ความชุกของการกลายพันธุ์ด้านปลายเอ็นของเอนไซม์ไพรูเวตไคเนสในกลุ่มเสี่ยงต่อการติดเชื้อมาลาเรียในเอเชียตะวันออกเฉียงใต้

นางสาวปัญญ์ชลี มังคลสุต

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PREVALENCE OF N-TERMINAL MUTATION OF PYRUVATE KINASE AMONG VULNERLABLE MALARIA-RISK POPULATION IN SOUTHEAST ASIA

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ปัญญ์ชลี มังคลสุต : ความชุกของการกลายพันธุ์ด้านปลายเอ็นของเอนไซม์ไพรูเวตไคเนสในกลุ่มเสี่ยงต่อการติดเชื้อมาลาเรียในเอเชียตะวันออกเฉียงใต้ (PREVALENCE OF N-TERMINAL MUTATION OF PYRUVATE KINASEAMONG VULNERLABLE MALARIA-RISK POPULATION IN SOUTHEAST ASIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ชาลิสา หลุยเจริญ ชีพสุนทร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.พูลลาภ ชีพสุนทร, 72 หน้า.

ไพรูเวตไคเนสเป็นเอนไซม์ที่สำคัญในกระบวนการไกลโคไลซิสซึ่งมีหน้าที่ผลิตพลังงานเพื่อการดำเนินไปของปฏิกิริยาต่างๆ ภายในเซลล์ ภาวะพร่องเอนไซม์ไพรูเวตไคเนสเป็นความผิดปกติทางโลหิตวิทยาที่เกิดจากการกลายพันธุ์ของยีน *PKLR* มีการค้นพบการกลายพันธุ์ใหม่ในเอเชียตะวันออกเฉียงใต้ซึ่งเกิดจากจากเปลี่ยนแปลงลำดับอะมิโนตำแหน่งที่ 41 ของโปรตีนไพรูเวตไคเนส จากอาร์จีนีน (R) เป็นกลูตามีน (Q) (*PKLR*^{R41Q}) จากการศึกษาก่อนหน้านี้มีการรายงานว่าความผิดปกติทางโลหิตวิทยาเป็นกุญแจที่สำคัญต่อการป้องกันการติดเชื้อมาลาเรีย อีกทั้งยังลดความรุนแรงของโรคมาลาเรียอีกด้วย อย่างไรก็ตามความชุกและความสัมพันธ์ของการกลายพันธุ์แบบ *PKLR*^{R41Q} ต่อโรคมาลาเรียในมนุษย์ยังไม่ชัดเจน จากการศึกษาผู้ป่วยมาลาเรียจำนวน 267 ราย พบความชุกของการกลายพันธุ์แบบ *PKLR*^{R41Q} ร้อยละ 4.87 คิดเป็นความถี่อัลลีลเท่ากับ 0.026 ผลการศึกษาความสัมพันธ์ของการกลายพันธุ์และโรคมาลาเรียชี้ให้เห็นว่าการกลายพันธุ์แบบ *PKLR*^{R41Q} เพิ่มจำนวนซ้ำของการติดเชื้อมาลาเรียอย่างมีนัยสำคัญทางสถิติ (p=0.001) โดยอัลลีล *PKLR*^{R41Q} ถูกพบในผู้ป่วยมาลาเรีย hyperparasitemia (*P. falciparum*: 0.100 and *P. vivax*: 0.104) ได้มากกว่าเมื่อเทียบกับผู้ป่วยมาลาเรียที่มีเชื้อในเลือดไม่สูง (*P. falciparum*: 0.051 and *P. vivax*: 0.023) นอกจากนี้ *PKLR*^{R41Q} ยังมีแนวโน้มที่จะมีความสัมพันธ์กับ hyperparasitemia (ฟาซิพารัม OR: 2.18, 95% CI: 0.340 - 14.096, p= 0.410, ไวแวกซ์ OR: 4.20, 95% CI: 0.709 - 24.880, p= 0.114) อีกทั้งยังพบว่าจำนวนเม็ดเลือดแดงตัวอ่อนมีแนวโน้มเพิ่มสูงขึ้นในผู้ป่วยมาลาเรียที่พบ *PKLR*^{R41Q} (p=0.700) ผลงานวิจัยแสดงให้เห็นว่าจำนวนเม็ดเลือดแดงตัวอ่อนที่มีแนวโน้มเพิ่มสูงขึ้นในผู้ป่วยมาลาเรียที่พบ *PKLR*^{R41Q} ซึ่งเป็นผลมาจากการติดเชื้อและภาวะพร่องเอนไซม์ไพรูเวตไคเนส อาจจะเป็นปัจจัยสำคัญต่อการเพิ่มขึ้นของปริมาณเชื้อมาลาเรียในกระแสเลือด ผลการศึกษาแสดงให้เห็นว่าการกลายพันธุ์แบบ *PKLR*^{R41Q} สามารถพบได้ในเอเชียตะวันออกเฉียงใต้ อีกทั้ง *PKLR*^{R41Q} มีแนวโน้มต่อการเพิ่มจำนวนครั้งต่อการติดเชื้อมาลาเรียและต่อปริมาณเชื้อในกระแสเลือด

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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PUNCHALEE MUNGKALASUT: PREVALENCE OF N-TERMINAL MUTATION OF PYRUVATE KINASEAMONG VULNERLABLE MALARIA-RISK POPULATION IN SOUTHEAST ASIA. ADVISOR: CHALISA LOUICHAROEN CHEEPSUNTHORN, Ph.D., CO-ADVISOR: ASSOC. PROF. POONLARP CHEEPSUNTHORN, Ph.D., 72 pp.

Pyruvate kinase (PK) is an important enzyme in glycolysis pathway, which has function in producing ATP for cellular activities. Mutation of *PKLR* gene causes a hematological disorder, known as PK deficiency (PKD). It has been observed that hemolytic disorder is the key to protect against malaria infection and to reduce the severity of malaria. Recently, a novel mutation at N-terminus of PK has been found in Southeast Asian population. This mutation results in a change of amino acid residue 41 from arginine (R) to glutamine (Q) and is designated as $PKLR^{R41Q}$. However, the prevalence and association between $PKLR^{R41Q}$ and malaria in human is not known. The results from 267 malaria patients showed that the prevalence of $PKLR^{R41Q}$ was 4.87% with allele frequency was 0.026. The association study demonstrated that the number of *parasite* attacks was significantly higher in patients with $PKLR^{R41Q}$ than in patients without $PKLR^{R41Q}$ (p=0.001). The allele frequency of $PKLR^{R41Q}$ with hyperparasitemia (*P. falciparum*: 0.100 and *P. vivax*: 0.104) was higher than none hyperparasitemia (*P. falciparum*: 0.051 and *P. vivax*: 0.023). Odds of hyperparasitemia was higher in patients with $PKLR^{R41Q}$ than in patients without $PKLR^{R41Q}$ (*P. falciparum;* OR: 2.18, 95% CI: 0.340 - 14.096, p= 0.410, *P. vivax;* OR: 4.20, 95% CI: 0.709 - 24.880, p= 0.114). Moreover, the reticulocyte count in patients with $PKLR^{R41Q}$ was increased, compared to that of patients without $PKLR^{R41Q}$ (p=0.700). It is speculated that increasing of reticulocyte in patients with $PKLR^{R41Q}$ could be due to hemolysis from infection and PKD and could contribute to proliferation of parasites. These findings suggest that $PKLR^{R41Q}$ is a common *PKLR* mutation in Southeast Asian population. The presence of $PKLR^{R41Q}$ may increase a tendency of number of parasite attacks and hyperparasitemia.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Medical Biochemistry	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

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CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Malaria is a life-threatening disease caused by *Plasmodium spp*. Transmission occurs through the bites of an infected Anopheles mosquito (1). In each year, 500 million clinical cases is estimated and at least a million people die from malaria (2). Malaria is widespread throughout the world and prevalent in the tropical and semitropical regions such as Middle and South America, Africa, Middle East, Pacific Island and Southeast Asia (3). There are evidence supporting that malaria endemic areas have coincidence with high level of heterozygous hematological disease (4). A previous study by Louicharoen *et al.* has led to propose that the historical evolution of human genome may be forced by malaria to mutate genes that causes changes in erythrocyte's phenotype to protect infected host against the severity and mortality of malaria (5). It has been discovered that several hematological diseases have evolved protective mechanisms against malaria. Such mechanisms include defect in cytoskeleton and membrane proteins (glycophorin C deficiency) (6-8) and southeast Asian ovalocytosis (SAO) (9, 10), hemoglobin (α-thalassemia) (11, 12), hemoglobin S (13), hemoglobin C (14-17) , hemoglobin E (18), and enzymatic activity (glucose 6-phosphate dehydrogenase (G6PD) deficiency and pyruvate kinase (PK) deficiency) (5, 19-26).

PK catalyses the last step of glycolysis by converting phosphoenolpyruvate (PEP) to pyruvate with the generation of adenosine triphosphate (ATP) (27). In mature erythrocytes lacking mitochondria, PK is essential for energy production. PK deficiency (PKD) is caused by mutations of pyruvate kinase gene (*PKLR*) located on chromosome 1q21 leading to hereditary non-spherocytic hemolytic anemia (28). Clinical PKD is associated with *PKLR* mutational spectrum (29). A novel mutation at N-terminal of PK was found in Southeast Asians. Recently, it has been reported that PKD is protective against malaria through the following mechanisms. First, invasion of *P. falciparum* is

reduced because of PK deficient erythrocytes. Second, phagocytosis of ring-stage–infected PKD erythrocytes is enhanced (30). Therefore, it is speculated that *PKLR* mutations could possibly play a protective role, if retained in malaria-endemic areas. To address this possibility, we investigated the prevalence and association between the heterozygous mutation at N-terminal of PK and risk of severe malaria in Southeast Asian patients with malaria.

1.2 Research Questions

2.1 Can N-terminal mutation of PK be detected in Southeast Asian patients with malaria?

2.2 Does N-terminal mutation of PK confer protection against malaria?

1.3 Objectives

3.1 To investigate the prevalence of N-terminal mutation of PK in Southeast Asian patients with malaria.

3.2 To determine the clinical impact of N-terminal mutation of PK on malaria disease.

1.4 Hypotheses

4.1 Allele frequency of N-terminal mutation of PK can be detected in Southeast Asian patients with uncomplicated malaria more frequently than severe malaria.

4.2 Clinical symptoms (parasitemia and severe malaria) in malaria patients with N-terminal mutation of PK are less severe than malaria patients without this mutation.

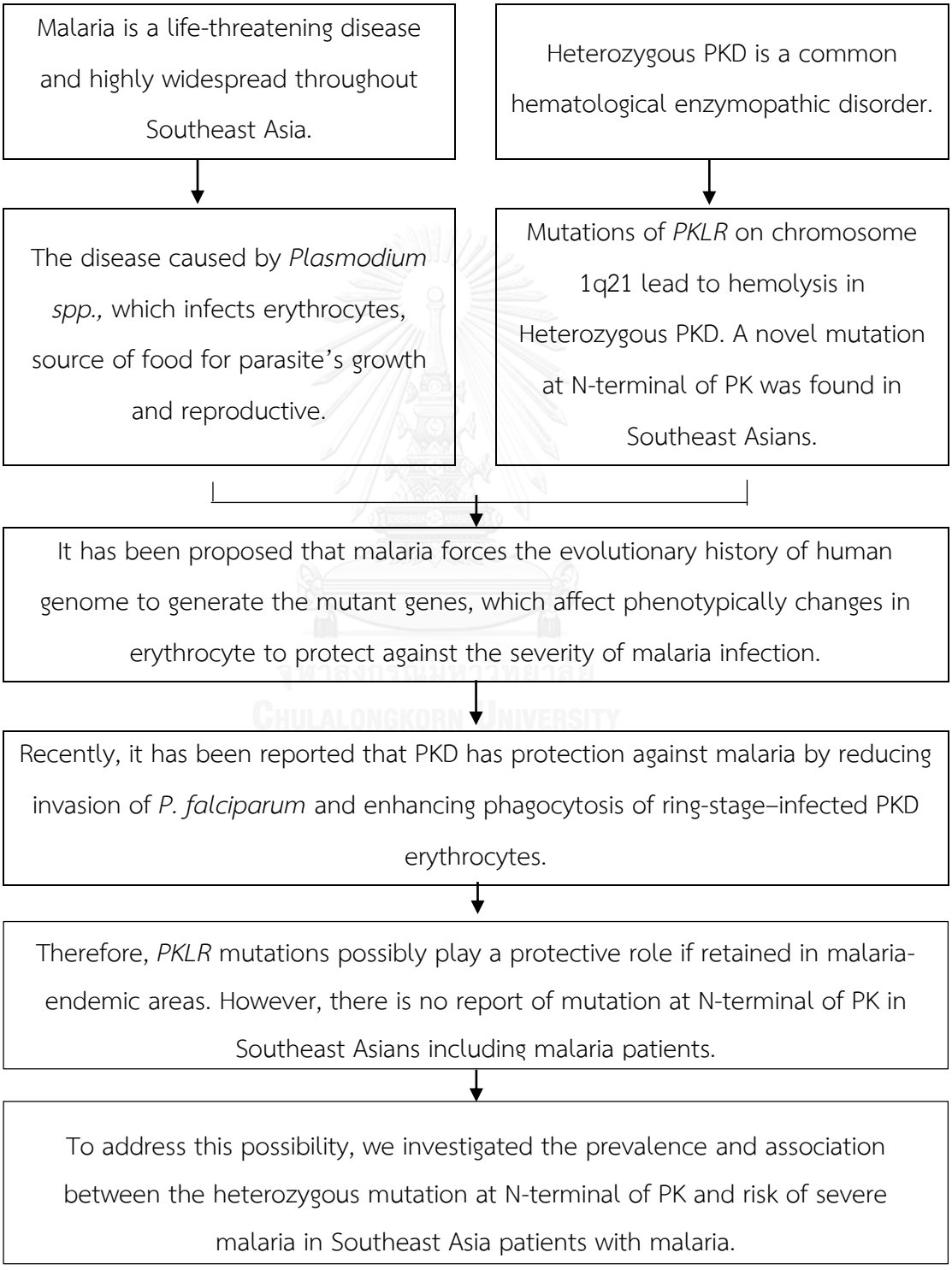
1.5 Keywords

Pyruvate Kinase Deficiency, N-Terminal Mutation, Severe Malaria, Parasitemia, Southeast Asia

1.6 Research Design

Analytical study

1.7 Conceptual Framework



1.8 Benefits of Study

This study reveals the hidden allele frequencies of N-terminal mutation of PK in Southeast Asians, which have never been estimated. It also provides basic knowledge of clinical impact of N-terminal mutation of PK on malaria infection and severity. This valuable data could support the positive selection effect of hematological diseases on protection against malaria and provide information for physician in concerning treatment of PKD patients with malaria.

1.9 Technical Term in the Research

PKLR^{R41Q} is a missense mutation on nucleotide position 158 from G to be A or amino acid residue 41, which changes arginine (R) to be glutamine (Q). This particular amino acid is located in N-terminal helical domain.

CHAPTER 2

LITERATURE REVIEWS

2.1 Functions of PK

Pyruvate kinase (PK; ATP: pyruvate 2-o-phosphotranferase, EC 2.7.1.40), an important enzyme in glycolysis, transfers a phosphoryl group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) yielding one molecule of pyruvate and ATP (27). The enzyme requires K^+ and Mg^{2+} (or Mn^{2+}) (31, 32) for reaction **(Figure 1).** PEP and pyruvate are involved in cellular energy and metabolism.

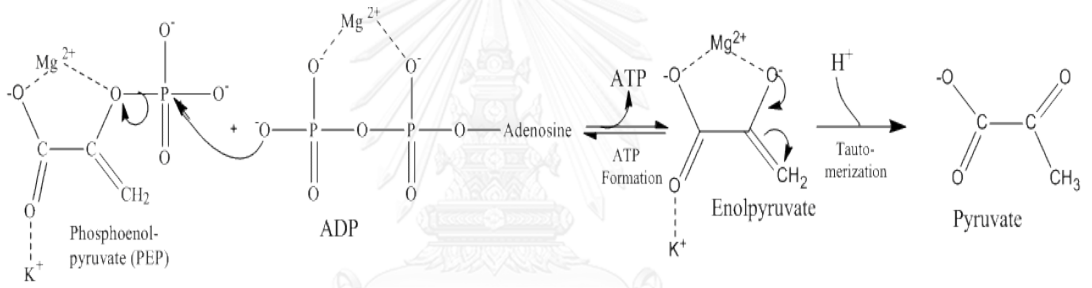


Figure 1 The mechanism of the reaction catalyzed by PK(33)

Since mature erythrocytes lack mitochondria, they are completely dependent on glycolysis pathway for maintaining cellular energy. PK deficiency (PKD) leads to accumulation of the glycolytic intermediates particularly 1, 3-bisphosphoglycerate, which can be converted to toxic 2, 3-bisphosphoglycerate (2, 3-BPG) impairing the glycolytic flux through the inhibition of hexokinase (34). Moreover, ATP depletion in PKD erythrocytes affects cell viability.

2.2 Structure of PK

PK is a tetramer with a molecular weight of approximately 200–240 kilodalton (kDa) with four identical subunits. Each subunit consists of four domains **(Figure 2)** (29);

1. A domain: $(\beta/\alpha)_8$ barrel topology
2. B domain: inserted between strand $\beta 3$ and helix $\alpha 3$ of the A domain
3. C domain: an $\alpha + \beta$ topology
4. Small N-terminal helical domain

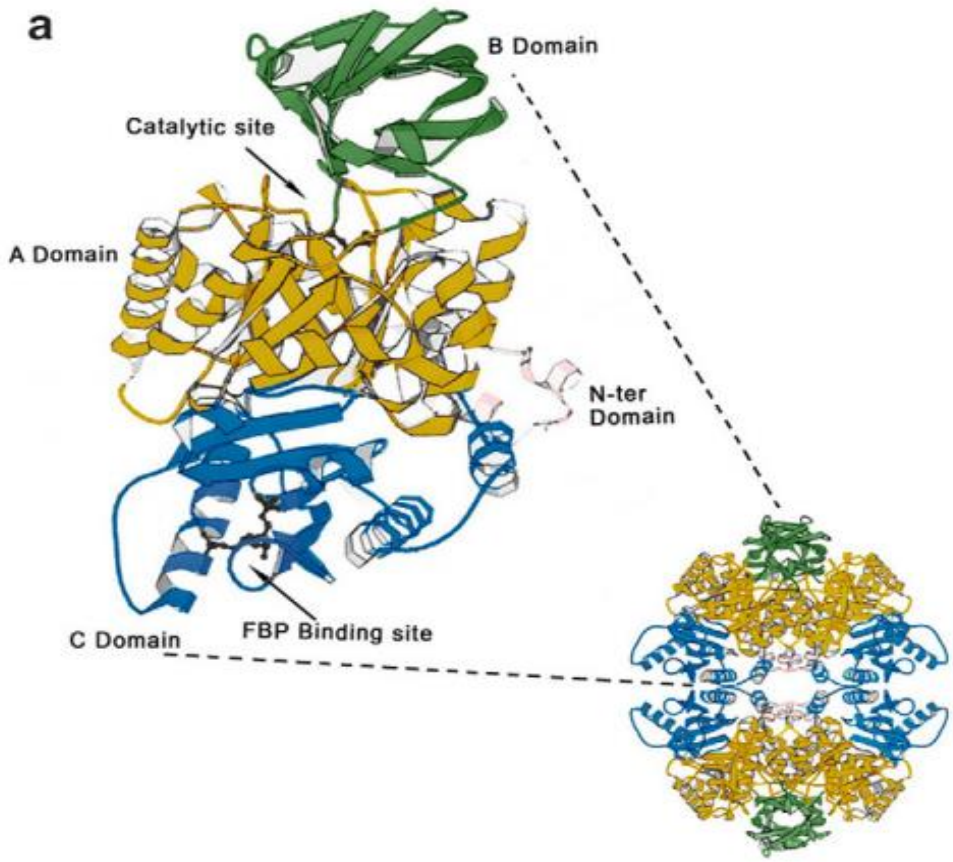


Figure 2 Three-dimensional (3D) structure of PK in monomer and tetramer form(29). The A domain is yellow, the B domain is green, the C domain is blue and the N-terminal domain is pink. Fructose 1, 6-bisphosphate (FBP) binding site and catalytic site are located on C and A domain, respectively.

