តែងតែមានសុខភាពល្អ លើកលែងតែពេលពួកគេប្រើប្រាស់ឱសថ ឬក៏សំដែលត្រូវសារធាតុគីមីមួយចំនួន ពួកគេនឹងទៅប្រែជាស្លេកស្លាំង និងទឹកនោមប្រែជាពណ៌ខ្មៅ។ ការផ្លាស់ប្តូរសែន G6PD មានឥទ្ធិពលលើកង្វះ G-6-PD។ ការផ្លាស់ប្តូរសែន G6PD មួយចំនួនមានទំនាក់ទំនងជាមួយក្រុមជនជាតិភាគតិចក្នុងតំបន់អាស៊ីអាគ្នេយ៍។ គោលបំណងនៃការសិក្សានេះ គឺប្រៀបធៀបពីទិន្នន័យនៃសែន G6PD ក្នុងចំណោមអ្នកជំងឺក្រុមជនជាតិភាគតិច និងមនុស្សដែលមានសុខភាពល្អ ដើម្បីពិពណ៌នាពីដំណើរវិវត្តរបស់សែននេះនៅតំបន់អាស៊ីអាគ្នេយ៍។

៦. គោលបំណង

1. កំណត់អត្តសញ្ញាណ microsatellite haplotypes នៃសែន G6PD ក្នុងជនជាតិថៃ ឡាវ ប្រុយនេ និងកម្ពុជា

2. ពិពណ៌នាអំពីការវិវត្តរបស់សែន G6PD នៃប្រជាជនក្នុងតំបន់អាស៊ីអាគ្នេយ៍។

៧. ចំណើរការនៃការចូលរួមស្រាវជ្រាវ

យើងនឹងយកឈាមម្តង ៧មីលីលីត្រ "២ស្លាបព្រា" ពីសរសៃឈាមរបស់អ្នក។ បរិមាណឈាមនេះនឹងត្រូវបានចែកចេញជាពីរផ្នែក: កម្មវិធីត្រួតពិនិត្យសុខភាពសំរាប់ការអនុញ្ញាតិអោយធ្វើការក្នុងប្រទេសថៃ និងធ្វើគំរោងស្រាវជ្រាវ។ យើងនឹងរៀបចំការធ្វើតេស្តដើម្បីត្រួតពិនិត្យថាតើកង្វះសែន G6PD ពិតជាថាមិនមានបញ្ហាចំពោះអ្នក។

៨. ការអនុវត្តន៍

1. ដឹងពីសេនេទិចរបស់ប្រជាជននៅតំបន់អាស៊ីអាគ្នេយ៍ ។

2. សាងសង់ឡើងវិញអំពីការវិវត្តទិន្នន័យសេនេទិចរបស់ប្រជាជននៅតំបន់អាស៊ីអាគ្នេយ៍

៩. ប្រសិទ្ធិភាពនៃការស្ម័គ្រចិត្តក្នុងគំរោងស្រាវជ្រាវ

បរិមាណឈាមនេះមិនបង្កភាពអន្តរាយដល់សុខភាពរបស់អ្នកទេ ។ ដោយសារតែអ្នកកំពុងតែទទួលបានឈាមក្នុងគោលបំណងផ្សេង មានតែឈាមលើសប៉ុណ្ណោះនឹងត្រូវបានប្រមូល ។

១០. សិទ្ធិក្នុងការចូលរួមក្នុងគំរោងស្រាវជ្រាវ

អ្នកអាចបដិសេធក្នុងការធ្វើជាអ្នកស្ម័គ្រចិត្ត បើសិនជាអ្នកមិនចង់ចូលរួម ។ ការបដិសេធរបស់អ្នកមិនមានផលប៉ះពាលដល់ការទទួលបាននូវសេវាថែទាំសុខភាពឡើយ ។

១១. រង្វាន់

អ្នកស្ម័គ្រចិត្ត និងមិនបានប្រាក់កំរៃទេ

១២. ទិន្នន័យរបស់អ្នកគឺសំងាត់

លទ្ធផលនៃការសិក្សានេះគឺជាគោលបំណងតែមួយគត់នៃការស្រាវជ្រាវ ។ ព័ត៌មានផ្ទាល់ខ្លួនរបស់អ្នកនឹងត្រូវបានទុកជាសំងាត់ និងបង្ហាញក្រោមទំរង់ជាការសន្និដ្ឋាននៃការស្រាវជ្រាវប៉ុណ្ណោះ ។

១៣. អ្នកពិគ្រោះយោបល់ក្នុងគំរោងស្រាវជ្រាវ

ប្រសិនបើអ្នកមានបញ្ហាពីការធ្វើជាអ្នកស្ម័គ្រចិត្ត អ្នកអាចទាក់ទងទៅ បណ្ឌិត Issarang Nuchprayoon តាមរយៈទូរស័ព្ទលេខ ០១-៦៤១-៤៧៩៧ ។

លិខិតព្រមព្រៀង

ទីកន្លែង.....

កាលបរិច្ឆេទ.....

ឈ្មោះ.....អាយុ.....អាស័យដ្ឋាន.....

.....ទីក្រុង.....ប្រទេស.....

ឯកសារនេះត្រូវបានរៀបចំឡើងដើម្បីទុកជាការសំងាត់

១. ខ្ញុំសូមអះអាងថា ខ្ញុំបានអាន និងយល់ពីព័ត៌មាននៃគំរោងស្រាវជ្រាវស្តីពី " ប្រភេទសេនេទិច នៃសែន G6PD របស់ប្រជាជនក្នុងតំបន់អាស៊ីអាគ្នេយ៍ " និង រាល់សំណួររបស់ខ្ញុំត្រូវបានឆ្លើយដោយការពេញចិត្ត។

២. ខ្ញុំយល់ព្រមចូលរួមក្នុងការសិក្សាខាងលើនេះ

៣. អ្នកស្រាវជ្រាវបានបញ្ជាក់ប្រាប់ខ្ញុំពី គោលបំណង វិធីសាស្ត្រ សុវត្ថិភាព ផលចំណេញ និងគ្រោះថ្នាក់នៃការស្រាវជ្រាវនេះ

៤. អ្នកស្រាវជ្រាវសន្យាថែរក្សាព័ត៌មានផ្ទាល់ខ្លួនរបស់ខ្ញុំជាសំងាត់ និងបង្ហាញតែក្រោមទំរង់ជាការសន្និដ្ឋាននៃការស្រាវជ្រាវប៉ុណ្ណោះ។

៥.ខ្ញុំយល់ថាការទទួលខុសត្រូវផ្ទាល់ខ្លួន ក្នុងការរៀបចំចុះឈ្មោះ និងបទបញ្ជ (មានន័យថាក្រុមប្រឹក្សាត្រួតពិនិត្យឡើងវិញនៃស្ថាប័ន) ប្រហែលជាមានលទ្ធភាពទទួលបានឯកសាររបស់ខ្ញុំ។

៦. ខ្ញុំយល់ថា ការចូលរួមរបស់ខ្ញុំគឺជាការស្ម័គ្រចិត្ត និងខ្ញុំមានសេរីភាពក្នុងការចាកចេញគ្រប់ពេលវេលាដោយមិនចាំបាច់ផ្តល់ហេតុផល និងមិនមានផលប៉ះពាល់ដល់សេវាកម្មផ្នែកសុខាភិបាល។

ហត្ថលេខា.....អ្នកស្ម័គ្រចិត្ត

(.....)

ហត្ថលេខា.....អ្នកដឹកនាំស្រាវជ្រាវ

(.....)

ហត្ថលេខា.....សាក្សី

(.....)

ហត្ថលេខា.....សាក្សី

(.....)

គំរោងសំណួរ

លេខ.....

សញ្ជាតិ:☐ភូមា ☐ចាន់ ☐ការ៉ែន ☐យ៉ាកែ ☐ចិន ☐មន ☐ឥណ្ឌា ☐Danu ☐កាជិន ☐Lisu

☐កម្ពុជា

☐ឡាវ

☐ផ្សេងៗ.....

ទីកន្លែងកំណើត.....

សញ្ជាតិរបស់

ម្តាយ:: ☐ភូមា ☐ចាន់ ☐ការ៉ែន ☐យ៉ាកែ ☐ចិន ☐មន ☐ឥណ្ឌា ☐Danu ☐កាជិន ☐Lisu

☐កម្ពុជា

☐ឡាវ

☐ផ្សេងៗ.....

ជីតា: : ☐ភូមា ☐ចាន់ ☐ការ៉ែន ☐យ៉ាកែ ☐ចិន ☐មន ☐ឥណ្ឌា ☐Danu ☐កាជិន ☐Lisu

☐កម្ពុជា

☐ឡាវ

☐ផ្សេងៗ.....

ជីដូន:: ☐ភូមា ☐ចាន់ ☐ការ៉ែន ☐យ៉ាកែ ☐ចិន ☐មន ☐ឥណ្ឌា ☐Danu ☐កាជិន ☐Lisu

☐កម្ពុជា

☐ឡាវ

☐ផ្សេងៗ.....

ເອກະສານຂໍ້ມູນຂອງອາສາສະມັກ

1. **ຫົວຂໍ້ຂອງການວິໄຈ:** ກຳມະພັນ ຂອງປະຊາກອນ G6DP ໂດຍສະເພາະ ປະຊາກອນຢູ່ໃນພນທີ່ອາຊີຕາເວັນອອກສ່ຽງໃຕ້
2. **ຜູ້ວິໄຈ:** ຮອງສາດຕາຈານ ດຣ. ອິສາລາງ ນຸພາຍູນ (Assos, Prof. Dr. Issarang Nuchprayoon) ແລະ ນາງ ຊາລິສາ ລຸ້ງຈາເລີນ (Miss Chalisa Louicharoen)
3. **ສະຖານທີ່ວິໄຈ:** ໂຮງພະຍາບານ ຕາກສິນ
4. **ທີ່ມາຂອງການວິໄຈ:** ສາຖາບັນວິໄຈຈຸລາພອນ ຫຼັກສູດປະລິນຍາເອກ (RGJ) ແລະ ກອງທຶນ ວິໄຈຂອງປະເທດໄທ (TRF)