Four subunits of the PK tetramer are assembled to form a D2 symmetric oligomer. The inter-subunit interactions define two large contact areas; the A/A′

interface involves the A domains of subunits related by the vertical 2-fold axis, whereas the C/C′ interface involves the C domains of subunits interacting along the horizontal axis (27). PK use fructose 1, 6-bisphosphate (FBP) as an activator to expose sigmoidal kinetics reaction. The PEP site (catalytic site) located in the latter between A and B domain and the FBP-binding site is inside the C domain (35).

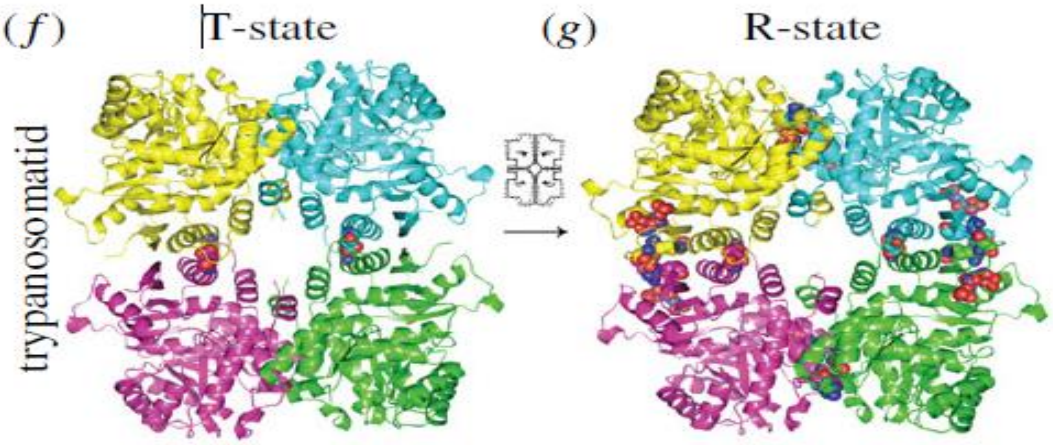


Figure 3 Three-dimensional (3D) structure of PK in T-state and R-state (36).

The enzyme activity is regulated by a combination of domain and subunit rotation coupled to change the active site geometry which can change conformation states. The conformation of PK can switch from a low-affinity tight state (T-state) to a high-affinity relaxed state (R-state) (**Figure 3**) (36).

There are four isoenzymes of PK in mammals, which expressed in tissue-specific: R, L, M_1 and M_2 (37). R type refers to red blood cells, L type is predominant in liver, renal cortex, small intestine and pancreatic beta cell, M1 type is conspicuous in skeletal muscle, heart and brain, and M2 type is mainly expressed in early fetal and proliferating tissue: kidney, leukocytes, platelets, lung, spleen and adipose tissue (38, 39). Each type shows different kinetic and regulatory properties that reflex to the particular metabolic requirement of the tissue. The erythorcytic PK in human was divided into 2 species, PK-R1 and PK-R2. PK-R1 which is the L_4' homotetramer is dominant in reticulocyte and young red blood cell. PK-R2 which is the L_2L_2' heterotetramer is prominent in mature red blood cell (35).

2.3 Molecular Genetics of PK

PK isozymes are encoded from 2 separate genes (*PKM* and *PKLR*) **(Figure 4)**. *PKM*, located on chromosome 15 (15q22), encodes two types of M (M1, M2) using an alternative splicing (40, 41). *PKLR,* located on chromosome 1 (1q21) **(Figure 5)**, encodes L and R type using different promoter (42, 43). *PKLR* gene, approximately 9.5 kilobase (kb) long, consists of 12 exons. The cDNA encoding R type is 2060 base pair (bp), encodes a protein of 574 amino acids (43). The amino acid residue 1-84 is the N-terminal helical domain, residue 85-159 and 263-431 is the A domain, residue 160-262 is B domain and residue 432-574 is C domains **(Figure 6)** (44). N-terminal domain is highly conserved in mammal (45) and A domain is more conserved than B and C domain (37).

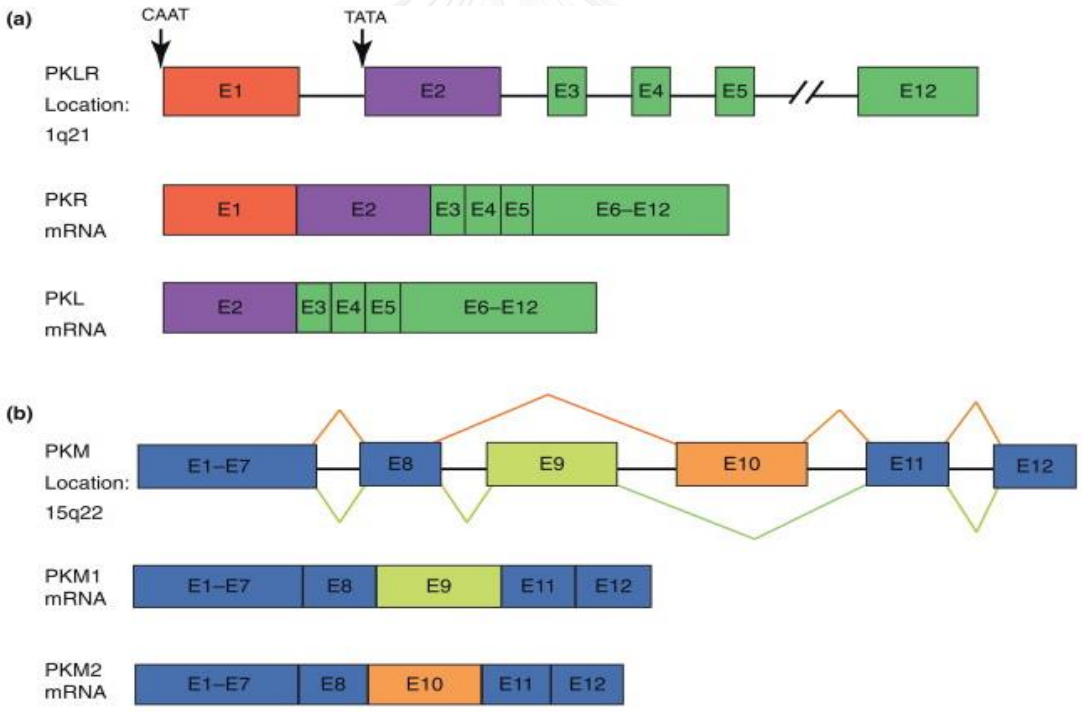


Figure 4 Schematic representation of the different pyruvate kinase (PK) isoforms (46)

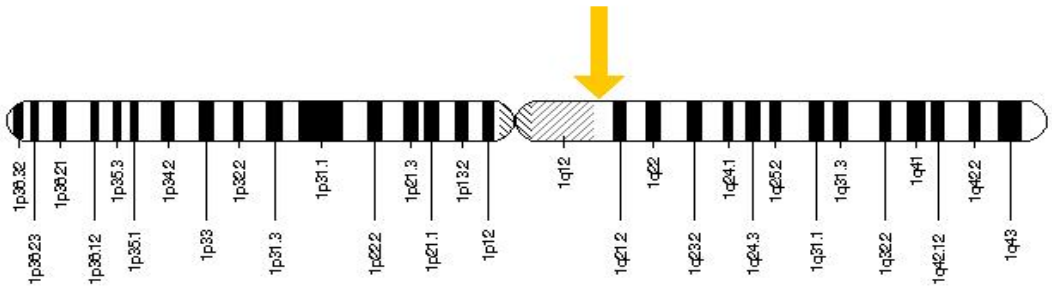


Figure 5 Location of *PKLR* on chromosome 1(47)

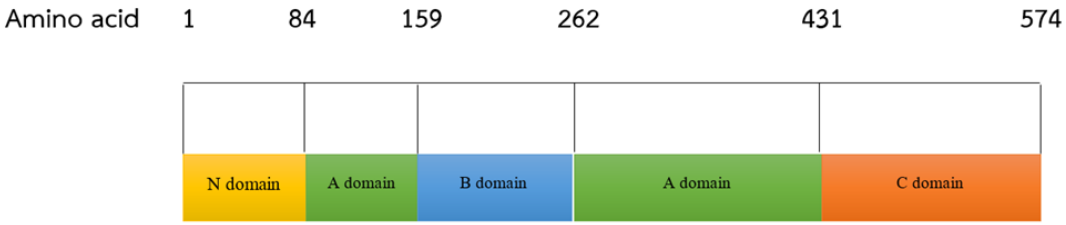


Figure 6 Schematic representation of PK

2.4 PKLR Mutations

In *PKLR* gene, there are more than 180 mutations including missense, splicing, stop codon, deletions, insertions and frame shift mutation have been reported (29). The mutations occur widespread all over the coding regions as well as promoter regions (48, 49) **(Figure 7)**. The mutations in *PKLR* cause of functional abnormality and/or instability of PK.

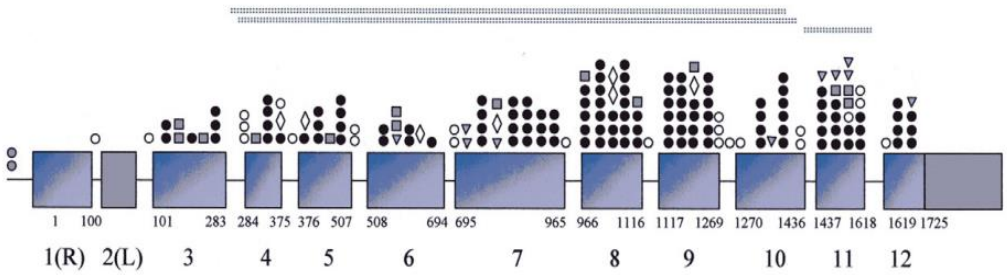


Figure 7 Mutations on coding regions of *PKLR*. ● is mutation in promoter region, ○ is mutation in splice site, • is missense mutation, ■ is frameshift mutation, ◊ is insertion/deletion mutation, ▽ is stop codon mutation, and ▪▪▪ is large deletion mutation (29).

2.5 *PKLR*^{R41Q} Mutations

A preliminary molecular genetics study of *PKLR* in Karen population revealed a new missense mutation on nucleotide position 158 from G to A (amino acid residue 41), which changes the amino acid from arginine (R) to glutamine (Q) as called '*PKLR*^{R41Q}'. This particular amino acid is located in N-terminal helical domain, which is highly conserved in mammalian organism **(Figure 8)**. Therefore, the properties of the particular mutant *PKLR* should be changed, which may have some effects of PKD. However, there is no prior report about the effects of this mutation.

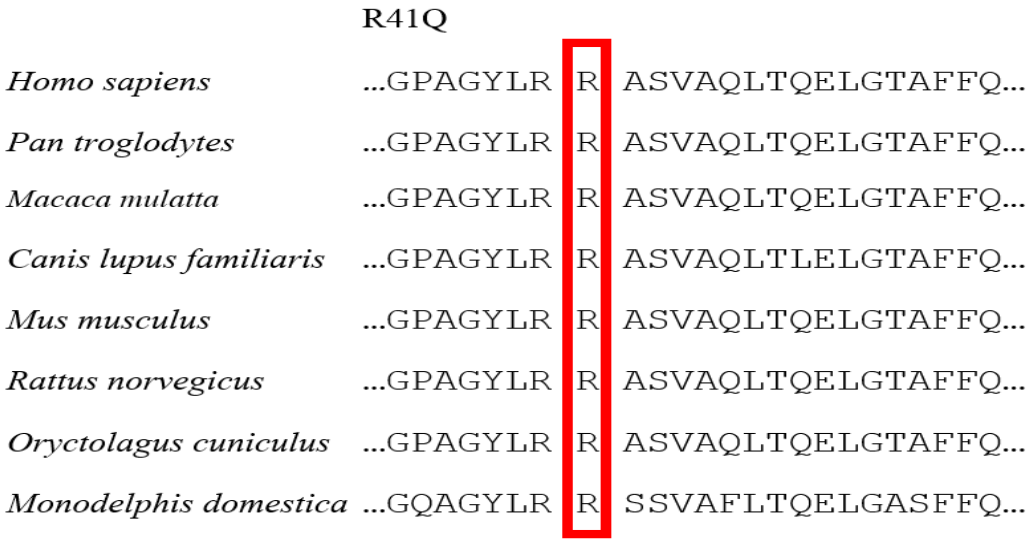


Figure 8 Multiple protein alignment of PK in mammalian organisms.

PKLR^{R41Q} mutation is located within the N-terminal domain of PK-R type. Moreover, arginine at residue 41 is highly conserved in mammals **(Figure 8)**. The enzyme activity is regulated by phosphorylation of serine at residue 43, which increases affinity for allosteric inhibitors (ATP, alanine) and decreases affinity for activators (PEP, FBP) (50, 51). Mutation of *PKLR*^{R41Q} that closes to the allosteric residues may affect the structural and functional aspects of PK.

The previous studies characterized the function of 2 mutations; *PKLR*^{A36G} and *PKLR*^{G37E}, which located in N-terminal domain and closed to *PKLR*^{R41Q} mutation. *PKLR*^{A36G} mutation causes a congenital nonspherocytic hemolytic anemia because of reducing enzyme activity (52). *PKLR*^{G37E} is gain-of function mutation by increasing the

enzyme activity and ATP levels. It is possibly linked to alterative phosphorylation of serine 37 (53, 54).

2.6 Pyruvate Kinase Deficiency (PKD)

PKD, an autosomal recessive trait, is caused by mutations in *PKLR* (55). The mutations reduce the stability and activity of PK. Losing activity of PK in erythrocytes cause a defect in the Embden-Meyerhof pathway of anaerobic glycolysis leading to ATP depletion and increases toxicity from glycolysis intermediates which is 1, 3-bisphosphoglycerate that can convert to 2, 3-bisphosphoglycerate (2, 3-BPG) (27). The insufficient ATP has an influence in intracellular electrolyte concentration and the high level of 2, 3-BPG effects on a right shift in the hemoglobin-oxygen dissociation curve. Moreover, the imbalance between ATP and erythrocyte's energy requirements results in the membrane damaged, distortion and dehydration the red blood cell, which affected the rigidity of erythrocyte. Therefore, PKD is the most common cause of nonspherocytic hemolytic anemia (55). Most PKD patients are asymptomatic because of heterozygous status of this gene. However, clinical manifestations of PKD are icterus (the white of the eye is yellow), jaundice, pale skin and chronic hemolysis (27).

2.7 Prevalence of PKD

PKD have been reported in northern Europe, Japan, and the USA **(Figure 9),** especially in the Old Order Amish population in Pennsylvania (27, 56). The estimated prevalence is 51 cases per million by allele frequency studies but the observed prevalence in one northern England region was 3.3 cases per million (57). Moreover, the incidence homozygous PKD is estimated at 1 per 20,000 people of the general white population (56). On the other hand, the prevalence of PKD was 1.4% in an American population (58), 0.24% in a Spanish population (59), 3.4% in Hong Kong (60), and slightly higher than 2% in Chinese infants (61).

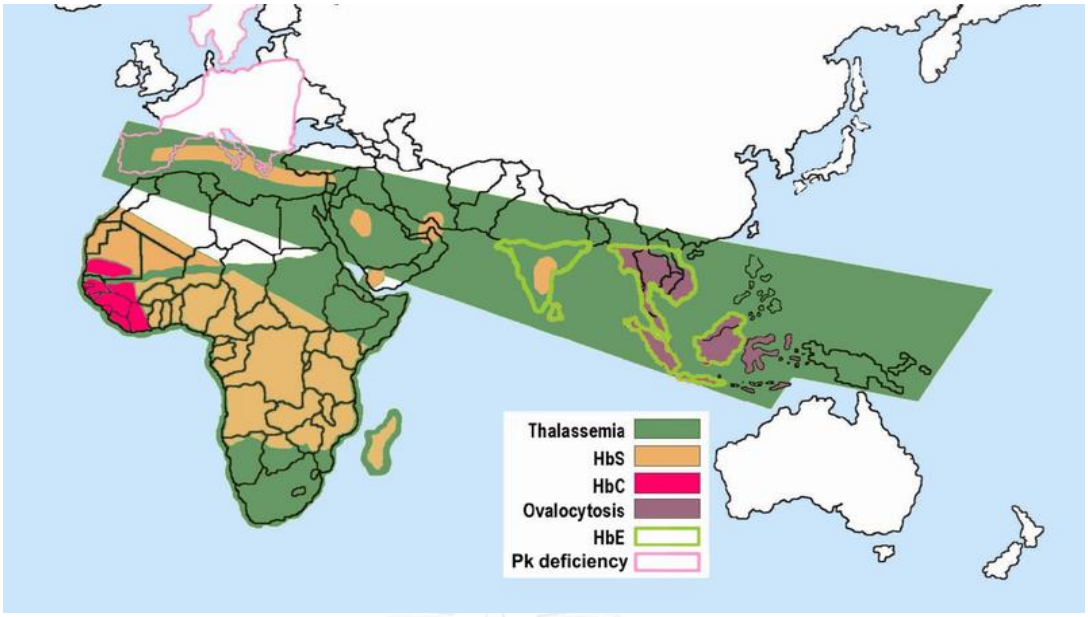


Figure 9 Distribution of red blood cell disorders(62)

2.8 Clinical Manifestations of PKD and Treatment

The hallmark of PKD is life-long chronic hemolysis. The clinical manifestations of PKD were described below (29)

1. Hemoglobin level is used to classify the severity of PKD
    - Severe phenotype : Hb < 8 g/dL
    - Moderate phenotype : Hb 8-10 g/dL
    - Mild phenotype : Hb ≥ 10 g/dL
2. Reticulocyte is increased by 5 – 15%
3. Bilirubin is higher than 100 µmol/L

There is no specific therapy for PKD. The treatment of this disease highlights on retrieving symptoms associated with anemia and hyperbilirubinemia (29). Blood transfusion may be given for the patient with low level of hemoglobin level. Folic acid supplement is often given to match the demand from increased erythropoiesis. Iron chelating therapy (29) and bone marrow transplantation may be considered as an alternative therapy (57).

2.9 Diagnosis of PKD

There are several techniques to diagnose PKD including hematological and biochemical testing. In addition, genetic testing in patients and their family could support diagnosis.

2.9.1 Hematological Testing

Prickle cells in blood film is typical in PKD (**Figure 10**) (20). The minimal tests including complete blood count (CBC), differential blood count, and serum bilirubin level are required to diagnose PKD. Moreover, types of anemia; normochromic, normocytic, or macrocytic anemia, together with reticulocytosis are necessary to rule out immune hemolysis.