ທ່ານແມ່ນຜູ້ທີ່ໄດ້ເຊື້ອເຊີນເຂົ້າຮ່ວມ ໃນໂຄງການວິໄຈ ຫົວຂໍ້ເລື່ອງ ກຳມະພັນຂອງປະຊາກອນທີ່ຂາດ G6DP ໃນຈຸລັງຂອງເມັດເລືອດ. ພວກຂ້າພະເຈົ້າ ຈະໄດ້ ອະທິບາຍ ແລະ ປະເມີນຜົນ ກ່ຽວກັບ ສາຍສືບພັນຂອງປະຊາກອນ ໃນຂົງເຂດອາຊີຕາເວັນອອກສ່ຽງໃຕ້ ຈາກບັນດາຂໍ້ມູນທີ່ໄດ້ສຶກສາວິໄຈ ດ້ານກຳມະພັນ. ກ່ອນທີ່ທ່ານຈະຕັດສິນໃຈເຂົ້າຮ່ວມໃນໂຄງການນີ້, ພວກຂ້າພະເຈົ້າ ສະເໜີ ລາຍງານ ໃຫ້ທ່ານຮັບຮູ້ເຖິງ ເຫດຜົນ ແລະ ລາຍລະອຽດຂອງການວິໄຈໃນຄັ້ງນີ້:

5. **ຄວາມເປັນມາຂອງໂຄງການວິໄຈ:**

Glucose-6-Phosphate dehydrogenase (G-6-DP) ແມ່ນຊື່ທາດທາງເຄມີຂອງຜູ້ທີ່ຂາດເລືອດ. ສ່ວນຫຼາຍແລ້ວ ແມ່ນເກີດຈາກໂລກໄພໄຂ້ເຈັບ ທີ່ເກີດຂຶ້ນໃນມະນຸດເຮົາ ຊຶ່ງພົບເຫັນເລື້ອຍໆ ໃນພາກພື້ນ ອາຊີຕາເວັນອອກສ່ຽງໃຕ້. ການຖ່າຍທອດທາງດ້ານກຳມະພັນ ແມ່ນສາຍເຫດໜຶ່ງ ທີ່ເຮັດໃຫ້ເກີດ ພະຍາດຂາດເລືອດ ນັ້ນໝາຍເຖິງຈຸລັງເມັດເລືອດແດງຕາຍກ່ອນອາຍຸກຳນົດ. ປະຊາກອນຜູ້ທີ່ເປັນພະຍາດ ຂາດເລືອດ G6DP ສ່ວນຫຼາຍແລ້ວແມ່ນບໍ່ໄດ້ຮັບການເອົາໃຈໃສດ້ານສຸຂະພາບ. ເມື່ອພວກເຂົາ ໄດ້ຮັບການຮັກສາດ້ວຍຢາ ຫຼື ນຳໃຊ້ສານເຄມີ, ພວກເຂົາຈະກາຍເປັນຄົນທີ່ອ່ອນແອ, ມີອາການ ສະແດງອອກຄື ຖ່າຍເບົາເປັນສີຊຸ້ມ, ນັ້ນແມ່ນການປ່ຽນແປງຂອງຕົວສືບພັນ G6DP ທີ່ເກີດຈາກ ຜົນກະທົບຂອງການຂາດເຂີນ G-6-DP ດັ່ງກ່າວ. ຈຸດປະສົງຂອງການສຶກສາສິໃຈຄັ້ງນີ້ ແມ່ນເພື່ອປຽບທຽບ ຂໍ້ມູນຂອງ G6DP ທັງໝົດໃນຫຼາຍກຸ່ມ ຊົນຊາດ, ຊົນເຜົ່າ ແລະ ສຸຂະພາບຂອງບຸກຄົນ ເພື່ອການອະທິບາຍ ແລະ ການປະເມີນຜົນ ກ່ຽວກັບສາຍເຫດດ້ານກຳມະພັນ ຂອງປະຊາກອນໃນຂົງເຂດພາກພື້ນ ອາຊີຕາເວັນ ອອກສ່ຽງໃຕ້.

6. ຈຸດປະສົງ:

1. ເພື່ອຊອກຫາ microsatellite haplotypes ຂອງ G6DP ໃນກຳມະພັນຂອງຄົນໄທ, ລາວ, ພະມ້າ ແລະ ກຳປູເຈ້ຍ.
2. ເພື່ອອະທິບາຍ, ປະເມີນຜົນ G6DP ຂອງສາຍສືບພັນໃນປະຊາກອນໃນຂົງເຂດພາກພື້ນອາຊີຕາເວັນອອກສ່ຽງໃຕ້.