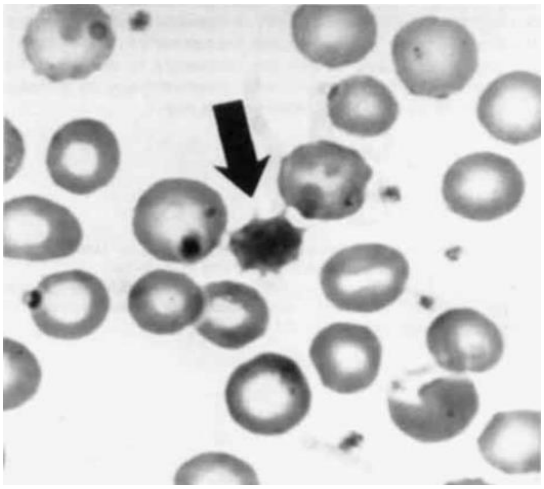


Figure 10 Prickle cell in blood film of PKD patient(63)

2.9.2 Biochemical Testing

PK quantitative assay was recommended by the International Committee for Standardization in Hematology (64). The reaction is determined in a lactate dehydrogenase coupled assay by measuring the absorbance at 340 nm of NADH (**Figure 11**). The reference range of PK activity is 6.7–14.3 U/g Hb. Moreover, screening test for PKD was also developed using long-wave UV light (**Figure 12**) (65).

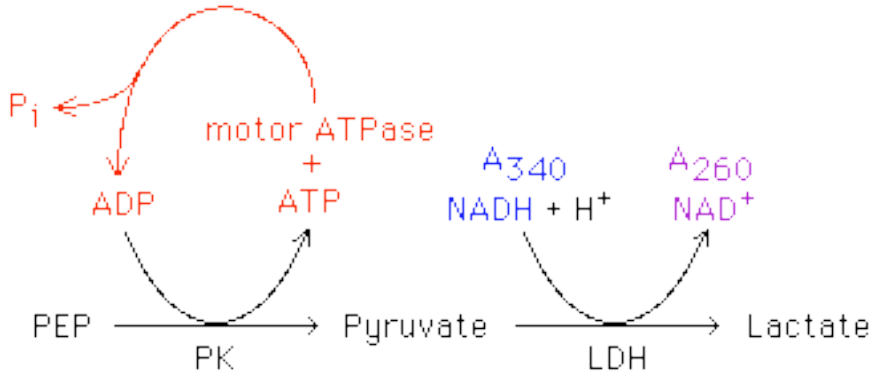


Figure 11 PK, lactate dehydrogenase coupled reaction(66)

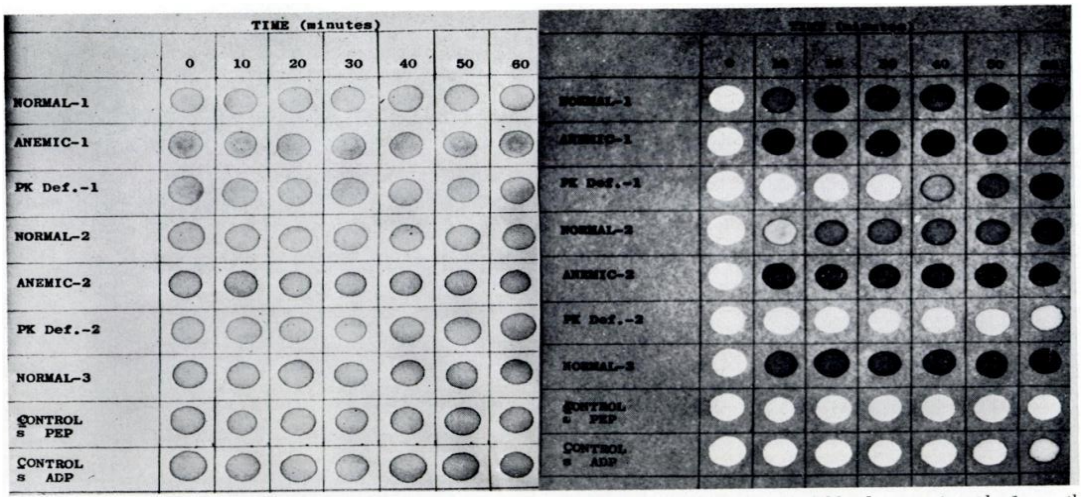


Figure 12 Screening test for PKD(65)

2.9.3 Molecular Genetic Testing

Molecular methods including high-resolution melting analysis (HRM), DNA sequencing, PCR-RFLP, and single-strand conformation polymorphism (SSCP) are currently available for PKD detection. They are used to confirm the diagnosis of PKD.

2.10 Malaria Life Cycle

Malaria, a major threat of human, is estimated more than 200 million patients and 660,000 deaths annually (67). The disease is transmitted by *Plasmodium spp.* carrying female Anopheles mosquito. *Plasmodium* parasite is divided into 5 species; *P.*

falciparum, P. vivax, P.malariae, P. ovale and *P. knowlesi* (1). Malaria is widespread in the tropical and subtropical regions such as Sub-Saharan Africa, Asia, and Latin America. Elimination of malaria is difficult because of lacking of efficient vaccine and drug resistance of parasite.

The ecology of malaria consists of 3 main cycles; exo-erythocytic cycle, erythocytic cycle, and sporogonic cycle (1). After infected female *Anopheles* mosquitoes take human blood meal, the parasites 'sporozoites' grow and asexually multiply to be 'shizonts' in parenchymal cells of human with asymptomatic symptoms. Then, shizonts invade and grow in erythrocytes. Throughout the erythrocytic cycle, the parasites develop from rings (immature trophozoite) to metabolically active 'trophozoites' and mature schizonts, which later rupture and release merozoites. Releasing merozoites and rupture of erythrocytes promote recurrent fever of malaria symptoms. When certain merozoites in bloodstream that differentiate into sexually gametocytes are ingested by female *Anopheles* mosquitos during taking blood meal, they are fertilized and grow to be ookinetes that penetrate the mosquito's midgut wall and form oocysts on the exterior surface. After 10-18 days the parasites 'sporozoites' are found in the mosquito's salivary glands. When the infected mosquitoes take blood meal on human, the sporozoites are transferred with mosquito's saliva **(Figure 13).**

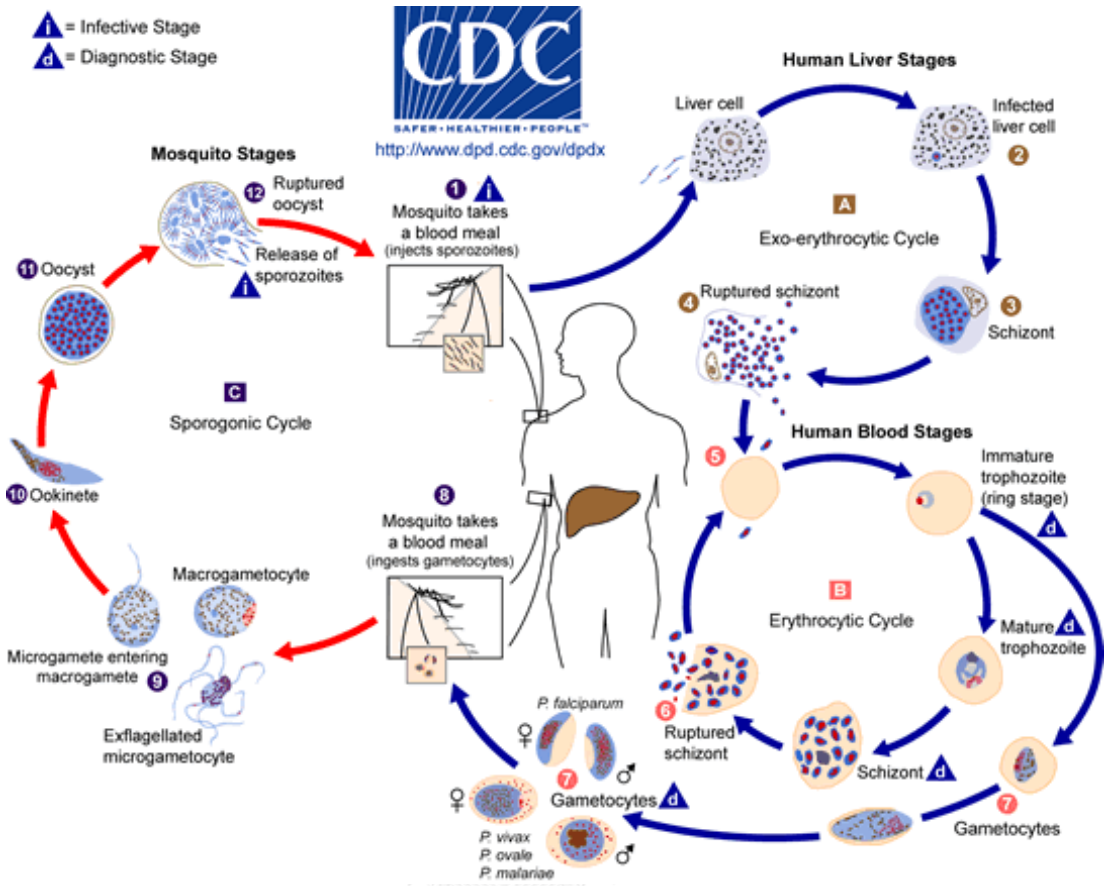


Figure 13 The malaria life cycle(68)

2.11 Clinical Symptoms of Malaria

The incubation period after malaria infection varies from 7 to 30 days. Malaria symptom can be divided into 2 categories; uncomplicated malaria and severe malaria (complicated) (22).

Uncomplicated malaria contains classic symptoms, which common to all malaria species such as fever, chills, sweats, headaches, nausea, vomiting, body aches, and general malaise. The classic symptom of malaria is a cyclical occurrence, consists of

1. Cold stage is caused by the rupture of infected erythrocytes that release swarms parasites into the bloodstream. The symptoms of this stage are sensation of cold and shivering, occurring every 2 days (tertian fever) for *P. falciparum, P. vivax,* and *P. ovale* infections and every 3 days (quatrain fever) in *P. malariae* infection.

2. Hot stage is caused by inflammation. The symptoms of this stage are fever, headaches, vomiting, and seizures in young children.
3. Sweating stage returns patient's body temperature to normal. The symptoms of this stage are sweating and tiredness.

Severe malaria (complicated), usually caused by *P. falciparum*, affects serious organ failure. The manifestations of severe malaria are shown in **Table 1**.

Table 1 Severe malaria manifestations (67)

NO.	Symptoms
1	Cerebral malaria: the clinical symptoms are abnormal behavior, impairment of consciousness, seizures, coma and other neurologic abnormalities.
2	Severe anemia is caused by destruction of erythrocytes
3	Hemoglobinuria due to hemolysis
4	Acute respiratory distress syndrome (ARDS), an inflammatory reaction in the lungs that inhibits oxygen exchange. It may occur even in period of decreasing parasitemia in response to the treatment.
5	Abnormalities in blood coagulation
6	Low blood pressure caused by cardiovascular collapse
7	Acute kidney failure
8	Hyperparasitemia, where more than 5% of erythrocytes are infected by malaria parasites
9	Metabolic acidosis often associated with hypoglycemia.
10	Hypoglycemia may occur in pregnant women with uncomplicated malaria, or after treatment with quinine.

2.12 Pyruvate Kinase Deficiency and Malaria

In 2003, Min-Oo Gandula and his team found that PKD (homozygous *PKLR*^{T269A}) in mice protects against *chabaudi* malaria by reduced parasitemia and mortality rate (**Figure 14**) (24). Then in 2008, they also found that PKD could protect against replication of *P. falciparum* in human erythrocytes by 2 mechanisms: defective invasion of PKD erythrocytes and macrophage clearance preference of ring-stage-infected PKD erythrocytes (**Figure 15, 16**) (30). Moreover, global distribution of PKD and malaria endemic regions overlaps in the area of Mediterranean and Southeast Asia (**Figure 9, 17**) (3).

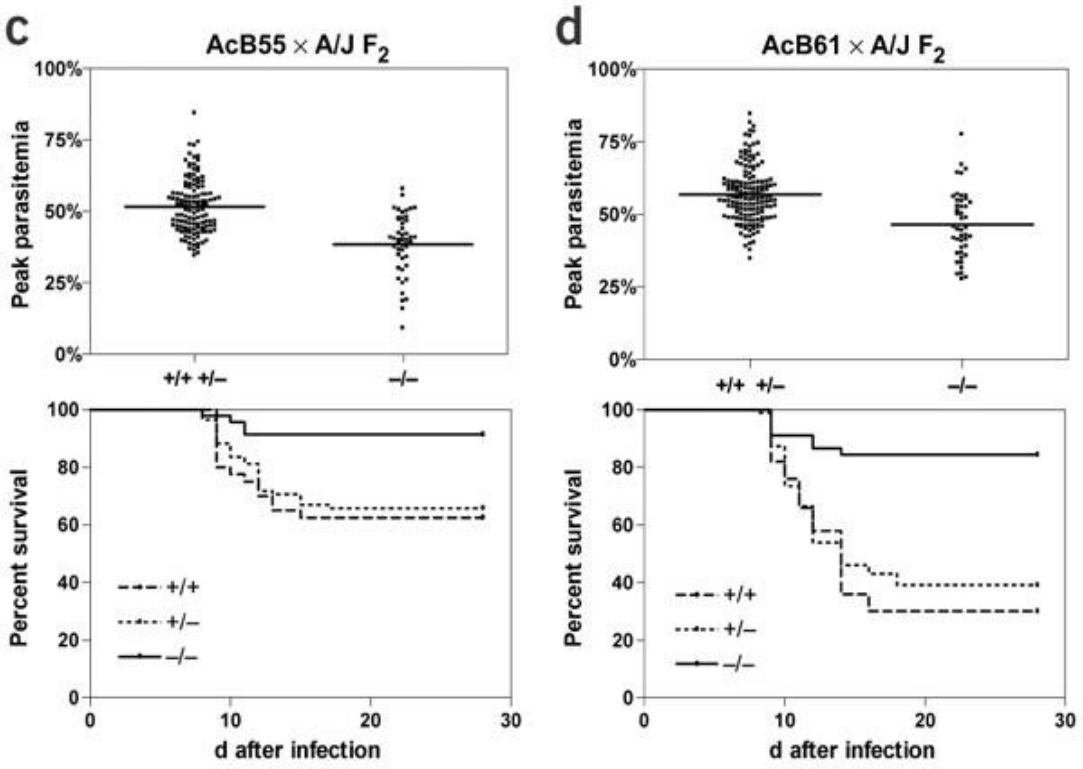


Figure 14 Association of *PKLR*^{T269A} (resulting in I90N) with protection against malaria on peak parasitemia and survival to infection with *P. chabaudi* (24).

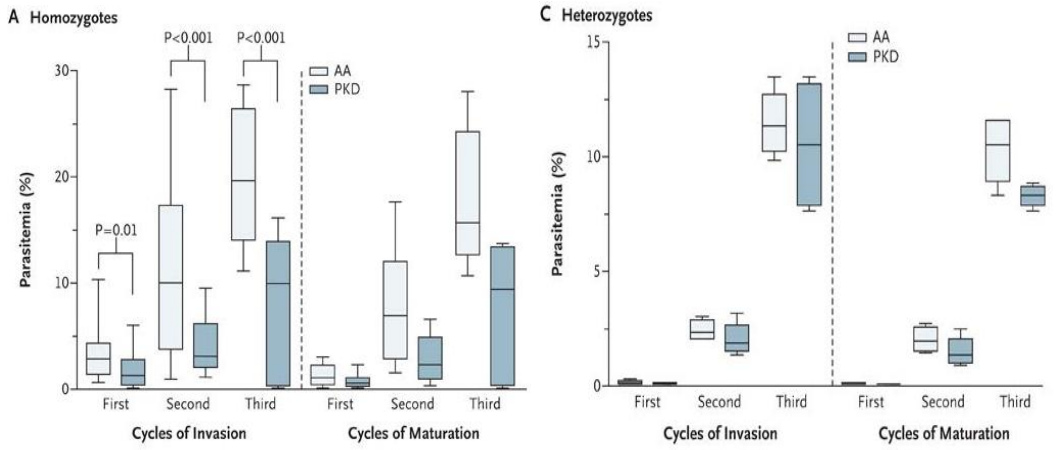


Figure 15 *Plasmodium falciparum* invasion of erythrocytes from PKD subjects and control subjects (30).

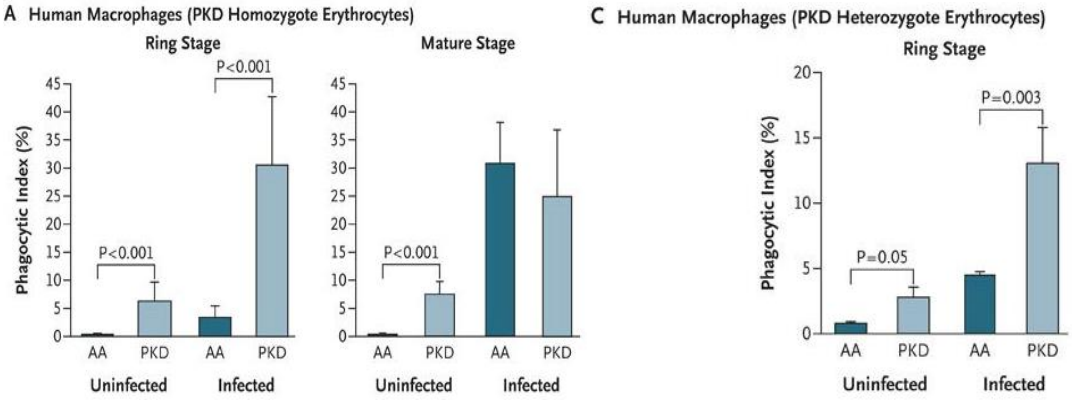


Figure 16 Phagocytosis of malaria-infected erythrocytes from PKD subjects and control subjects (30).

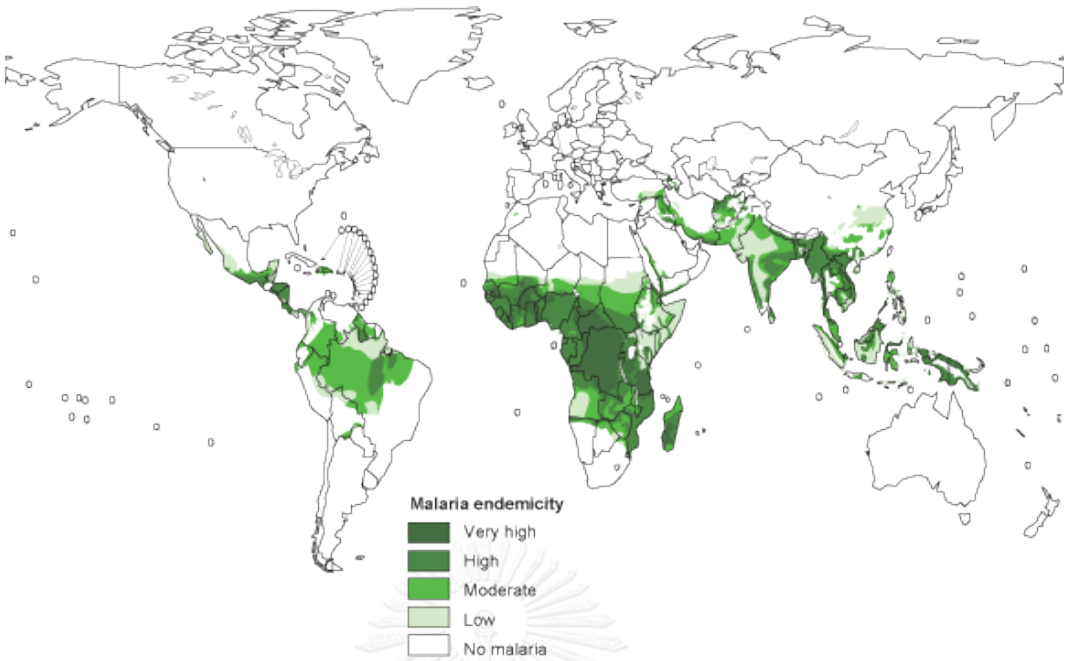


Figure 17 World distribution of malaria endemic areas (69)

CHAPTER 3

MATERIALS AND METHODS

3.1 Population and Sample Size

Two hundred and sixty seven leftover frozen DNA / blood samples of volunteers from the previous project, entitled "Prevalence of alpha-thalassemia, HbE and their clinical correlation in malaria patients in Southeast Asia" (COA No. 040/2013 IRB No. 459/55) were used in this study. All volunteers were malaria patients, admitted to the Hospital for Tropical Diseases, Mahidol University between 2011 and 2012. Samples were enrolled, if patients have provided informed consent. This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (IRB 432/57). There were several ethnic groups of samples consisted of 192 Burmeses, 36 Thais, 16 Karens, 9 Cambodians, 4 Mons, 2 Laotians, and 8 cases with no data of ethnic groups.