7. ຂັ້ນຕອນຂອງຜູ້ເຂົ້າຮ່ວມການວິໃຈ:

ພວກຂ້າພະເຈົ້າຈະໃຊ້ເລືອດ 7 ມິລິລິດ (ປະມານ 2 ບ່ວງຊາ) ຈາກເສັ້ນເລືອດດຳຂອງທ່ານ. ຊຶ່ງເລືອດທັງໜົດນັ້ນແມ່ນຈະແບ່ງ ເປັນ 2 ພາກສ່ວນຄື: ນຳໄປວິໃຈຕາມໂປແກມວິເຄາະສຸຂະພາບ ສຳລັບການ ຂໍອະນຸຍາດເຮັດວຽກຢູ່ປະເທດໄທ ແລະ ການເຮັດວິໃຈໂຄງການ. ໃນການທົດສອບ ແມ່ນເພື່ອໃຫ້ຮູ້ກ່ຽວກັບ ການຂາດເຂີນ G6DP ໂດຍທ່ານບໍ່ຕ້ອງເສັຍຄ່າໃຊ້ຈ່າຍ ໃດໆ.

8. ຜົນໄດ້ຮັບ:

1. ເພື່ອຮູ້ກ່ຽວກັບກຳມະພັນ ຂອງປະຊາກອນໃນຂົງເຂດອາຊີຕາເວັນອອກສ່ຽງໃຕ້
2. ສ້າງຄົນໃໝ່ກ່ຽວການປະເມີນ ຖານຂໍ້ມູນກຳມະພັນຂອງປະຊາກອນໃນອາຊີຕາເວັນ ອອກສ່ຽງໃຕ້

9. ຜົນກະທົບທີ່ຈະເກີດຈາກການເປັນອາສາສະມັກຂອງໂຄງການ:

ເລືອດທີ່ໄດ້ໃຊ້ເຂົ້າໃນການທົດສອບ ແມ່ນບໍ່ມີຜົນກະທົບຫຍັງ ຕໍ່ສຸຂະພາບຂອງທ່ານ.

10. ສິດທິຂອງການເຂົ້າຮ່ວມໂຄງການວິໃຈ:

ທ່ານຈະສາມາດປະຕິເສດການເປັນອາສາສະມັກໄດ້ ຖ້າທ່ານບໍ່ສະມັກໃຈເຂົ້າຮ່ວມ ແລະ ກໍ່ຈະບໍ່ມີ ຜົນກະທົບໃນການບໍລິການດ້ານຮັກສາສຸພະພາບ.

11. ຜົນຕອບແທນ:

ຜູ້ທີ່ເປັນອາສາສະມັກແມ່ນຈະບໍ່ມີຄ່າໃຊ້ຈ່າຍຫຍັງ.

12. ຂໍ້ມູນຂອງທ່ານແມ່ນຈະເປັນຄວາມລັບ:

ຜົນຂອງການສຶກສານີ້ ແມ່ນມີຈຸດປະສົງສຳລັບການເຮັດວິໃຈ, ຊຶ່ງຂໍ້ມູນສ່ວນຕົວຂອງທ່ານ ແມ່ນຈະ ຮັກສາເປັນຄວາມລັບ ແລະ ຈະໃຊ້ພຽງແຕ່ນຳສະເໜີໃນແບບຟອມຂອງບົດສະຫຼຸບການເຮັດ ວິໃຈເທົ່ານັ້ນ.

13. ຜູ້ໃຫ້ຄຳປຶກສາໃນໂຄງການ:

ຖ້າທ່ານໃດມີບັນຫາ ກ່ຽວກັບການເປັນອາສາສະມັກ, ທ່ານສາມາດຕິດຕໍ່ ດຣ. ອິສາລາງ ນຸພາຍູນ (Assos, Prof. Dr. Issarang Nuchprayoon), ໝາຍເລກໂທລະສັບ 01-641-4797

ແບບຟອມຍິນຍອມ

ສະຖານທີ່:.....
ວັນທີ:.....

ຊື່ ແລະ ນາມສະກຸນ:..... ອາຍຸ:..... ທີ່ຢູ່: ....
....ເມືອງ:..... ປະເທດ:.....

ເອກະສານດັ່ງກ່າວນີ້ສ້າງຂຶ້ນເພື່ອເປັນພະຍານຫຼັກຖານ

1. ຂ້າພະເຈົ້າຂໍຢັ້ງຢືນວ່າໄດ້ອ່ານ ແລະ ຮັບຮູ້ ກ່ຽວກັບຂໍ້ມູນຂ່າວສານຂອງໂຄງການວິໄຈ "ການພັນ ຂອງປະຊາກອນ G6DP ໂດຍສະເພາະ ປະຊາກອນຢູ່ໃນຂົງເຂດ ພື້ນທີ່ອາຊີຕາເວັນອອກສ່ຽງໃຕ້ " ແລະ ໄດ້ຕອບຄຳຖາມທັງໝົດດ້ວຍຄວາມພໍໃຈ.
2. ຂ້າພະເຈົ້າຕົກລົງ ເຫັນດີໃຫ້ເອົາເລືອດອອກຕາມຈຸດປະສົງ ຂອງໂຄງການວິໄຈ ທີ່ໄດ້ກ່າວມານັ້ນ.
3. ນັກວິໄຈໄດ້ຊີ້ແຈງລາຍລະອຽດໃຫ້ຂ້າພະເຈົ້າຊາບ ກ່ຽວກັບຈຸດປະສົງ, ວິທີການ, ຄວາມປອດໄພ, ຜົນປະໂຫຍດ ແລະ ຜົນເສັຍ ຂອງ ໂຄງການວິໄຈນີ້
4. ນັກວິໄຈໄດ້ໃຫ້ສັນຍາວ່າ ຈະເກັບຂໍ້ມູນສ່ວນຕົວເປັນຄວາມລັບ ແລະ ຈະສະເໜີໃນຮູບແບບຂອງຜົນ ການວິໄຈເທົ່ານັ້ນ.
5. ຂ້າພະເຈົ້າຮັບຮູ້ວ່າ ການສະມັກ ແມ່ນຄວາມຮັບຜິດຊອບສ່ວນບຸກຄົນ ແລະ ຖືກຕ້ອງຕາມລະ ບຽບການ.
6. ຂ້າພະເຈົ້າຮັບຮູ້ ການເຂົ້າຮ່ວມໃນຄັ້ງນີ້ ແມ່ນຄວາມສະມັກໃຈ ແລະ ສາມາດຖອນຕົວ ອອກຈາກການເປັນອາສາສະມັກໄດ້ທຸກເວລາ ໂດຍ ປົດສະຈາກເຫດຜົນຕ່າງໆ ແລະ ບໍ່ໄດ້ຮັບການດູແລຮັກສາອື່ນໆ.

ລາຍເຊັນ..... ອາສາສະມັກ
(.....)
ລາຍເຊັນ .....ຫົວໜ້າທີມຂອງນັກວິໄຈ
(.....)
ລາຍເຊັນ .....ພະຍານ
(.....)
ລາຍເຊັນ..... ພະຍານ
(.....)

ແບບສອບຖາມ

ເລກທີ່:.....

ເຊື້ອຊາດ: □ ມ້ຽນມາ □ຈາມ □ ກາເລມ □ ຢາໄກ □ຈີນ □ ມອນ □ ອິນເດຍ □ ດານູ □ ກາຊີນ □ ລີຊູ
□ ກຳປູເຈຍ
□ ລາວ
□ ອື່ນໆ.....

ສະຖານທີ່ເກີດ:.....

ເຊື້ອຊາດ ຂອງ

ແມ່: □ ມ້ຽນມາ □ຈາມ □ ກາເລມ □ ຢາໄກ □ ຈີນ □ ມອນ □ ອິນເດຍ □ ດານູ □ ກາຊີນ □ ລີຊູ
□ ກຳປູເຈຍ
□ ລາວ
□ ອື່ນໆ.....

ພໍ່ເຖົ້າ: □ ມ້ຽນມາ □ຈາມ □ ກາເລມ □ ຢາໄກ □ຈີນ □ ມອນ □ ອິນເດຍ □ ດານູ □ ກາຊີນ □ ລີຊູ
□ ກຳປູເຈຍ
□ ລາວ
□ ອື່ນໆ.....

ແມ່ເຖົ້າ: □ ມ້ຽນມາ □ຈາມ □ ກາເລມ □ ຢາໄກ □ ຈີນ □ ມອນ □ ອິນເດຍ □ ດານູ □ ກາຊີນ □ ລີຊູ
□ ກຳປູເຈຍ
□ ລາວ
□ ອື່ນໆ.....

စေတနာ့ဝန်ထမ်းများသိသာရန်အချက်အလက်များ

၁။ သုတေသနအမည်

အရှေ့တောင်အာရှရှိ **G6PD** ကိန်းအောင်းသောလူမျိုးစုများ၏ မျိုးပွားပုံကို လေ့လာသော ဘာသာရပ်။

၂။ သုတေသီများ၏အမည်

တွဲဖက်ပါမောက္ခ ဒေါက်တာ အစွရန် နာချိုပရုယသွန်း
မစ္စ ချုလိစာ လူဝီကျုရန်း

၃။ သုတေသနပြုလုပ်မည့်နေရာ

တာခ်စင်ဒေသရှိ

၄။ သုတေသနပြုလုပ်သောအဖွဲ့အစည်း

The Royal Golden Jubilee Ph.D. Program(RGJ), The Thailand Research Found(TRF).

သွေးဥထဲတွင်ရှိသော G6PDမျိုးစေ့၏မျိုးပွားပုံကို လေ့လာသော ဘာသာရပ်အား သုတေသနပြုလုပ်သည့် စီမံကိန်းတွင် သင့်အား ပါဝင်ကူညီပါရန် ဖိတ်ခေါ်အပ်ပါသည်။ ထို့နောက် ဤမျိုးစေ့ အရှေ့တောင်အာရှရှိ လူမျိုးစုများအတွင်း၌ အဆင့်ဆင့်တိုးတက်ဖြစ်ပေါ်ပြောင်းလဲလာပုံကို မျိုးပွားပုံ သတင်းအချက်အလက်များမှ ဖော်ပြပေးမည်ဖြစ်ပါသည်။ ဤသုတေသနလုပ်ငန်း၌ သင်ပါဝင်ကူညီရန် သဘောတူဆုံးဖြတ်ချက် မချမီ၊ ဤလုပ်ငန်း လုပ်ဆောင်ရခြင်း၏အကြောင်းအရင်းနှင့် အကြောင်းအရာအစုံအလင်ကို ရှေ့ဦးစွာ အသိပေးလိုပါသည်။