Required sample size for this study was calculated according to the statistic formula (see below), resulting in at least 203 samples.

Sample size =

$$n = \frac{(Z_{\alpha/2})^2 \ pq}{d^2}$$

~ 203

Where	Z = 95 % confidence level	=	1.96
	p = Expected proportion of PKD	=	0.05
	q = 1-p	=	0.95
	d = Margin of error in estimating p	=	0.03

Inclusion Criteria were as follows: patients infected with *P. falciparum* or *P. vivax*, patients were Southeast Asian, and patient's age ranging from 14-60 years.

Exclusion Criteria were as follows: patients co-infected with *P. falciparum* and *P. vivax*, G6PD deficient patients, and Thalassemic patients

3.2 Data Collection

The hematological and general data of patients (see below) were collected from the patient's medical records of the Hospital for Tropical Disease.

3.2.1 General Information: sex, age, ethnic, geographic location

3.2.2 Clinical Information

3.2.2.1 ***Parasitology***: species of parasite, parasitemia and number of infection

3.2.2.2 ***Hematology***: complete blood count (CBC), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), hemoglobin, reticulocyte count, bilirubin

3.2.2.3 ***Complication***: parasitemia (*P. falciparum* density ≥50,000/µl, *P. vivax* density ≥20,000/µl), renal insufficiency (serum creatinine concentration >1.5 mg/µl), reticulocyte count (>2%), anemia (hemoglobin <10 g/dL) and hyperbilirubinemia (total bilirubin >2.5 mg/dL)

3.3 Identification G6PD deficiency

In previous study, G6PD deficiency had been identified in all collected ACD blood samples using gold standard G6PD spectrophotometric assay (70). First, hemoglobin was measured using the following protocol. One ml of ACD blood was centrifuged at 3000 rpm for 5 min. Then, 100 µl of red blood cells was washed with 0.9% normal saline. The solution was centrifuged at 3000 rpm for 5 min and the supernatant was removed. The packed red cells was washed with 0.9% normal saline 3 times. In the last times of washing by normal saline, the supernatant was removed only 1/3 of the red cell pellet to measure hemoglobin in absorbance at 570 nm using Hemocue® Hemoglobin Photometer.

After that, 950 µl of water was added into 50 µl of red blood cells and the mixture was frozen at -20°C for 10 min to break down cell membrane. Next, the mixture was centrifuge at 14,000 rpm for 10 min. G6PD activity in hemolysate was measured by spectrophotometry, which monitoring the rate of NADPH formation as an

increase in absorbance at 340 nm. Fifty µl of hemolysate was added into 850 µl of stock solution (2 mM NADP, 1 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$) and incubated at room temperature for 5 min. Next, 100 µl of G 6-P (6 mM glucose 6-phosphate) was added. Finally, the solution was measured for NADPH level at 340 nm for 5 min. The average absorbance was calculated by the following formula.

$$\text{Activity (IU) / g Hb} = \frac{\mathbf{1 \times O.D/min \times dilution \times 100 \times TCF}}{\mathbf{6.22 \times 0.05 \times Hb\ (g/dL)}} \quad \text{Hb gm\%}$$

1	=	The volume of solution (mL)
6.22	=	Millimolar absorptivity of reduced NADP
Dilution	=	50 µL of red blood cell: 950 µL of water= 20
1.00	=	Factor (IU/g Hb)
0.05	=	The volume of hemolysate (mL)
Hb	=	Hemoglobin (g/dL)
TCF	=	Temperature correction factor = 1.66

G6PD activity less than 1.40 IU/gHb was classified as G6PD deficiency.

3.4 DNA Extraction

Genomic DNA were extracted from buffy coat of samples using standard phenol-chloroform method (71). Frozen blood samples were lysed on iced for 5 min with 10 ml lysis buffer [0.32 M sucrose (USB), 10 mM Tris-HCL pH 7.5 (Fisher Scientific, J. T. BAKER), 5 mM $MgCl_2$ (BIO BASIC INC), 1% Triton X-100 (SIGMA)] to remove red blood cells. After that, the suspension was centrifuged at 4°C for 10 min at 1,000 g. Next, the supernatant was removed and pellet was collected. The pellet was washed with 5 ml lysis buffer and centrifuged at 4°C for 10 min at 1,000g. If the solution was dark red, the pellet should be washed with lysis buffer until it was white.

White blood cells were lysed with 1 ml SE buffer [0.075 M NaCl (UNIVAR), 0.024 M EDTA pH 8.0 (Plusone® Amersham Biosciences)], 4 ml Proteinase K digestion buffer [27% sucrose (USB), 1X SSC, 1 mM EDTA (Plusone® Amersham Biosciences), 1% SDS

(GE Healthcare)] and 150 ml of 20 mg/ml proteinase K (Amresco) and then incubated at 50°C for 16-18 hours. Following by addition 5 ml phenol-chloroform-isoamylalcohol (25:24:1) and the solution was inverted for 30 min – 2 hours. Next, the mixture was centrifuged at 2,500 rpm for 10 min to remove protein layer from DNA layer. Next, the solution was precipitated with isopropanol (MERCK) and incubated at room temperature for 30 min before centrifuged at 4,000 rpm for 30 min. If the DNA pellets were invisible, it should be centrifuged again at 4,000 rpm for 10 min. After that, the supernatant was removed and DNA pellets was washed with 5 ml 70% ethanol and centrifuged at 4,000 rpm at 10 min. Following this step, the supernatant was removed and the DNA pellets were air dried at room temperature. Later, The DNA pellets were dissolved with 100-200 µl of 1X TE buffer and measured DNA concentration using Nano drop 1000 Spectrophotometer (Thermo Scientific). Then, working solution was diluted with 1X TE buffer to be 50 ng/µl.

3.5 Identification *G6PD* Mutation Using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP)

Common *G6PD* mutations including *G6PD*^{Mahidol} (c.487G→A), *G6PD*^{Viangchan}(c.871G→A), *G6PD*^{Union}(c.1360C→T), *G6PD*^{Canton} (c.1376G→T), *G6PD*^{Kaiping}(c.1388G→A), *G6PD*$^{Chinese-4}$(c.392G→T), *G6PD*^{Coimbra}(c.592C→T) and *G6PD*$^{Chinese-5}$ (c.1024C→T) were determined in deficient samples by PCR-RFLP (72). The reaction was carried out in a 20 µl reaction containing DNase free water, PCR buffer, 0.5U Taq DNA Polymerase (Invitrogen), 200 µM dNTPs (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 µM primer (**Table 2**) (Bio Basic Inc.) and approximate 50 ng DNA. PCR was performed to generate the PCR fragments under 5 min of initial denaturation DNA at 94°C, then 35 cycles of 94°C for 45 sec, 45 sec of the annealing temperature for each primer pair (**Table 2**), 72°C for 45 sec, followed by a final extension at 72°C for 10 min in Veriti® 96-well thermal cycler (Applied Biosystems). PCR fragments were digested with appropriate enzymes (**Table 3**). The reaction was carried out in a 20 µl reaction containing DNase free water, 1U restriction enzyme (Fermentas), 1X reaction buffer (Fermentas) and 10 µl PCR products and then the reaction was incubated at an appropriate temperature (**Table 3**) for 12–14 hours. PCR-RFLP products were analyzed

using gel electrophoresis with 8% polyacrylamide gel (29% acrylamide + 1% N, N' methylenebisacrylamide (Bio-RAD), (10X) TBE buffer (Bio-RAD), 10% ammonium persulfate (APS) (Pharmacia Biotech), TEMED (Bio-RAD) and water (71). Five µl of PCR-RFLP product was mixed with 0.8 µl of novel juice (Genedirex). Then, mixture was loaded on 8% polyacrylamide gel and run at 100 volt for 60 min. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).

Table 2 Sequence of *G6PD* primers

G6PD **Mutation**	**Primer Name**	**Sequence of primer (5′–3′)**	**Annealing Temperature(°C)**
G6PD$^{\text{Mahidol}}$	487F	5′-GCGTCTGAATGATGCAGCTCTGAT-3′	56
	487R	5′-CTCCACGATGATGCGGTTCAAGC-3′	
G6PD$^{\text{Viangchan}}$	871F	5′-TGGCTTTCTCTCAGGTCTAG-3′	60
	9R	5′-GTCGTCCAGGTACCCTTTGGGG-3′	
G6PD$^{\text{Union}}$	1360F	5′-ACGTGAAGCTCCCTGACGC-3′	65
	1360R	5′-GTGAAAATACGCCAGGCCTTA-3′	
G6PD$^{\text{Canton}}$	1360F	*G6PD*$^{\text{Union}}$ primer	65
	1360R	*G6PD*$^{\text{Union}}$ primer	
G6PD$^{\text{Kaiping}}$	1388F	5′-ACGTGAAGCTCCCTGACGC-3′	65
	1388R	5′-GTGCAGCAGTGGGGTGAACATA-3′	
G6PD$^{\text{Chinese-4}}$	392F	5′GGACTCAAAGAGAGGGGCTG-3′	65
	392R	5′-GAAGAGGCGGTTGGCCGGTGAC-3′	
G6PD$^{\text{Coimbra}}$	592F	5′-GAGGAGGTTCTGGCCTCTACTC-3′	65
	592R	5′-TTGCCCAGGTAGTGGTCGCTGC-3′	
G6PD$^{\text{Chinese-5}}$	1024F	5′-GTCAAGGTGTTGAAATGCATC-3′	65
	1024R	5′-CATCCCACCTCTCATTCTCC-3′	

Table 3 Sequence of *G6PD* restriction enzymes and product size

G6PD Mutation	Restriction Enz.	Buffer	Restriction Size	Product Size (bp)	Temperature (°C)
G6PD^{Mahidol}	*Hin*dIII	R	5′-A^AGCTT-3′ 3′-TTCGA^A-5′	N: 104 M: 82 + 22	37
G6PD^{Viangchan}	*Xba*I	Tango	5′-T^CTAGA-3′ 3′-AGATC^T-5′	N: 126 M: 106+20	37
G6PD^{Union}	*Hha*I	Tango	5′-GCG^C-3′ 3′-C^GCG-5′	N: 142+45+27 M: 187+27	37
G6PD^{Canton}	*Afl*II	O	5′-C^TTAAG-3′ 3′-GAATT^C-5′	N: 214 M: 194+20	37
G6PD^{Kaiping}	*Nde*I	O	5′-CA^TATG-3′ 3′-GTAT^AC-5′	N: 227 M: 206+21	37
G6PD$^{Chinese-4}$	*BstE*II	O	5′-G^GTNACC-3′ 3′-CCANTG^G-5′	N: 188+15 M: 203	37
G6PD^{Coimbra}	*Pst*I	O	5′-CTGCA^G-3′ 3′-G^ACGTC-5′	N: 157+83 M: 157+63+20	37
G6PD$^{Chinese-5}$	*Mbo*II	B	5′-GAAGA$(N)_8$^-3′ 3′-CTTCT$(N)_7$^-5′	N: 187 M: 150+37	37

3.6 Identification Globin Gene Variants Using Multiplex Gap-PCR (multiplex Gap-PCR) and PCR-RFLP

3.6.1 Alpha Thalassemia

Alpha (α)-globin gene variants including -$\alpha^{3.7}$, -$\alpha^{4.2}$, --^{SEA} and –^{FIL} were investigated by multiplex gap-PCR. Target DNAs were amplified using several primer pairs (**Table 4**). The PCR reaction was carried out in a 25 µl reaction containing DNase free water, 1X PCR buffer, 1.25 U Platinum Taq DNA Polymerase (Invitrogen), 200 µM dNTPs (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 µM primer (Bio Basic Inc.), 1 M Betaine (Sigma) and 100 ng DNA template. PCR was performed to generate the PCR fragments of the α-globin gene variants under 5 min of initial denaturation DNA at 94°C, then 30

cycles of 97°C for 45 sec, 65°C for 1.15 min, 72°C for 2.30 min, following by a final extension at 72°C for 5 min in Veriti® 96-well thermal cycler (Applied Biosystems). PCR products were analyzed using 1% agarose gel electrophoresis (73). Five µl of PCR product was mixed with 0.8 µl of novel juice (Genedirex). Then, mixture was loaded on 1% agarose gel and run at 100 volt for 60 min. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).

Table 4 Sequence of α-globin primers and product size

α -globin variants	**Primer Name**	**Sequence of primer (5′–3′)**	**Product Size (bp)**
Positive control	LIS1-F	5′-GTC GTC ACT GGC AGC GTA GAT C-3′	2503
	LIS1-R	5′-GAT CCA GGT TGT AGA CGG ACT G-3′	
$-\alpha^{3.7}$	α2/3.7-F	5′-CCC CTC GCC AAG TCC ACC C -3′	2022
	3.7-R	5′-AAA GCA CTC TAG GGT CCA GCG-3′	
αα	α2/3.7-F	5′-CCC CTC GCC AAG TCC ACC C -3′	1800
	α2-R	5′-AGA CCA GGA AGG GCC GGT G-3′	
$-\alpha^{4.2}$	4.2-F	5′-GGT TTA CCC ATG TGG TGC CTC-3′	1628
	4.2-R	5′-CCC GTT GGA TCT TCT CAT TTC CC-3′	
$-\alpha^{SEA}$	SEA-F	5′-CGA TCT GGG CTC TGT GTT CTC-3′	1349
	SEA-R	5′- AGC CCA CGT TGT GTT CAT GGC-3′	
$-\alpha^{FIL}$	FIL-F	5′- TGC AAA TAT GTT TCT CTC ATT CTG TG-3′	1166
	FIL-R	5′-ATA ACC TTT ATC TGC CAC ATG TAG C-3′	

3.6.2 HbCS

HbCS variant was detected by PCR-RFLP technique using HbCS forward primer and HbCS reverse primer (**Table 5**). The reaction was carried out in a 25 µl reaction containing DNase free water, 1X PCR buffer, 0.5 U Taq DNA Polymerase (Invitrogen), 200 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 0.2 µM primer (Bio Basic Inc.) and approximate 50 ng DNA. PCR was performed to generate the PCR fragments of the

HbCS variant under 5 min of initial denaturation DNA at 94°C, then 30 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min, following by a final extension at 72°C for 10 min in Veriti® 96-well thermal cycler (Applied Biosystems). The amplicon was digested with *Tru1*I (5'-T^TAA-3') (Fermentas), according to manufacturer's protocols. The reaction was carried out in a 20 µl reaction containing DNase free water, 1U *Tru1*I (Fermentas), 1X reaction buffer (Fermentas) and 10 µl PCR products and then the reaction was incubated at 65°C for 12–14 hours. PCR-RFLP products were analyzed on 2.5% agarose gel electrophoresis (73). Five µl of PCR product was mixed with 0.8 µl of novel juice (Genedirex). Then, mixture was loaded on 2.5% agarose gel and run at 100 volt for 60 min. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).

Table 5 Sequence of HbCS, HbE, and *PKLR*^{R41Q} primers and product size

Mutation	**Name of primer**	**Sequences of primer (5′–3′)**	**Size (bp)**
HbCS	HbCS-F	5′- TGC GGG CCT GGG CCG CAC TGA -3′	N: 165+111
	HbCS-R	5′-GCC GCC CAC TCA ACT TTA TT-3′	M: 276
HbE	HbE-F	5′-CAT TTG CTT CTG ACA CAA CTG-3′	N: 233+171+135+59
	HbE-R	5′– TTG AGG TTG TCC AGG TGA G –3′	M: 233+135+59
PKLR^{R41Q}	PKLR-R41Q-F2	5′–GCC AAC GGG GTA TCT ACG GC–3′	N: 102+19
	PKLR-R41Q-R2	5′–GCA GAG GTG TTC CAG GAA GG–3′	M: 121

3.6.3 HbE

HbE variant was detected by PCR-RFLP technique using HbE forward primer and HbE reverse primer (T**able 5**) (74). The reaction was carried out in a 25 µl reaction containing DNase free water, 1X PCR buffer, 0.5 U Taq DNA Polymerase (Invitrogen), 200 µM dNTPs (Invitrogen), 1.5 mM MgCl2 (Invitrogen), 0.2 µM primer (Bio Basic Inc.) and approximate 50 ng DNA. PCR was performed to generate the PCR fragments of the HbE variant under 5 min of initial denaturation DNA at 95°C, then 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, following by a final extension at 72°C for 10 min

in Veriti® 96-well thermal cycler (Applied Biosystems). The amplicon was digested with *Mnl*I (5'-CCTC(N)$_7$^-3') (Fermentas), according to manufacturer's protocols. The reaction was carried out in a 20 µl reaction containing DNase free water, 1U *Mnl*I (Fermentas), 1X reaction buffer (Fermentas) and 10 µl PCR products and then the reaction was incubated at 37°C for 12–14 hours. PCR-RFLP products were analyzed on 2.5% agarose gel electrophoresis (73). Five µl of PCR product was mixed with 0.8 µl of novel juice (Genedirex). Then, mixture was loaded on 2.5% agarose gel and run at 100 volt for 60 min. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).

3.7 Identification *PKLR*^{R41Q} Mutation Using PCR-RFLP

PKLR^{R41Q} was detected by PCR-RFLP technique using PKLR-R41Q-F2 forward primer and PKLR-R41Q-R2 reverse primer (**Table 5**). The reaction was carried out in 20 µl reaction containing DNase free water, 1x PCR buffer (RBC Bioscience), 0.2 mM dNTPs (RBC Bioscience), 1.5 mM $MgCl_2$ (RBC Bioscience), 0.5 U Taq DNA Polymerase (RBC Bioscience), 200 nM of primer (Biolabs) and approximate 50 ng DNA. PCR was performed to generate the PCR fragments of the *PKLR*^{R41Q} mutation under 5 min of initial denaturation DNA at 94°C, then 35 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 7 min in Veriti® 96-well thermal cycler (Applied Biosystems). PCR fragments were digested with *Aci*I (5'...C^CGC...3') (Fermentas), according to manufacturer's protocols and analyzed on 8% polyacrylamide gel electrophoresis. The reaction was carried out in a 20 µl reaction containing DNase free water, 1U *Aci*I (Fermentas), 1X reaction buffer (Fermentas) and 10 µl PCR products and then the reaction was incubated at 37°C for 12–14 hours. PCR-RFLP products were analyzed on 8% polyacrylamide gel electrophoresis. Five µl of PCR-RFLP product was mixed with 0.8 µl of novel juice (Genedirex). Then, mixture was loaded on 8% polyacrylamide gel and run at 100 volt for 60 min. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).

3.8 Identification *PKLR*^{R41Q} Mutation using Direct Sequencing

PKLR^{R41Q} mutation was re-confirmed by direct sequencing. First, the *PKLR*^{R41Q} locus was amplified using PCR with primers in **Table 5.** PCR condition was performed according to 3.7. Then, the PCR product was analyzed via 2% agarose gel electrophoresis at 100 volt for 45 min before purified using Gel/PCR DNA Fragments Extraction Kit Cat No.YDF300 Lot.No.PG-913-09362 (RBC Bioscience). Finally, the purified product was measured its concentration using Nano drop 1000 Spectrophotometer (Thermo Scientific) and sequenced.

A hundred µl of DF buffer was added into PCR product and then vortexed. Next, the mixture was pipetted into DF column in Collection tube and centrifuged at 13,000 rpm for 30 sec. The solution in Collection tube was removed. Next, 600 µl of Wash buffer was added into DF column. Next, centrifuged the column at 13,000 rpm for 30 sec, removed the solution in Collection tube and centrifuged again at 13,000 rpm for 2 min to discard the solution. The DF column was placed into new 1.5 ml microcentrifuge tube and 30 µl of elution buffer was added. Lastly, the column was centrifuged at 13,000 rpm for 2 min. Sanger sequencing results were analyzed by comparing with genomic reference sequence of Human *PKLR* (NM_000298.5) using BioEdit version 7.2.5.

3.9 Data Analysis

All clinical data of subjects were collected from Case Report Form (CRF). Deviation of genotype and allele frequency from Hardy-Weinberg equilibrium (HWE) was tested by Chi-square test. The association between *PKLR*^{R41Q} mutation and susceptible or protective against malaria infection and complicated malaria were tested by Mann-Whitney U-test and odd ratio. Ninety five percent of confidence interval (95% CI) was set as a significance level. GraphPad Prism 5 and SPSS statistics 22.0 were used for all statistical analysis.

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RESULTS

4.1 Population Information

A total of 267 Southeast Asian malaria patients admitted to Hospital for Tropical Diseases were enrolled. They consisted of 192 Burmeses (71.9%), 36 Thais (13.5%), 16 Karens (6.0%), 9 Cambodians (3.4%), 4 Mons (1.5%), 2 Laotians (0.7%), and 8 cases with no data of ethnic groups (3.0%) (**Figure 18**). There was 252 (94.4%) male and 15 (5.6%) female, which ratio male:female was 17:1. The average age of all subjects was 28.0 ± 10.2 (range 14-60) years (**Table 6**).

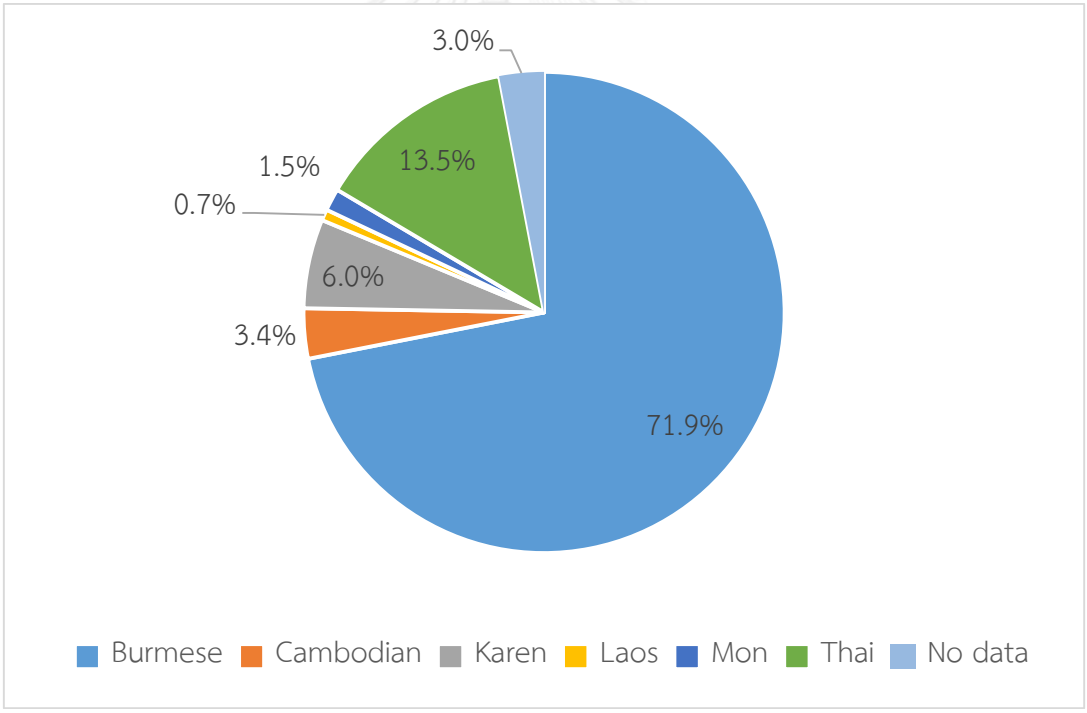

Figure 18 The proportion of ethnicity in this study

The general information of each ethnic group was shown in **Table 6.** There was no statistical difference of age between ethnic groups. *P. vivax*, the prominent parasite infection, was found 58.2% (145/249) in these subjects (**Table 6**).

Table 6 General information of malaria patients in this study

ETHNICITY	TOTAL	SEX		AGE		PARASITE INFECTION (NO.)		
		Male	Female	Mean ± SD	Range	*P. falciparum*	*P. vivax*	ND
BURMESE	192	185	7	28.0 ± 10.3	14 - 58	79	104	9
THAI	36	30	6	31.9 ± 10.8	16 - 60	14	22	0
KAREN	16	16	0	22.7 ± 7.9	14 - 40	7	9	0
CAMBODIAN	9	9	0	24.4 ± 3.8	19 - 30	1	7	1
MON	4	4	0	22.8 ± 2.2	21 - 26	1	3	0
LAOTIAN	2	1	1	28.0 ± 5.7	24 - 32	2	0	0
ND	8	7	1	27.4 ± 11.6	15 - 48	0	1	7
TOTAL	267	252	15	28.0 ± 10.2	14 - 60	104	146	17

ND: no data

4.2 Other Hematological Variants

One hundred and forty three patients, who had G6PD deficiency, α-thalassemia and other hemoglobinopathies were excluded from association study with malaria because they are confounding factors. Only 124 patients were studied the association with clinical manifestation of malaria infection.

4.2.1 G6PD Mutations

G6PD^{Mahidol} (**Figure 19**), *G6PD*^{Viangchan} (**Figure 20**), and *G6PD*^{Kaiping} (**Figure 21**) were found in this population **(Table 7)**. Others common *G6PD* mutations in Southeast Asia were not found in this study.

Table 7 Allele frequency and amount of malaria patients with *G6PD* variants

G6PD variants	Amount (Hemizygote)	Allele frequency
G6PD^{Mahidol}	19	0.073
G6PD^{Viangchan}	3	0.012
G6PD^{Kaiping}	1	0.004

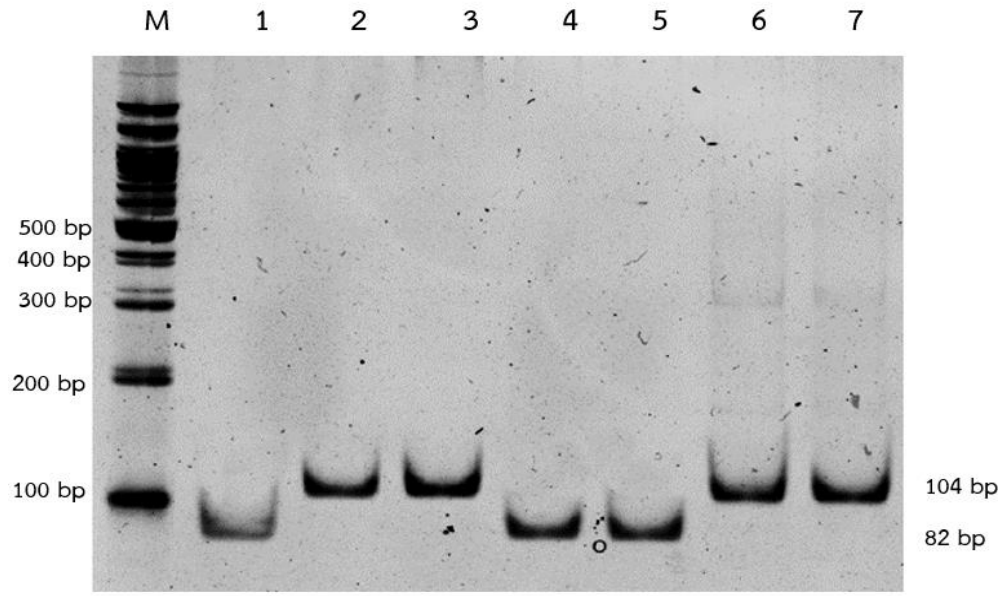

Figure 19 Detection of *G6PD*$^{\text{Mahidol}}$ mutation Lane M; 100 bp marker, Lane 1; Positive control; 104 bp, Lane 2, 3, 6, 7; 104 bp after digested with *Hind*III represent non-*G6PD*$^{\text{Mahidol}}$, Lane 4, 5; 82 bp after digested with *Hind*III represent *G6PD*$^{\text{Mahidol}}$.

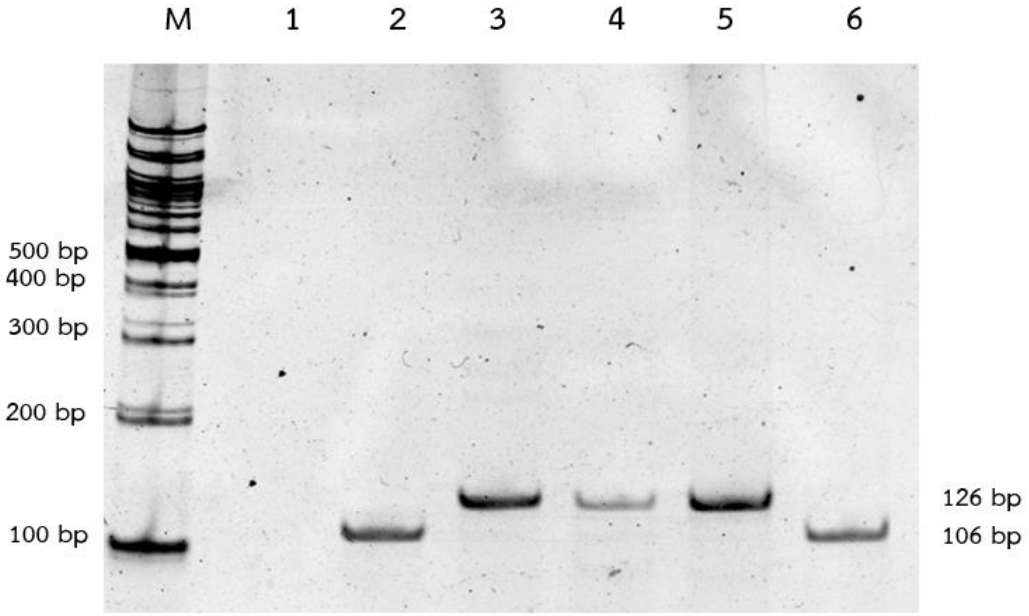

Figure 20 Detection of *G6PD*$^{\text{Viangchan}}$ mutation Lane M; 100 bp marker, Lane 1; Negative control (H_2O), Lane 2; Positive control; 106 bp, Lane 3, 4, 5; 126 bp after digested with *Xba*I represent non-*G6PD*$^{\text{Viangchan}}$, Lane 6; 106 bp after digested with *Xba*I represents *G6PD*$^{\text{Viangchan}}$.

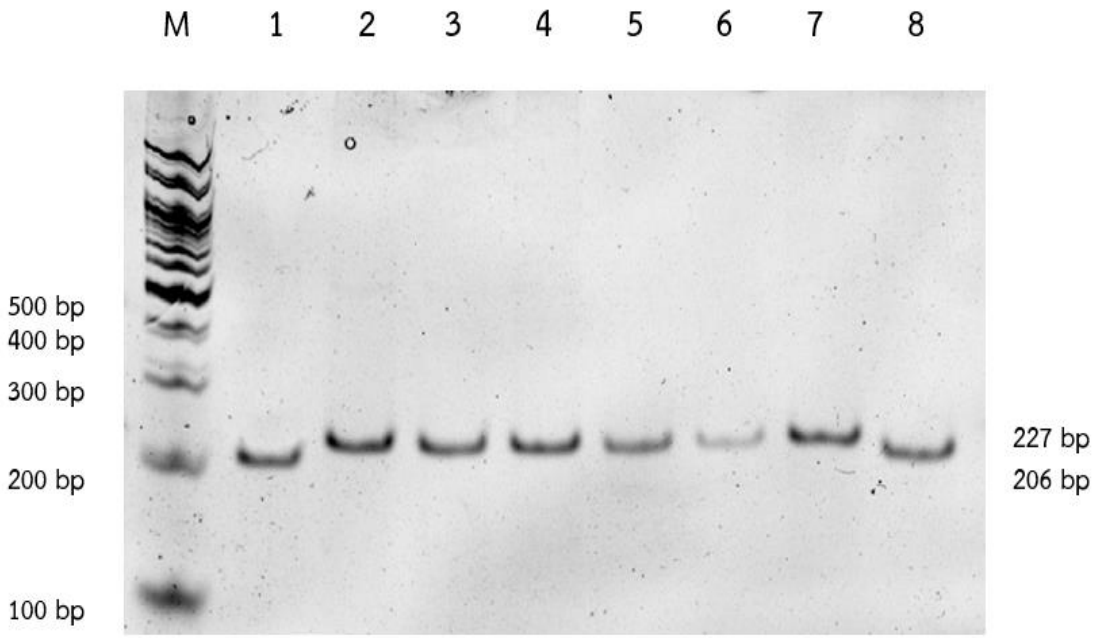

Figure 21 Detection of *G6PD*^{Kaiping} mutation Lane M; 100 bp marker, Lane 1; Positive control; 206 bp, Lane 2-7; 227 bp after digested with *Nde*I represent non-*G6PD*^{Kaiping}, Lane 8; 206 bp after digested with *Nde*I represents *G6PD*^{Kaiping}.

4.2.2 α-Thalassemia

Based on multiplex-PCR analysis of α-globin gene variants, we found -$\alpha^{3.7}$, –$\alpha^{4.2}$, –^{SEA} and HbCS **(Figure 22)**. However, --^{FIL} variant was not found in this population *(Table 8)*.

Table 8 Alelle frequency and amount of malaria patients with α-globin gene variant

α-globin gene variants	Amount		Allele frequency
	Homozygote	Heterozygote	
-$\alpha^{3.7}$	20	84	0.246
–$\alpha^{4.2}$	-	2	0.004
–^{SEA}	-	2	0.004
--^{FIL}	-	-	0.000
HbCS	1	4	0.012

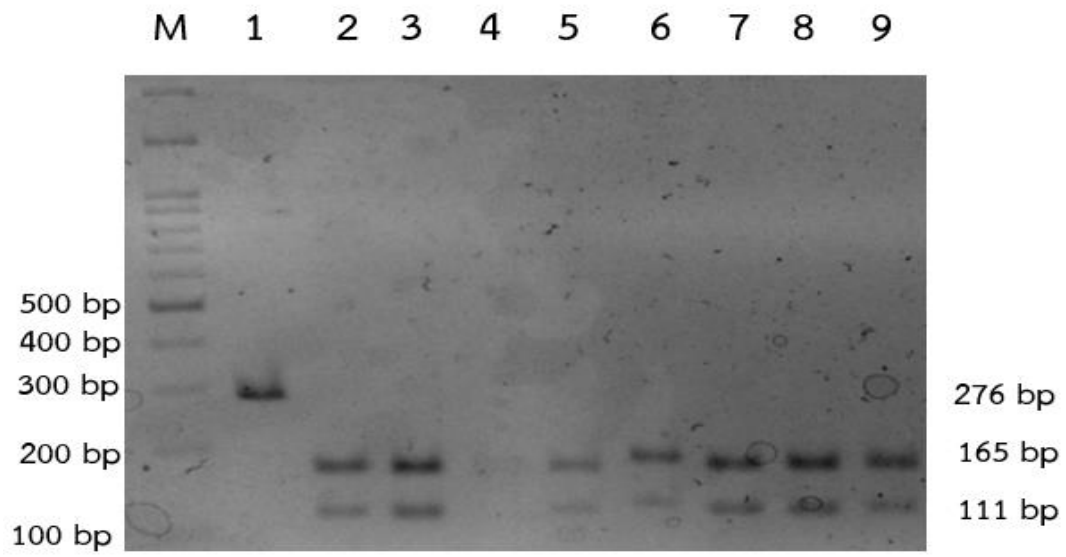

Figure 22 Detection of HbCS Lane M; 100 bp marker, Lane 1; 276 bp after digested with *Tru1*I represents $\alpha^{CS}\alpha/\alpha^{CS}\alpha$, Lane 2-9; 165+111 bp after digested with *Tru1*I represent **αα/αα.**

4.2.3 Hemoglobin E codon 26 (G ⟶ A) (HbE)

HbE was found in 49 malaria patients (**Figure 23**). There were 8 homozygous HbE and 41 heterozygous HbE. The allele frequency was 0.1135.

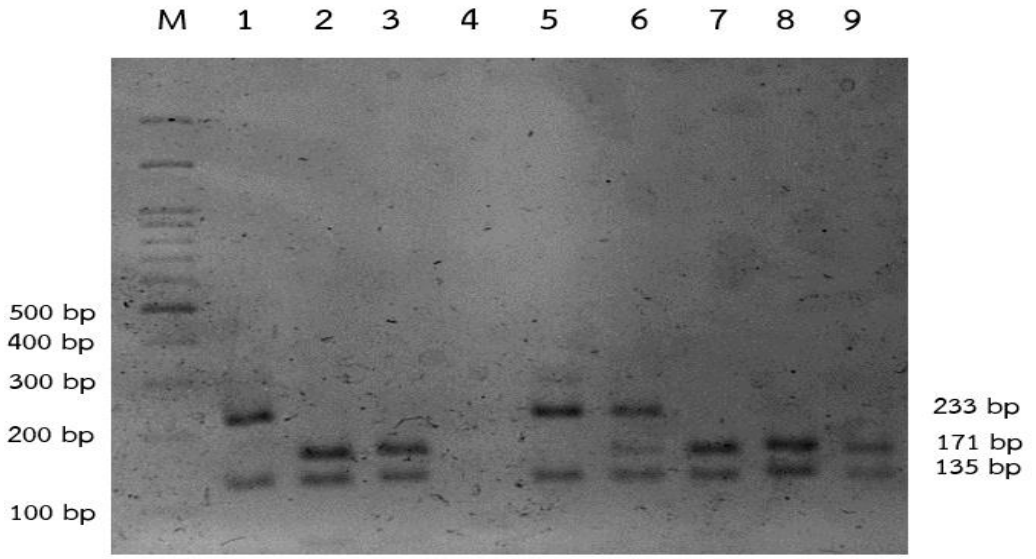

Figure 23 Detection of HbE Lane M; 100 bp marker, Lane 1, 5; 233+135 bp after digested with *Mnl*I represent β^E/β^E, Lane 2, 3, 7, 8, 9; 171+135 bp after digested with *Mnl*I represents β^A/β^A and Lane 6; 233+171+135 bp after digested with *Mnl*I represents β^A/β^E.