၅။ သုတေသနစီမံကိန်း၏ နောက်ခံအကြောင်းအရင်း

အရှေ့တောင်အာရှတွင် အများဆုံးတွေ့ရတတ်သော လူအများစု၏ချို့ယွင်း မှု့မှာ ဂလူးကို့စ်(၆) ဖောစ်ဖိတ် ဒီဟိုက်ဒရိုဂျင်နိစ်ဖေ့ (G-6-PD) ချို့တဲ့မှု့ပင်ဖြစ်ပါသည်၊ ထိုအင်ဇိုင်းချို့တဲ့ မှု့၏ ဆိုးမွေကြောင့် ဟေမိုလတ်တစ်အနီးမီးယား (Hemolytic Anemia) ခေါ် အသွေးယင်ပတိုင်ပို သွေးနီဥများ သေဆုံးသောရောဂါ ဖြစ်ပွားစေနိုင်ပါသည်။ (G-6-PD) ချို့တဲ့သောလူများမှာ များသောအားဖြင့် ကျန်းကျန်းမာမာ ရှိနေတတ်ကြပါသည်၊ သို့ပါသော်လည်း ထိုလူများမှာ အချို့သောဆေးဝါးများကို သောက်သုံးမိခြင်း ကြောင့်လည်းကောင်း သို့မဟုတ် အခြားသော အန္တရာယ်ဖြစ်စေနိုင်သည့်ဓါတုဗေဒပစ္စည်းများ ကို ကိုင်တွယ်ထိတွေ့မိခြင်းကြောင့်လည်းကောင်း သွေးဆုတ်၍ ဖြူဖတ်ဖြူရော်ဖြစ်ပြီး ဆီးအရောင် မဲမှောင်လာတတ်ပါသည်။ တိကျစေရန်ပြောရလျှင် (G-6-PD) ပြောင်းလဲဖြစ်ပေါ်မှု့များသည် အရှေ့တောင်အာရှအတွင်းရှိ လူမျိုးစုငယ်များ၏ အချို့သောအုပ်စုငယ်များနှင့် ပတ်သက်ဆက်စပ်နေပါသည်။ ဤသုတေသနလုပ်ငန်း၏ ရည်ရွယ်ချက်မှာ လူမျိုးစုအုပ်စုငယ်များအတွင်းမှ နာမကျန်းဖြစ်နေသူများနှင့် ကျန်းမာသန်စွမ်းသူများ၏ (G6PD) အချက်အလက်များကို နှိုင်းယှဉ်၍ အရှေ့တောင်အာရှအတွင်းရှိ ဤ (G6PD) မျိုးစေ့ အဆင့်ဆင့်တိုးတက်ဖြစ်ပေါ်ပြောင်းလဲလာပုံကို ဖော်ပြရန် ဖြစ်ပါသည်။

၆။ ရည်ရွယ်ချက်

(၁) ထိုင်း၊ လားအို၊ မြန်မာ၊ ကန်ဘောဒီးယားလူမျိုးများအတွင်းရှိ (G6PD) မျိုးစေ့၏ မိုက်ခရိုဆက်တလိုက် ဟပလိုတိုက်(ပ်) (microsatellite haplotypes) ကို တိကျမှန်ကန်စွာ သက်သေပြနိုင်ရန်၊

(၂) အရှေ့တောင်အာရှရှိ လူမျိုးစုများအတွင်း၌ (G6PD) မျိုးစေ့၏ အဆင့်ဆင့်တိုးတက်ဖြစ်ပေါ်ပြောင်းလဲလာပုံကို သေချာစွာဖော်ပြနိုင်ရန် တို့ဖြစ်သည်။

၇။ သုတေသနတွင် ပါဝင်ကူညီလုပ်ဆောင်ပေးရမည့် လုပ်ငန်းစဉ်

သင်၏သွေးကျောမှ သွေးပမာဏ ၇၀ မီလီ လီတာ (လွှက်ရည်ဇွန်း ၂ ဇွန်းခန့်) ကို တစ်ကြိမ်ဖောက်ယူပါမည်။ ထိုသွေးပမာဏကို နှစ်ပိုင်းခွဲ၍၊ ထိုင်းနိုင်ငံအတွင်း အလုပ်လုပ်ခွင့်တင်သူများအား ကျန်းမာရေးစစ်ဆေးသည့်အစီအစဉ် နှင့် သုတေသနပြုလုပ်သောစီမံကိန်း လုပ်ဆောင်ခြင်း တို့၌ အသုံးပြုမည် ဖြစ်ပါသည်။ ကျန်ပ်တို့မှ သင့်အား G6PD ချို့တဲ့ခြင်းရှိမရှိကို အခမဲ့စမ်းသပ်ပေးမည်ဖြစ်ပါသည်။