4.3 Prevalence of *PKLR*^{R41Q}

It was found that 13 out of 267 samples carried *PKLR*^{R41Q} with 1 homozygous *PKLR*^{R41Q} (**Figure 24**) and 12 heterozygous *PKLR*^{R41Q} (**Figure 25**). *PKLR*^{R41Q} was re-comfirmed by direct sequencing (**Figure 26**). After Hardy Weinberg equilibrium (HWE) testing, Chi-square equaled 0.1662 with 1 degree of freedom (df). The observed frequency was not significantly different from the expected frequency, which illustrated that the allele and genotype frequency of *PKLR*^{R41Q} in this population followed the rule of Hardy-Weinberg equilibrium (**Table 9**). The allele frequency of *PKLR*^{R41Q} was 0.026.

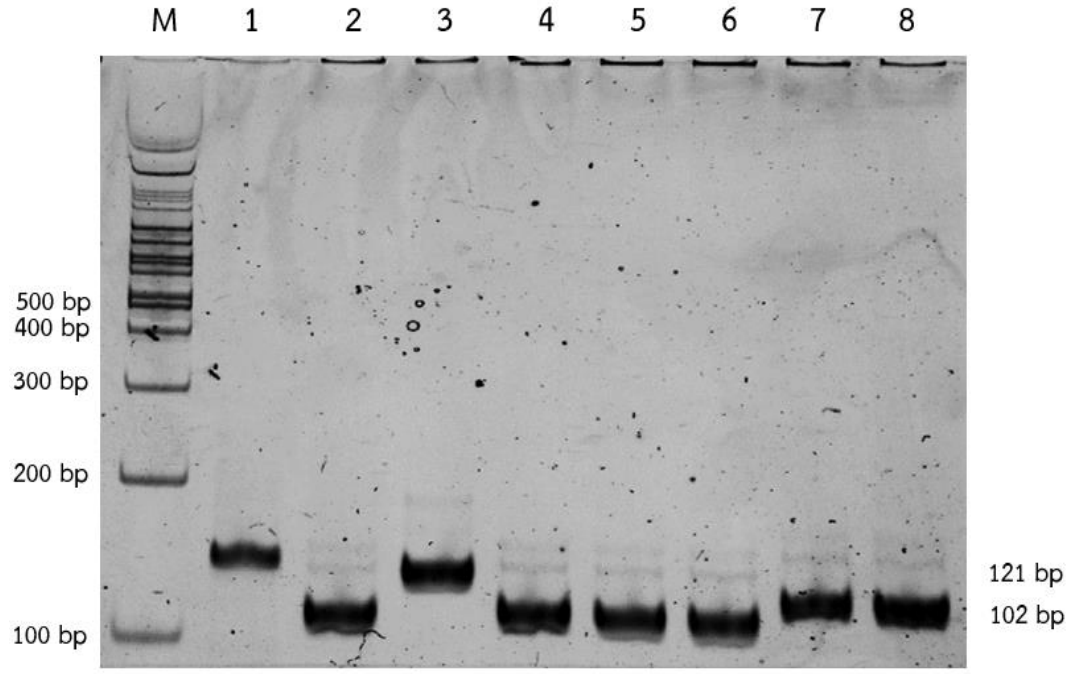

Figure 24 Detection of *PKLR*^{R41Q} Lane M; 100 bp marker, Lane 1; 121 bp of PCR product without *Aci*I, Lane 2 and 4-8; 102 bp after digested with *Aci*I represent homozygous *PKLR*^{R41R} normal, Lane 3; 121 bp after digested with *Aci*I represents homozygous *PKLR*^{R41Q}.

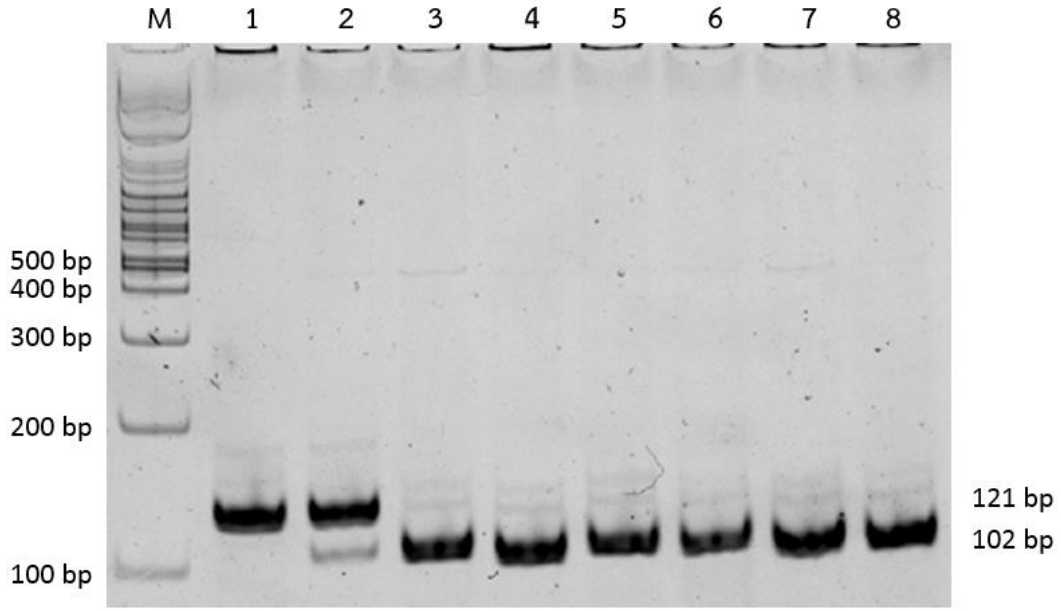

Figure 25 Detection of *PKLR*^{R41Q}Lane M; 100 bp marker, Lane 1; 121 bp of PCR product without *Aci*I, Lane 2; 121, 102 bp after digested with *Aci*I represents heterozygous *PKLR*^{R41Q}, Lane 3-8; 102 bp after digested with *Aci*I represent homozygous *PKLR*^{R41R} normal.

Table 9 The genotype frequency of *PKLR*^{R41Q}

Genotype	Observed frequency	Expected frequency	*p*-value
G G	254	254.2	0.050
G A	12	12.7	
A A	1	0.2	
Total	267	267	

PKLR^{R41Q} was found in Burmese (5.8%; 11/192), Cambodian (11.1%; 1/9) and Thai (5.6%; 2/36) ethnics. The allele frequency of *PKLR*^{R41Q} in Burmese, Cambodian, and Thai ethnics were 0.032, 0.056 and 0.028, respectively (**Figure 27**). There was no *PKLR*^{R41Q} in Karen, Laos, and Mon.

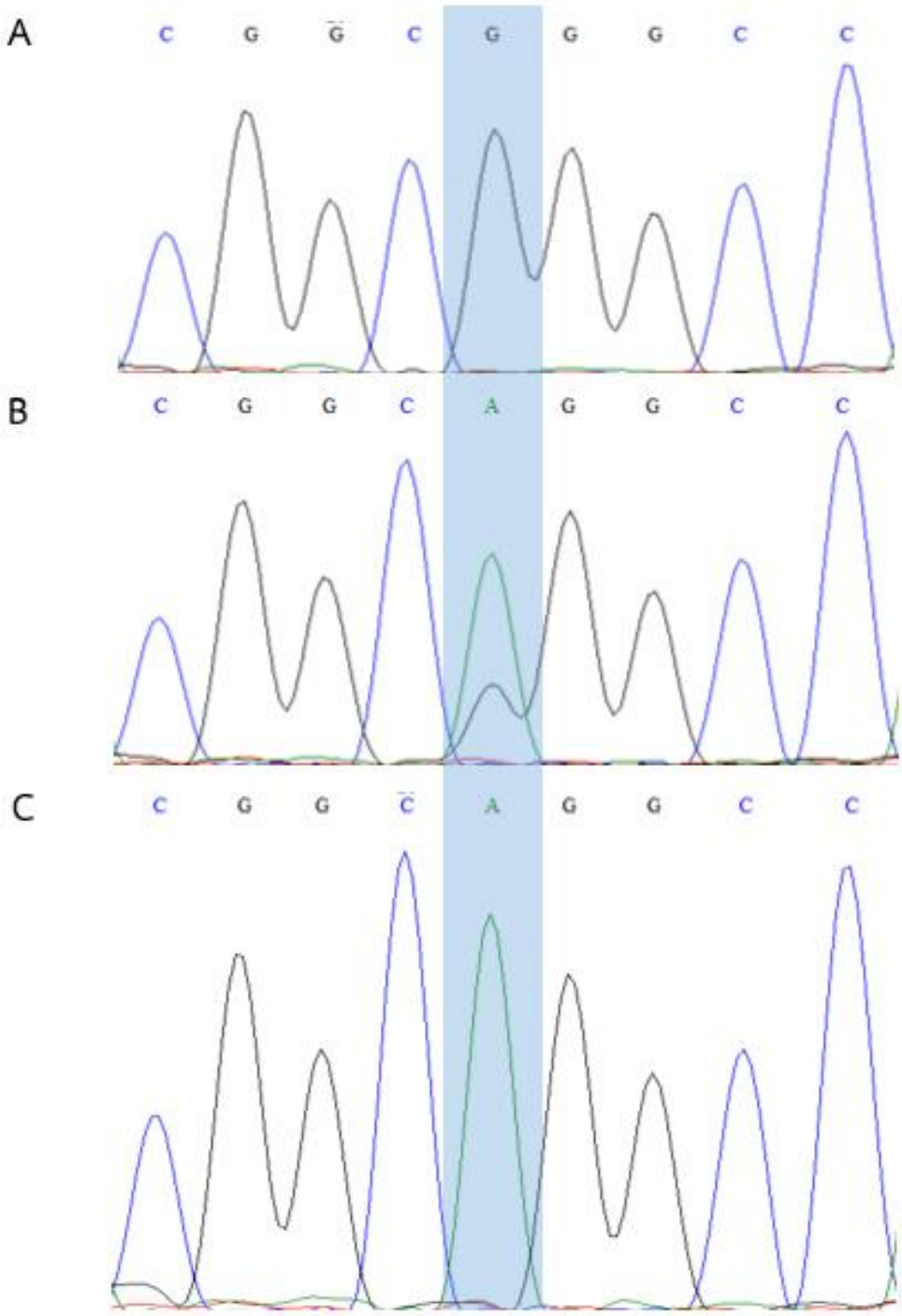

Figure 26 Chromatogram in sequencing of *PKLR*^{R41Q}; (A) Homozygous *PKLR*^{R41R} (B) Heterozygous *PKLR*^{R41Q} (C) Homozygous *PKLR*^{R41Q}

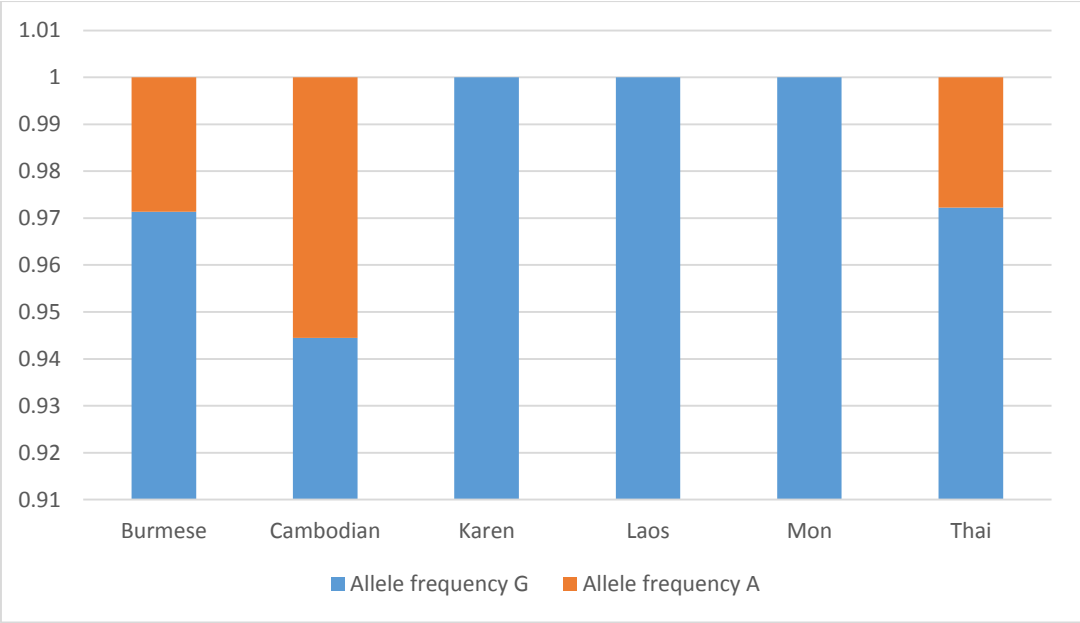

Figure 27 The allele frequency of *PKLR*^{R41Q} in individual ethnicity

4.4 Hematological Information of *PKLR*^{R41Q}

The hematological information of *PKLR*^{R41Q} patients was shown in **Table 10**. Mean of red blood cell (RBC) was 5.20 ± 0.49 10^6/μL (reference range: 4.50–5.90 10^6/μL), hemoglobin (Hb) was 13.2 ± 1.8 g/dL (reference range: 14.0–18.0 g/dL), mean corpuscular volume (MCV) was 79.4 ± 9.3 fL (reference range: 82.0-96.0 fL), mean corpuscular hemoglobin (MCH) was 25.5 ± 3.6 pg (reference range: 26.0-32.0 pg), mean corpuscular hemoglobin concentration (MCHC) was 32.1 ± 2.0 g/dL (reference range: 32.0–36.0 g/dL), reticulocyte count was 1.5 ± 0.9 % (reference range: 0.5-2.5 %), creatinine was 0.9 ± 0.1 mg/dL (reference range: 0.0-1.5 mg/dL), and total bilirubin was 0.83 ± 0.44 mg/dL (reference range: 0.0 – 1.0 mg/dL).

There was one patient who carried *G6PD*^{Mahidol} with *PKLR*^{R41Q}, one case of heterozygous -**α**$^{3.7}$, heterozygous Hb E with *PKLR*^{R41Q} and one case of homozygous -**α**$^{3.7}$ with *PKLR*^{R41Q}. Moreover, there were 4 patients with heterozygous -**α**$^{3.7}$ and *PKLR*^{R41Q} **(Table 10)**.

Table 10 *General and hematological data of malaria patients with PKLR^{R41Q}*

No.	Sex	Ethnic group	Geno type	*Plasmodium* spp.	RBC	Hb	MCV	MCH	MCHC	Reticulocyte count (%)	Creatinine	Total bilirubin	α-Thalas-semia	Hb E	*G6PD*
1	Male	Burmese	G A	*P. vivax*	4.95	13.1	80.6	26.6	33.0	1.5	0.8	0.55	ND	ND	Mahidol
2	Male	Burmese	G A	ND	ND	ND	ND	ND	ND	ND	ND	ND	αα/αα	β^A/β^A	*G6PD*
3	Male	Burmese	G A	*P. falciparum*	5.38	12.9	69.7	24.1	34.5	0.8	1.0	1.04	αα /-$\alpha^{3.7}$	β^A/β^A	*G6PD*
4	Male	Burmese	G A	*P. falciparum*	4.86	13.2	81.1	27.1	33.4	0.7	0.9	0.45	αα /-$\alpha^{3.7}$	β^A/β^A	*G6PD*
5	Male	Burmese	G A	*P. vivax*	5.26	12.9	73.6	24.5	33.3	1.8	1.1	2.00	αα /-$\alpha^{3.7}$	β^A/β^E	*G6PD*
6	Male	Burmese	G A	*P. falciparum*	5.04	8.2	58.9	16.2	27.5	2.9	0.7	0.57	αα /-$\alpha^{3.7}$	β^A/β^A	*G6PD*
7	Male	Burmese	G A	*P. falciparum*	6.31	13.8	71.6	21.9	30.6	1.7	0.9	0.64	-$\alpha^{3.7}$/-$\alpha^{3.7}$	β^A/β^A	*G6PD*
8	Male	Thai	G A	*P. falciparum*	5.08	14.1	89.0	27.9	31.3	1.3	0.7	1.12	αα/αα	β^A/β^A	*G6PD*
9	Male	Burmese	A A	*P. vivax*	4.87	13.3	90.0	27.3	30.3	1.4	0.9	0.33	αα/αα	β^A/β^A	*G6PD*
10	Male	Burmese	G A	*P. vivax*	4.93	13.6	85.5	27.6	32.4	0.7	0.7	0.98	αα/αα	β^A/β^A	*G6PD*
11	Male	Cambodian	G A	*P. vivax*	5.41	15.3	87.7	28.3	32.2	1.0	1.0	0.85	αα/αα	β^A/β^A	*G6PD*
12	Male	Thai	G A	*P. falciparum*	5.79	14.9	81.5	25.7	31.6	0.9	0.8	0.55	αα /-$\alpha^{3.7}$	β^A/β^A	*G6PD*
13	Male	Burmese	G A	*P. vivax*	4.47	13.1	83.7	29.3	35.0	3.8	0.8	0.88	αα/αα	β^A/β^A	*G6PD*

ND: no data

Reference ranges for hematological parameters; RCB 4.50 – 5.90 10^6/μL, Hb 14.0 – 18.0 g/dL, MCV 82.0 - 96.0 fL, MCH 26.0 -32.0 pg, MCHC 32.0 – 36.0 g/dL, Reticulocyte count 0.5 -2.5 %, Creatinine 0.0 -1.5 mg/dL and Total bilirubin 0.0 – 1.0 mg/dL.

4.5 Clinical Correlation between *PKLR*^{R41Q} and Malaria

4.5.1 Parasite Density

We characterized the associations of this variant with malaria phenotypes. Median log parasitemia (Interquartile range) of *P. falciparum* in the patients with and without *PKLR*^{R41Q} were 9.772/μL (3.990) and 8.940/μL (2.456), respectively (**Figure 25**). There was no significant difference of *P. falciparum* densities between patients with *PKLR*^{R41Q} and without *PKLR*^{R41Q} (p=0.714). Median log parasitemia of *P. vivax* in the patients with and without *PKLR*^{R41Q} were 9.046/μL (3.343) and 9.343/μL (2.109), respectively (**Figure 28**). Median parasitemia of *P. vivax* was not significantly different in patients with *PKLR*^{R41Q} and without *PKLR*^{R41Q} (p=0.992).

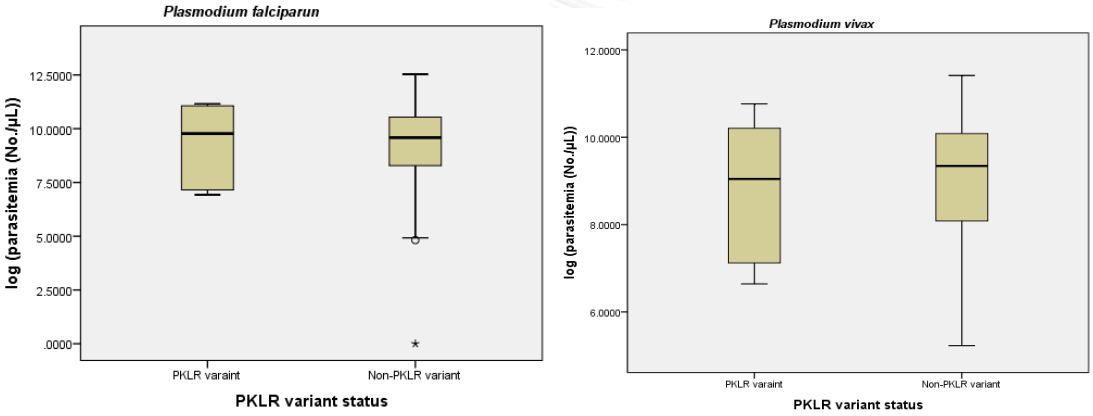

Figure 28 Effect of *PKLR*^{R41Q} on log parasitemia of *P. falciparum* (left) and *P. vivax* (right)

4.5.2 Number of Parasite Attack

PKLR^{R41Q} significantly increased the number of *Plasmodium* attack (p=0.001). Median of the number of *Plasmodium* attack in the patients with *PKLR*^{R41Q} was 4 time higher in patients with *PKLR*^{R41Q} than in patients without *PKLR*^{R41Q} (**Figure 29**).

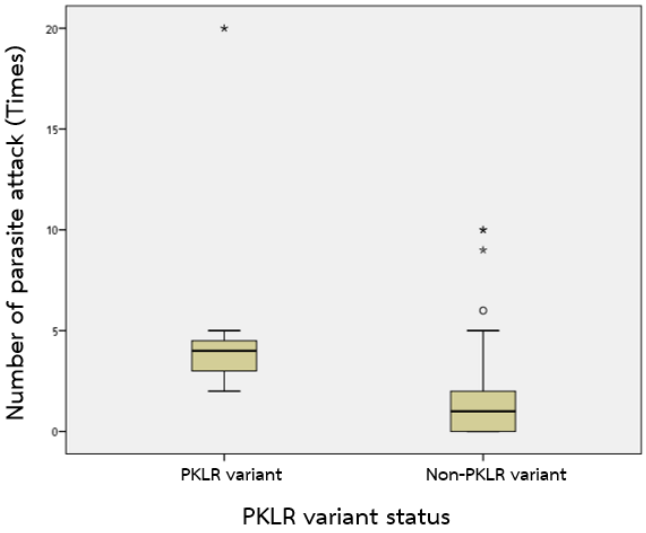

Figure 29 Effect of *PKLR*^{R41Q} on number of parasite attack

4.5.3 Hyperparasitemia

Hyperparasitemia of *P. falciparum* in *PKLR*^{R41Q} and non-*PKLR*^{R41Q} patients was tested by Odds ratio (**Table 11**). Odds ratio showed that the patients with *PKLR*^{R41Q} increased odds of hyperparasitemia (≥50,000/μL) of *P. falciparum* more than patients without *PKLR*^{R41Q} (OR: 2.18, 95% CI: 0.340 - 14.096). However, there was no significant difference between patients with *PKLR*^{R41Q} and without *PKLR*^{R41Q} (p= 0.410). In the patients with *P. vivax,* 4 of 6 patients with *PKLR*^{R41Q} had hyperparasitemia (≥20,000/μL). On the other hand, 20 of 62 malaria patients without *PKLR*^{R41Q} had hyperparasitemia (**Table 11**). The number of patients with *PKLR*^{R41Q}, whom had hyperparasitemia of *P. vivax* were higher than of patients without *PKLR*^{R41Q} (OR: 4.20, 95% CI: 0.709 - 24.880). However, there was no significant difference between patients with *PKLR*^{R41Q} and without *PKLR*^{R41Q} (p= 0.114).

Allele frequency of *PKLR*^{R41Q} in patients with hyperparasitemia (≥50,000/μL) of *P. falciparum* was 0.100 that was higher than in patients without hyperparasitemia (allele frequency = 0.051). Allele frequency of *PKLR*^{R41Q} in patients with hyperparasitemia (≥20,000/μL) of *P. vivax* was 0.104 that was higher than in patients without hyperparasitemia (allele frequency = 0.023).

Table 11 The association between *PKLR*^{R41Q} and severe parasitemia

Genotype	*P. falciparum*				*P. vivax*			
	Parasitemia ≥50,000/μL (%)	**Parasitemia <50,000/μL (%)**	**OR (95% CI)**	***p*-value**	**Parasitemia ≥20,000/μL (%)**	**Parasitemia <20,000/μL (%)**	**OR (95% CI)**	***p*-value**
R41Q	2 (33.3)	4 (66.7)	2.18	0.410	4 (66.7)	2 (33.3)	4.20	0.114
Non-R41Q	8 (18.6)	35 (81.4)	(0.340 - 14.096)		20 (32.3)	42 (67.7)	(0.709 - 24.880)	
Total	10 (20.4)	39 (79.6)			24 (35.3)	44 (64.7)		
Allele Freq. (G)	0.900	0.949			0.896	0.977		
Allele Freq. (A)	0.100	0.051			0.104	0.023		

***** The clinical data in some patients were lost to collect and analyze.

4.5.4 Reticulocyte Count

Reticulocytes are slightly immature red blood cells, which determine bone marrow activity. The high level of reticulocyte indicates the high rate of red blood cell synthesis in bone marrow, which occurs after heavy bleeding or certain types of hemolytic anemia. The higher reticulocyte count was found in the *falciparum* malaria patients without *PKLR*^{R41Q} more than in the patients with *PKLR*^{R41Q} (OR: 0.56, 95% CI: 0.058 - 5.395) (p=0.616) (**Table 12**). Odds ratio demonstrated that the *vivax* malaria patients with *PKLR*^{R41Q} increased odds of high reticulocyte count more than patients without *PKLR*^{R41Q} (OR: 2.90, 95% CI: (0.270 - 31.151) (p=0.379) (**Table 12**).

Table 12 The association between *PKLR*^{R41Q} and reticulocyte count

Genotype	*P. falciparum:* Reticulocyte Count				*P. vivax:* Reticulocyte Count			
	> 2 % (%)	≤ 2 % (%)	OR (95%CI)	*p*-value	> 2 % (%)	≤ 2 % (%)	OR (95%CI)	*p*-value
R41Q	1 (16.7)	5 (83.3)	0.56	0.616	1 (16.7)	5 (83.3)	2.90	0.379
Non-R41Q	10 (26.3)	28 (73.7)	(0.058 - 5.395)		4 (11.9)	58 (88.1)	(0.270 - 31.151)	
Total	11 (25.0)	33 (75.0)			5 (7.4)	63 (92.6)		

* The clinical data in some patients were lost to collect and analyze.

Mean of reticulocyte count in patients with *PKLR*^{R41Q} was 1.35% and in patients without *PKLR*^{R41Q} was 1%. *PKLR*^{R41} was inclined to increase reticulocyte count (*p*=0.700) (**Figure 30**).

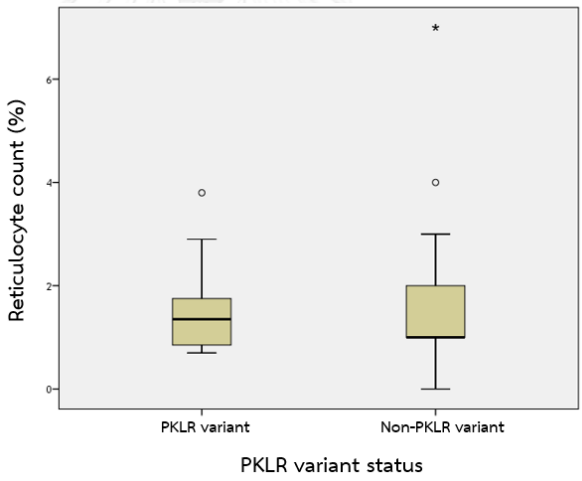

Figure 30 Effect of *PKLR*^{R41Q} on reticulocyte count (%)

4.5.5 Anemia

There was no significant difference in risk of anemia between the *falciparum* malaria patients with *PKLR*^{R41Q} and without *PKLR*^{R41Q} (*p*=0.743). We found 1 patient with *PKLR*^{R41Q} and 5 patients without *PKLR*^{R41Q} had hemoglobin (Hb) lower than 10 g/dL (**Table 13**). The patients with *PKLR*^{R41Q} might increase risk of anemia more than the patients without *PKLR*^{R41Q} (OR: 1.48, 95% CI: 0.142 - 15.386). We found none *vivax*

patients who carried *PKLR*^{R41Q} had Hb lower than 10 g/dL but we found 2 out 62 in patients without *PKLR*^{R41Q} (**Table 13**).

Table 13 The association between *PKLR*^{R41Q} variant and anemia

Genotype	*P. falciparum:* Anemia				*P. vivax:* Anemia			
	Hb≤10 g/dL (%)	Hb>10 g/dL (%)	OR (95%CI)	*p*-value	Hb≤10 g/dL (%)	Hb>10 g/dL (%)	OR (95%CI)	*p*-value
R41Q	1 (16.7)	5 (83.3)	1.48 (0.142 - 15.386)	0.743	0 (0.0)	6 (100)	-	-
Non-R41Q	5 (11.9)	37 (88.1)			2 (8.6)	60 (91.4)	-	
Total	6 (17.5)	42 (82.5)			2 (8.2)	66 (91.8)		

* The clinical data in some patients were lost to collect and analyze.

4.5.6 Hyperbilirubinemia

In the patients with *P. falciparum,* we found 3 of 42 *falciparum* malaria patients without *PKLR*^{R41Q} had total bilirubin more than 2.5 mg/dL but we not found it in patients with *PKLR*^{R41Q} (**Table 14**). None of *P. vivax* infected patients with *PKLR*^{R41Q} had total bilirubin more than 2.5 mg/dL. On the other hand, eight *P. vivax* infected patients without *PKLR*^{R41Q} had total bilirubin more than 2.5 mg/dL.

Table 14 The association between *PKLR*^{R41Q} variant and hyperbilirubinemia

Genotype	*P. falciparum:* Total bilirubin				*P. vivax:* Total bilirubin			
	≥2.5 mg/dL (%)	<2.5 mg/dL (%)	OR (95%CI)	*p*-value	≥2.5 mg/dL (%)	<2.5 mg/dL (%)	OR (95%CI)	*p*-value
R41Q	0 (0.0)	6 (100)	-	-	0 (0.0)	6 (100)	-	-
Non-R41Q	3 (7.1)	39 (92.9)	-		8 (13.1)	53 (86.9)	-	
Total	3 (6.3)	45 (93.7)			8 (13.1)	59 (96.9)		

* The clinical data in some patients were lost to collect and analyze.

4.5.7 Renal Insufficiency

In the patients with *P. falciparum,* we found 4 of 41 *P. falciparum* infected patients who not carried *PKLR*^{R41Q} had creatinine less than 1.5 mg/dL but we not found it in patients with *PKLR*^{R41Q} (**Table 15**). None of *P. vivax* infected patients with *PKLR*^{R41Q} had creatinine less than 1.5 mg/dL. Other the other hand, only one *P. vivax* infected patients without *PKLR*^{R41Q} had total creatinine less than 1.5 mg/dL.

Table 15 The association between *PKLR*^{R41Q} variant and renal insufficiency (creatinine)

Genotype	***P. falciparum:* Creatinine**				***P. vivax:* Creatinine**			
	≥1.5 mg/dL (%)	**<1.5 mg/dL (%)**	**OR (95%CI)**	***p*-value**	**≥1.5 mg/dL (%)**	**<1.5 mg/dL (%)**	**OR (95%CI)**	***p*-value**
R41Q	0 (0.0)	6 (100)	-	-	0 (0.0)	6 (100)	-	-
Non-R41Q	4 (5.2)	37 (94.8)	-		1 (0.7)	56 (99.3)	-	
Total	4 (4.9)	43 (95.1)			1 (1.4)	62 (98.6)		

***** The clinical data in some patients were lost to collect and analyze.

CHAPTER 5

DISCUSSION

Malaria, a life-threaten disease, is estimate more than 500 million patients and 660,000 deaths annually (67). According to J.S.H. Haldane, many hematological disorders were prominent in tropical regions where malaria was endemic. Therefore, it has been proposed that hematological disorders could reduce the mortality and severity of malaria (75). Erythrocyte is one of malaria's habitats, where *Plasmodium* parasite grows and develops. Evidence has suggested that malaria likely to have strong selective pressure on the hematological genes of human, leading to increase prevalence of inherited hematological disorders (76). Louicharoen et al. (2009) have proposed that *G6PD*$^{\text{Mahidol}}$ mutation has been positively selected for the last 1500 years to reduce *P. vivax* parasite density in humans (5). Although the homozygous hematological diseases such as G6PD deficiency, sickle cell anemia, and thalassemia get worse by their own clinical symptoms, the heterozygote could protect against the severity and mortality of malaria. Previous studies have showed that homozygosity at the *PKLR* protects against *chabaudi* malaria in mice by reducing parasitemia and mortality rate (24). Likewise, *in vitro* study of *P. falciparum* infected human erythrocytes, homozygosity for PK-deficiency which causes by G-to-A mutation at position 1269 could protect against parasite replication by 2 mechanisms: invasion defection of PKD erythrocytes and macrophage clearance preference of ring-stage-infected PKD erythrocytes (30).

The aims of this study were to identify the prevalence of *PKLR*$^{\text{R41Q}}$, which is a novel mutation, and to investigate the clinical correlation between *PKLR*$^{\text{R41Q}}$ and the severity of malaria in Southeast Asian volunteers, who stayed in areas of malaria-endemic regions. Results from previous study in these populations have demonstrate the effect of thalassemia (-$\alpha^{3.7}$, -$\alpha^{4.2}$, --$^{\text{SEA}}$, --$^{\text{FIL}}$, HbCS and HbE) on susceptibility/protection against *P. falciparum* or *P. vivax* infection (77-80).

In this study, all 267 malaria patients, admitted to Hospital for Tropical Diseases, Mahidol University since March, 2011 to November, 2012 (19 months), were enrolled. There were several ethnic groups consisted of Thai, Laotian, Mon, Burmese, Karen, and Cambodian. The major ethnicity was Burmese, which found 71.9% of this population, followed by Thai (13.5%), Karen (6.0%), Cambodian (3.4%), Mon (1.5%), and Laos (0.7%). We found male (94.4%) more than female (5.6%) in these malaria patients because most of them were immigrant labors workers in malaria endemic area, Tak province, Thailand. The average age of all subjects was 28.0±10.2 (range 14-60) years. However, the median age of male patients were significantly different from that of female patients (Mann-whiney U-Test, p=0.008). Median age of female patients (38 years) was higher than median age of male patients (25 years). There was no significant difference of age between ethnic groups. There are 143 malaria patients were excluded from this analyses because

1. The hemotological and general data of patients were not recorded.
2. The malaria patients were infected with co-infected with *P. falciparum* and *P. vivax,* which interfered the interpretation by cross immunity (81).
3. The malaria patients with G6PD deficiency or thalassemia were excluded. This is because evidence suggests that G6PD deficiency and thalassemia have an influence in protection against malaria (5, 11, 18).

According to these reasons, the total number of subjects used in the analyses of clinical correlation between *PKLR*^{R41Q} and malaria were reduced.

5.1 *PKLR*^{R41Q} Mutation

PK is composed of highly conserved N-terminal domain with a helix-turn-helix motif (45), followed by A domain and B domain containing binding residues of the phosphoenolpyruvate (PEP) substrate and the C-terminal domain that binds to the allosteric regulator fructose-1, 6-bisphosphate (FBP). Structural studies have shown that the enzyme activity is regulated by a combination of domain and rotation of subunit, which change the active site for alternation of conformation. The conformation of PK can switch from a low-affinity tight state (T-state) to a high-affinity

relaxed state (R-state) (**Figure 3**) (36). *PKLR*^{R41Q} mutation is located within the N-terminal domain of PK-R. Moreover, arginine at residue 41 is highly conserved in mammals. The enzyme activity is regulated by phosphorylation of serine at residue 43 that increases affinity for allosteric inhibitors (ATP and alanine) and decreases affinity for activators (PEP and FBP) (50, 51). *PKLR*^{R41Q} is the mutation near the allosteric residues; therefore this mutation may affect the structural and functional aspects of PK. Functional experiment of *PKLR*^{R41Q} mutation was not available because we used leftover DNAs from the previous studies. Therefore, recollection of fresh blood samples from these patients was impractical for phenotyping experiments.

Previous studies characterized the function of 2 mutations; *PKLR*^{A36G} and *PKLR*^{G37E}, which located in N-terminal domain and closed to *PKLR*^{R41Q} mutation. First, *PKLR*^{A36G} mutation causes a congenital nonspherocytic hemolytic anemia because this mutation reduces enzyme activity (52). Second, *PKLR*^{G37E} is gain-of function mutation by increasing the enzyme activity and ATP levels. It is possibly linked to alterative phosphorylation of serine 37 (53, 54). All of supporting evidence suggests that the N-terminal domain of PK, carried the *PKLR*^{R41Q} may affect the enzyme activity of PK.

5.2 The Prevalence of *PKLR*^{R41Q} Mutation

In 1997, the first case of PKD was reported in Thailand. It was a 10-year-old girl, who was initially diagnosed with Hb H disease. The patient had a history of neonatal jaundice, severe anemia, and mild splenomegaly, which required blood transfusion, phototherapy, and iron chelation (82). PKD was found 1.4% in an American population (58), 0.24% in a Spanish population (59), 3.4% in Hong Kong population (60), and 2.2% in Chinese infants (61).

Based on the results obtained from PCR-RFLP and direct sequencing analysis of novel N-terminal mutation (*PKLR*^{R41Q} mutation) in Southeast Asian, we found *PKLR*^{R41Q} mutation in 12 heterozygotes and 1 homozygote of all 267 patients. The hematological data in homozygous *PKLR*^{R41Q} was normal and not different from heterozygous *PKLR*^{R41Q}. However, the homozygous *PKLR*^{R41Q} patient had Hb more than 10 g/dl indicating as mild phenotype of PKD **(CHAPTER 2, Topic 2.7)** (29)**.** However, *PKLR*^{R41Q} patients had many mutations of hematological gene such as *G6PD*, **α**-globin

and β-globin, therefore the complete blood count (CBC) were not in normal reference. Analysis of Hardy-Weinberg equilibrium (HWE) illustrated that the allele and genotype frequencies of *PKLR*^{R41Q} did not deviate from theory. According to HWE, the allele frequency was balance and fit to the ideal state of Hardy-Weinberg theory; no mutation, random mating, no selection, no genetic drift, no migration, and population was not small. In each ethnicity, *PKLR*^{R41Q} mutation was found 11.1% (1/9) in Cambodians, 5.8% (11/192) Burmeses, and 5.6% (2/36) Thais. In this study, *PKLR*^{R41Q} was investigated in only malaria patients admitted to Hospital for Tropical Diseases but not in healthy controls or non-malaria patients. Comparison of the prevalence between healthy controls and malaria patients who stay in the same place was limited. Therefore, distinction of *PKLR*^{R41Q} allele frequency in each population might be influenced from ethnic groups and exposure to the outbreak of malaria in each region. The results revealed that *PKLR*^{R41Q} mutation was present in Southeast Asian population with an allele frequency approximately 0.026 (14/534).