၈။ ထပ်ဆင့်ပြန်လည်အသုံးပြုမှု

(၁) အရှေ့တောင်အာရှလူမျိုးစုများ၏ မျိုးပွားပုံကို သိနားလည်လေ့လာရာ၌လည်းကောင်း၊

(၂) လူမျိုးစုများ၏ မျိုးပွားပုံကို အခြေခံသော အကြောင်အချက်အလက်များမှတဆင့် အရှေ့တောင်အာရှလူမျိုးစုများ၏ အဆင့်ဆင့်တိုးတက်ဖြစ်ပေါ်ပြောင်းလဲလာပုံကို ပြန်လည်တည်ဆောက်ရာ၌လည်းကောင်း အသုံးပြုမည်ဖြစ်ပါသည်။

၉။ သုတေသနစီမံကိန်းတွင် ပါဝင်လုပ်ဆောင်ပေးမည့် စေတနာဝန်ထမ်း၏ အကျိုးသက်ရောက်မှု

သင့်ထံမှ ထုတ်ယူမည့်သွေးပမာဏသည် သင်၏ကျန်းမာရေးကို ထိခိုက်အန္တရာယ်ဖြစ်စေခြင်း လုံးဝ မရှိပါ။ အဘယ်ကြောင့်ဆိုသော် သင်သည် သွေးကိုအခြားသောလုပ်ဆောင်မှုများမှ ရရှိနေရာ ကျန်ပ်တို့ ရယူမည့်ပမာဏသည် သင့်ထံတွင် ပိုနေသောသွေးပမာဏဖြစ်ခြင်းကြောင့် ဖြစ်ပါသည်။

၁၀။ သုတေသနစီမံကိန်းတွင် ပါဝင်လုပ်ဆောင်ပေးမည့် စေတနာဝန်ထမ်း၏ လုပ်ပိုင်ခွင့်

သင်သည် ပါဝင်လုပ်ဆောင်ပေးရန် လက်မခံလိုပါက၊ စေတနာဝန်ထမ်းအဖြစ်လုပ်ဆောင်ရန် ငြင်းပယ်နိုင်ပါသည်။ ထိုကဲ့သို့ ငြင်းဆိုခြင်းကြောင့် သင်၏ကျန်းမာရေးဆေးဝါးကုသခံယူခွင့်နှင့် ပတ်သက်သော ကိစ္စများတွင် မည်သို့မျှ ထိခိုက်စေခြင်း မရှိပါ။

၁၁။ လုပ်အားခ

စေတနာဝန်ထမ်းအား လုပ်အားခ ပေးမည်မဟုတ်ပါ။

၁၂။ သင်၏မှတ်တမ်းမှတ်ရာများကို လျှို့ဝှက်ထားမည်ဖြစ်သည်

ဤသုတေသနလုပ်ငန်းမှ အဖြေများကို ဤသုတေသနလုပ်ငန်း၏ ရည်ရွယ်ချက်တွင်သာရှိစေရမည်။ သင်တစ်ဦးတစ်ယောက်၏ ကိုယ်ရေးကိုယ်တာအချက်အလက်များကို လျှို့ဝှက်စွာ သိမ်းဆည်းထားပြီး၊ သုတေသနလုပ်ငန်း၏ နိဂုံးချုပ်ပုံစံ၌တွင်သာ ဖော်ပြမည်ဖြစ်သည်။

၁၃။ သုတောသနစီမံကိန်းရှိ အကြံပေးပုဂ္ဂိုလ်

အကယ်၍ စေတနာ့ဝန်ထမ်းအဖြစ် ပါဝင်ကူညီရန် ပြဿနာရှိပါက ဒေါက်တာ အစ္စရန် နာချုပရယူနိုး ဖုန်းနံပါတ် (၀၁-၆၄၁-၄၇၇၂) သို့ ဆက်သွယ်မေးမြန်းနိုင်ပါသည်။

သဘောတူညီချက် ပုံစံ

နေရာ
နေ့စွဲ

အမည်
အသက်
နေရပ်လိပ်စာ

ဤစာရွက်စာတမ်းသည် သက်သေခံအထောက်အထား ဖြစ်စေရမည်။

၁။ ကျွန်ုပ်သည် အရှေ့တောင်အာရှလူမျိုးစုများအတွင်း G6PD မျိုးစေ့ဇီဝမျိုးပွားပုံကို သုတေသနပြုလုပ်သော စီမံကိန်း၏ အကြောင်းအချက်အလက်များကို ဖတ်ရှုနားလည်သဘောပေါက်ပြီးဖြစ်သည် နှင့်အတူ မိမိ၏မေးခွန်းများအားလုံးကို စိတ်ကျေနပ်မှုရသည်အထိ ဖြေကြားပေးပြီးဖြစ်ပါသည်။

၂။ ကျွန်ုပ်သည် အထက်ဖေါ်ပြပါသုတေသနလုပ်ငန်းတွင် ပါဝင်ကူညီလုပ်ဆောင်ရန် သဘောတူညီပါသည်။

၃။ ဤသုတေသနလုပ်ငန်း၏ ရည်ရွယ်ချက်၊ လုပ်ထုံးလုပ်နည်း၊ လုံခြုံမှု၊ အကျိုးအမြတ် နှင့် အန္တရာယ်တို့ကို သုတေသနပြုလုပ်သူမှ ကျွန်ုပ်အား အသိပေးပြီးဖြစ်ပါသည်။

၄။ ကျွန်ုပ်၏ ကိုယ်ရေးကိုယ်တာအချက်အလက် လျှို့ဝှက်ချက်များကို သိမ်းဆည်းထားရန်နှင့် ထိုအချက်အလက်များကို သုတေသနလုပ်ငန်း၏ နိဂုံးချုပ်ပုံစံ၌တွင်သာ ထည့်သွင်းဖော်ပြမည်ဖြစ်ကြောင်းကို သုတေသနပြုလုပ်သူမှ ကျွန်ုပ်အား ကတိပေးပြီးဖြစ်ပါသည်။

၅။ မှတ်တမ်းနှင့် အုပ်ချုပ်မှုဆိုင်ရာကို စီမံခန့်ခွဲသော အာဏာပိုင် တာဝန်ရှိသူတစ်ဦးတစ်ယောက် (အဖွဲ့အစည်း၏ ပြန်လည်သုံးသပ်သောအဖွဲ့)မှနေ၍ ကျွန်ုပ်၏မှတ်တမ်းမှတ်ရာများကိုဝင်ရောက်ကြည့်ရှုနိုင်ကြောင်းကို သဘောပေါက်နားလည်ပြီးဖြစ်ပါသည်။

၆။ ကျွန်ုပ်၏ပါဝင်ကူညီလုပ်ဆောင်ပေးမှုမှာ စေတနာအလျောက်သာဖြစ်ပြီး၊ မည်သို့သောအကြောင်းပြချက်မျှ တင်ပြရန် မလိုဘဲ ကျွန်းမာရေးဆေးဝါးကုသခံယူခွင့်နှင့် ပတ်သက်သော ကိစ္စများ တွင် မည်သို့မျှ ထိခိုက်စေခြင်း မရှိဘဲ လွတ်လပ်စွာ နှုတ်ထွက်ပိုင်ခွင့်ရှိကြောင်းကို သဘောပေါက်နားလည်ပြီးဖြစ်ပါသည်။

လက်မှတ် (စေတနာဝန်ထမ်း)
( )

လက်မှတ် (သုတေသနပြုသူများ၏အကြီးအကဲ)
( )

လက်မှတ် (မျက်မြင်သက်သေ)
( )

လက်မှတ် (မျက်မြင်သက်သေ)
( )

ဖြေဆိုရမည့်မေးခွန်းများ

နိုင်ငံသား ☐ မြန်မာ(ဗမာ) ☐ ရှမ်း ☐ ကရင် ☐ ရခိုင် ☐ တရုတ် ☐ အိန္ဒိယ ☐ ဓနု ☐ ကချင်
☐ လီဈူး
☐ ကန်ဗောဒီးယား
☐ လာအို
☐ အခြားလူမျိုး

မွေးရပ်ဒေသ

နိုင်ငံသား(အမိ) ☐ မြန်မာ(ဗမာ) ☐ ရှမ်း ☐ ကရင် ☐ ရခိုင် ☐ တရုတ် ☐ အိန္ဒိယ ☐ ဓနု ☐ ကချင်
☐ လီဈူး
☐ ကန်ဗောဒီးယား
☐ လာအို
☐ အခြားလူမျိုး

နိုင်ငံသား(အဘိုး) ☐ မြန်မာ(ဗမာ) ☐ ရှမ်း ☐ ကရင် ☐ ရခိုင် ☐ တရုတ် ☐ အိန္ဒိယ ☐ ဓနု ☐ ကချင်
☐ လီဈူး
☐ ကန်ဗောဒီးယား
☐ လာအို
☐ အခြားလူမျိုး

နိုင်ငံသား(အဘွား)☐ မြန်မာ(ဗမာ) ☐ ရှမ်း ☐ ကရင် ☐ ရခိုင် ☐ တရုတ် ☐ အိန္ဒိယ ☐ ဓနု ☐ ကချင်
☐ လီဈူး
☐ ကန်ဗောဒီးယား
☐ လာအို
☐ အခြားလူမျိုး

BIOGRAPHY

Name	Miss Chalisa Louicharoen	**Date of birth**	March 4, 1980
Gender	Female	**Nationality**	Thai
Religion	Buddhism	**Country of birth**	Thailand
E-mail	chalisa_loui@yahoo.com		
Address	296/16 Soi 14, Rama III Rd, Bangkholaem, Bangkok 10120, THAILAND		

Degree	**Graduation**	**Institute**	**GPAX**
B. Sc. (Health Science)	1997-2000	Thammasat University, Thailand	3.14
M. Sc. (Medical Science)	2001-2003	Chulalongkorn University, Thailand	3.88

Thesis: Mutations and Haplotype Analysis of the G6PD Locus in the Southeast Asia Population.

Ph.D. (Biomedical Sciences)	2004-2008	Chulalongkorn University, Thailand	4.00

Thesis: A Role for G6PD Mahidol in Protection against Malaria in Southeast Asia.

Scholarships:
- 2002 Teacher Assistant Funding
- 2004 The Royal Golden Jubilee Ph.D program (RGJ), Thailand Research Fund (TRF)
- 2007 The French Embassy in Thailand
- 2008 EGIDE- Centre Français pour l'Accueil et des Echanges Internationaux

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