5.3 The Association between *PKLR*^{R41Q} Mutation and Malaria Manifestations

The results showed that *P. falciparum* and *P. vivax* density in *PKLR*^{R41Q} patients was slightly lower than non-*PKLR*^{R41Q} patients, but there was no significant (**Figure 28**). The previous *in vitro* study showed that the *P. falciparum* density in heterozygous PKD blood was slightly lower than the control, but there was no statistically significant difference (**Figure 31**) (20). The present study suggests that *PKLR*^{R4Q} mutation does not protect against *P. falciparum* and *P. vivax* malaria proliferation.

Besides, this finding showed a significant association between *PKLR*^{R41Q} mutation and an increased number of *Plasmodium* attacks (**Figure 29**). Because there were no records of previous malaria infection in these patients, including species of parasite, reappearance of *P. vivax* infection might be relapse, the number of attacks (infections) in malaria patients might be higher than normal.

Moreover, the allele frequency of *PKLR*^{R41Q} in malaria patients with hyperparasitemia (*P. falciparum:* 0.100 and *P. vivax*: 0.104) was higher than in malaria patients with none hyperparasitemia (*P. falciparum:* 0.051 and *P. vivax:* 0.023). Although this finding was no significant difference, it implied that higher reticulocyte in

patients with *PKLR*^{R41Q} was proned to increase the hyperparasitemia in *Plasmodium spp.* This is because both of *P. vivax* and *P. falciparum* can infect reticulocyte.

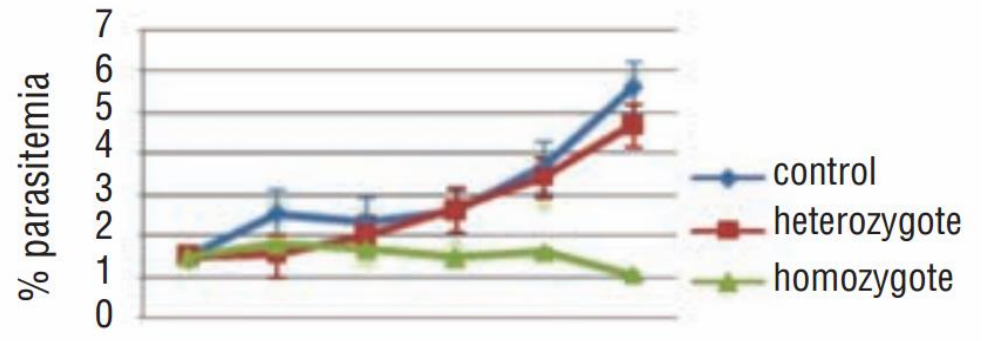

Figure 31 Parasite density in each genotype of PKD *in vitro* (20)

Odds ratio revealed that *PKLR*^{R41Q} tends to increase the odds of hyperparasitemia of both *P. falciparum* and *P. vivax* (*P. falciparum: p*=0.4102 and *P. vivax*: *p*=0.1139). The previous study reported that the average of hyperparasitemia of *P. falciparum* and *P. vivax* is approximately 50,000/μL and 20,000/μL, respectively (83). The number of parasites in hyperparasitemia of *P. falciparum* was higher than of *P. vivax* because *P. falciparum* is able to infect both of mature erythrocytes and reticulocytes while *P. vivax* specifically infects reticulocytes. In addition, high level of reticulocyte count was inclined in *vivax* patients with *PKLR*^{R41Q}. Due to ATP depletion and the high level of 2, 3-BPG of PKD erythrocytes which cause of hemolysis, reticulocytes are synthesized to compensate the damage red blood cells. Moreover, it may due to reticulocytes are indispensable for the growth and development of *P. vivax* (84), while *P. falciparum* could infect both of mature and immature red blood cells. Therefore the high level of *P. vivax* parasite density may increase with reticulocyte count.

The other complicated malaria such as anemia, hyperbilirubinemia and renal insufficiency were not associated with *PKLR*^{R41Q}. Although, one of *falciparum* patient with *PKLR*^{R41Q} had lower hemoglobin than the normal range but the patient had no symptoms of severe malaria.

However, there are several shortcomings of the present study. First, this analytical study with a relatively small sample size could affect the finding of *PKLR*^{R41Q} cases and analysis the effects of this mutation. Second, we could not measure the PK

enzyme activity because all blood samples were frozen making it impractical for the assay. Third, the clinical data of patients is incomplete; therefore affecting correlation analysis. Fourth, we have no data of patients before admission to the Hospital for Tropical Diseases. Further analysis regarding the function of *PKLR*^{R41Q} is required to prove the impact of *PKLR*^{R41Q} mutation in malaria patients.

Taken together, these findings suggest that *PKLR*^{R41Q} in Southeast Asian is a common enzymopathic mutation in malaria-endemic regions with allele frequency of 0.026. The allele frequency of *PKLR*^{R41Q} was higher in malaria patient with hyperparasitemia. The mutation showed a tendency to increase the number of *P. vivax* attacks and odds of hyperparasitemia.

REFERENCES

1. Walker NF, Nadjm, B., Whitty, C.J.M.. . Malaria. Medicine 2010;38:41-6.
2. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature. 2005;434(7030):214-7.
3. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria Diagnosis: A Brief Review. Korean J Parasitol. 2009;47(2):93-102.
4. Williams TN. Red blood cell defects and malaria. Molecular and biochemical parasitology. 2006;149(2):121-7.
5. Louicharoen C, Patin E, Paul R, Nuchprayoon I, Witoonpanich B, Peerapittayamongkol C, et al. Positively selected G6PD-Mahidol mutation reduces Plasmodium vivax density in Southeast Asians. Science. 2009;326(5959):1546-9.
6. Maier AG, Duraisingh MT, Reeder JC, Patel SS, Kazura JW, Zimmerman PA, et al. Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med. 2003;9(1):87-92.
7. Patel SS, King CL, Mgone CS, Kazura JW, Zimmerman PA. Glycophorin C (Gerbich antigen blood group) and band 3 polymorphisms in two malaria holoendemic regions of Papua New Guinea. Am J Hematol. 2004;75(1):1-5.
8. Patel SS, Mehlotra RK, King CL, Mgone CS, Kazura JW, Zimmerman PA. The association of the glycophorin C exon 3 deletion to ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea. Blood. 2001;98(11):3489–91.
9. Cortes A, Benet A, Cooke BM, Barnwell JW, Reeder JC. Ability of Plasmodium falciparum to invade Southeast Asian ovalocytes varies between parasite lines. Blood. 2004;104(9):2961-6.
10. Cortes A, Mellombo M, Mgone CS, Beck HP, Reeder JC, Cooke BM. Adhesion of Plasmodium falciparum-infected red blood cells to CD36 under flow is enhanced by the cerebral malaria-protective trait South-East Asian ovalocytosis. Mol Biochem Parasit. 2005;142(2):252-7.

11. Wambua S, Mwangi TW, Kortok M, Uyoga SM, Macharia AW, Mwacharo JK, et al. The effect of alpha(+)-thalassaemia on the incidence of malaria and other diseases in children living on the coast of Kenya. Plos Med. 2006;3(5):643-51.

12. Williams TN, Wambua S, Uyoga S, Macharia A, Mwacharo JK, Newton CRJC, et al. Both heterozygous and homozygous alpha(+) thalassemias protect against severe and fatal Plasmodium falciparum malaria on the coast of Kenya. Blood. 2005;106(1):368-71.

13. Cabrera G, Cot M, Migot-Nabias F, Kremsner PG, Deloron P, Luty AJF. The sickle cell trait is associated with enhanced immunoglobulin G antibody responses to Plasmodium falciparum variant surface antigens. J Infect Dis. 2005;191(10):1631-8.

14. Agarwal A, Guindo A, Cissoko Y, Taylor JG, Coulibaly D, Kone A, et al. Hemoglobin C associated with protection from severe malaria in the Dogon of Mali, a West African population with a low prevalence of hemoglobin S. Blood. 2000;96(7):2358-63.

15. Fairhurst RM, Baruch DI, Brittain NJ, Ostera GR, Wallach JS, Hoang HL, et al. Abnormal display of PfEMP-1 on erythrocytes carrying haemoglobin C may protect against malaria. Nature. 2005;435(7045):1117-21.

16. Fairhurst RM, Fujioka H, Hayton K, Collins KF, Wellems TE. Aberrant development of Plasmodium falciparum in hemoglobin CC red cells: implications for the malaria protective effect of the homozygous state. Blood. 2003;101(8):3309-15.

17. Modiano D, Luoni G, Sirima BS, Simpore J, Verra F, Konate A, et al. Haemoglobin C protects against clinical Plasmodium falciparum malaria. Nature. 2001;414(6861):305-8.

18. Chotivanich K, Udomsangpetch R, Pattanapanyasat K, Chierakul W, Simpson J, Looareesuwan S, et al. Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria. Blood. 2002;100(4):1172-6.

19. Cappadoro M, Giribaldi G, O'Brien E, Turrini F, Mannu F, Ulliers D, et al. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by plasmodium falciparum may explain malaria protection in G6PD deficiency. Blood. 1998;92(7):2527-34.

20. Durand PM, Coetzer TL. Pyruvate kinase deficiency protects against malaria in humans. Haematologica. 2008;93(6):939-40.

21. Howes RE, Dewi M, Piel FB, Monteiro WM, Battle KE, Messina JP, et al. Spatial distribution of G6PD deficiency variants across malaria-endemic regions. Malaria J. 2013;12: 418.

22. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malaria J. 2012;11.

23. Machado P, Manco L, Gomes C, Mendes C, Fernandes N, Salome G, et al. Pyruvate kinase deficiency in sub-Saharan Africa: identification of a highly frequent missense mutation (G829A;Glu277Lys) and association with malaria. PloS one. 2012;7(10):e47071.

24. Min-Oo G, Fortin A, Tam MF, Nantel A, Stevenson MM, Gros P. Pyruvate kinase deficiency in mice protects against malaria. Nature genetics. 2003;35(4):357-62.

25. Min-Oo G, Tam M, Stevenson MM, Gros P. Pyruvate kinase deficiency: correlation between enzyme activity, extent of hemolytic anemia and protection against malaria in independent mouse mutants. Blood cells, molecules & diseases. 2007;39(1):63-9.

26. Williams TN. Human red blood cell polymorphisms and malaria. Curr Opin Microbiol. 2006;9(4):388-94.

27. Zanella A, Fermo E, Bianchi P, Valentini G. Red cell pyruvate kinase deficiency: molecular and clinical aspects. British journal of haematology. 2005;130(1):11-25.

28. van Wijk R, van Solinge WW. The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis. Blood. 2005;106(13):4034-42.

29. Zanella A, Fermo E, Bianchi P, Chiarelli LR, Valentini G. Pyruvate kinase deficiency: the genotype-phenotype association. Blood reviews. 2007;21(4):217-31.

30. Ayi K, Min-Oo G, Serghides L, Crockett M, Kirby-Allen M, Quirt I, et al. Pyruvate kinase deficiency and malaria. New Engl J Med. 2008;358(17):1805-10.

31. Boyer PD, Lardy, H. A., Phillips, P.H. . The role of potassium in muscle phosphorylations. J Biol Chem. 1942;146:673-82.

32. Gupta RK, Oesterling, R. M., Mildvan, A. S. . Dual divalent cation requirement for activation of pyruvate kinase: essential roles of both enzyme- and nucleotide-bound metal ions. Biochemistry Biochemistry 1976;15:2881-7.

33. Pyruvate kinase [Internet]. 2014. Available from: http://proteopedia.org/wiki/index.php/Pyruvate_Kinase.

34. Jacobasch G. Biochemical and genetic basis of red cell enzyme deficiencies. Best Pract Res Cl Ha. 2000;13(1):1-20.

35. Zanella A, Bianchi P. Red cell pyruvate kinase deficiency: from genetics to clinical manifestations. Best Pract Res Cl Ha. 2000;13(1):57-81.

36. Morgan HP, Zhong W, McNae IW, Michels PAM, Fothergill-Gilmore LA, Walkinshaw MD. Structures of pyruvate kinases display evolutionarily divergent allosteric strategies. Royal Society Open Science. 2014;1(1):140120.

37. Munoz ME, Ponce E. Pyruvate kinase: current status of regulatory and functional properties. Comp Biochem Phys B. 2003;135(2):197-218.

38. Fothergillgilmore LA, Michels PAM. Evolution of Glycolysis. Prog Biophys Mol Bio. 1993;59(2):105-235.

39. Hall ERC, G. L. . Isozymes of pyruvate kinase in vertebrates: their physical, chemical, kinetic and immunological properties. Int J Biochem. 1978;9:785-93.

40. Noguchi T, Inoue H, Tanaka T. The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. J Biol Chem. 1986;261(29):13807-12.

41. Tani K, Yoshida MC, Satoh H, Mitamura K, Noguchi T, Tanaka T, et al. Human M2-type pyruvate kinase: cDNA cloning, chromosomal assignment and expression in hepatoma. Gene. 1988;73(2):509-16.

42. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J Biol Chem. 1987;262(29):14366-71.

43. Satoh HT, K.; Yoshida, M.C.; Sasaki, M.; Miwa, S.; Fujii, H. . The human liver-type pyruvate kinase (PKL) gene is on chromosome 1 at band q21. Cytogenetic and Cellular Genetic 1988;47:132-3.

44. van Wijk R, Huizinga EG, van Wesel AC, van Oirschot BA, Hadders MA, van Solinge WW. Fifteen novel mutations in PKLR associated with pyruvate kinase (PK) deficiency: structural implications of amino acid substitutions in PK. Human mutation. 2009;30(3):446-53.

45. Berghout J, Higgins S, Loucoubar C, Sakuntabhai A, Kain KC, Gros P. Genetic diversity in human erythrocyte pyruvate kinase. Genes and immunity. 2012;13(1):98-102.

46. Chaneton B, Gottlieb E. Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. Trends in biochemical sciences. 2012;37(8):309-16.

47. PKLR [Internet]. 2014. Available from: http://ghr.nlm.nih.gov/gene/PKLR.

48. Kugler W, Laspe P, Stahl M, Schroter W, Lakomek M. Identification of a novel promoter mutation in the human pyruvate kinase (PK) LR gene of a patient with severe haemolytic anaemia. Brit J Haematol. 1999;105(3):596-8.

49. van Solinge WW, Kraaijenhagen RJ, Rijksen G, van Wijk R, Stoffer BB, Gajhede M, et al. Molecular modelling of human red blood cell pyruvate kinase: structural implications of a novel G1091 to a mutation causing severe nonspherocytic hemolytic anemia. Blood. 1997;90(12):4987-95.

50. Kahn A, Marie J. Pyruvate kinases from human erythrocytes and liver. Methods in enzymology. 1982;90 Pt E:131-40.

51. Marie J, Buc H, Simon MP, Kahn A. Phosphorylation of human erythrocyte pyruvate kinase by soluble cyclic-AMP-dependent protein kinases. Comparison with human liver L-type enzyme. European journal of biochemistry / FEBS. 1980;108(1):251-60.

52. Fermo E, Bianchi P, Chiarelli LR, Cotton F, Vercellati C, Writzl K, et al. Red cell pyruvate kinase deficiency: 17 new mutations of the PK-LR gene. British journal of haematology. 2005;129(6):839-46.

53. Beutler E, Westwood B, van Zwieten R, Roos D. G-->T transition at cDNA nt 110 (K37Q) in the PKLR (pyruvate kinase) gene is the molecular basis of a case of hereditary increase of red blood cell ATP. Human mutation. 1997;9(3):282-5.

54. Zurcher C, Loos JA, Prins HK. Hereditary high ATP content of human erythrocytes. Folia haematologica. 1965;83(4):366-76.

55. Meza NW, Quintana-Bustamante O, Puyet A, Rio P, Navarro S, Diez A, et al. In Vitro and in vivo expression of human erythrocyte pyruvate kinase in erythroid cells: A gene therapy approach. Hum Gene Ther. 2007;18(6):502-14.

56. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. Blood. 2000;95(11):3585-8.

57. Carey PJ, Chandler J, Hendrick A, Reid MM, Saunders PW, Tinegate H, et al. Prevalence of pyruvate kinase deficiency in northern European population in the north of England. Northern Region Haematologists Group. Blood. 2000;96(12):4005-6.

58. Tanaka KR, Paglia DE. Pyruvate kinase deficiency. Seminars in hematology. 1971;8(4):367-96.

59. Garcia SC, Moragon AC, Lopez-Fernandez ME. Frequency of glutathione reductase, pyruvate kinase and glucose-6-phosphate dehydrogenase deficiency in a Spanish population. Human heredity. 1979;29(5):310-3.

60. Fung RH, Keung YK, Chung GS. Screening of pyruvate kinase deficiency and G6PD deficiency in Chinese newborn in Hong Kong. Archives of disease in childhood. 1969;44(235):373-6.

61. Wu ZL, Yu WD, Chen SC. Frequency of erythrocyte pyruvate kinase deficiency in Chinese infants. American journal of hematology. 1985;20(2):139-44.

62. Pyruvate kinase deficiency [Internet]. 2015. Available from: http://en.wikipedia.org/wiki/Pyruvate_kinase_deficiency.

63. Wei DC, Chan LC, Li A. Homozygous pyruvate kinase deficiency in Hong Kong ethnic minorities. Journal of paediatrics and child health. 1992;28(4):334-6.

64. Beutler E. Red cell metabolism "A manual of biochemical methods". 2 ed. New York: Grune & Straton; 1975.

65. Beutler E. A series of new screening procedures for pyruvate kinase deficiency, glucose-6-phosphate dehydrogenase deficiency, and glutathione reductase deficiency. Blood. 1966;28(4):553-62.

66. Steady State ATPase Assays Coupled Enzyme System [Internet]. 2002 Available from: http://labs.cellbio.duke.edu/kinesin/Methods/ATPase_assay.html.

67. Organization WH. Management of severe malaria: a practical handbook. 3 ed: WHO Press; 2012.

68. malaria [Internet]. 2010. Available from: http://www.cdc.gov/dpdx/malaria/

69. Map of malaria distribution [Internet]. 2015. Available from: http://www.biology.as.nyu.edu/object/JaneCarlton.html.

70. Betke KB, E.; Brewer, G.; Kirkman, H.; Luzzatto, L.; Motulsky, A.; et al. Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. . World World Health Organization Technical Report Series. 1967;366:3818-38.

71. Sambrook JF, EF.; Maniatis, T. Molecular cloning. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.

72. Louicharoen C. A role for G6PD Mahidol in Protection against malaria in Southeast Asia. Bangkok: Chulalongkorn University; 2008.

73. Para S. Prevalence of alpha-thalassemia,HbE and their clinical correlation in malaria patients in Southeast Asia. Bangkok: Chulalongkorn University; 2013.

74. Makonkawkeyoon L, Sanguansermsri T, Asato T, Nakashima Y, Takei H. Rapid detection of chain termination mutations in the alpha 2 globin gene. Blood. 1993;82(11):3503-4.

75. Haldane JB. Disease and evolution. La Ricerca Sci. 1949;19:68-76.

76. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet. 2005;77:171-92.

77. Rosanas-Urgell A, Senn N, Rarau P, Aponte JJ, Reeder JC, Siba PM, et al. Lack of associations of alpha(+)-thalassemia with the risk of Plasmodium falciparum and Plasmodium vivax infection and disease in a cohort of children aged 3-21 months from Papua New Guinea. International journal for parasitology. 2012;42(12):1107-13.

78. Lopera-Mesa TM, Doumbia S, Konate D, Anderson JM, Doumbouya M, Keita AS, et al. Impact of red blood cell variants on childhood malaria in Mali: a prospective cohort study. The Lancet Haematology. 2015;2(4):e140-e9.

79. Opoku-Okrah C, Gordge M, Kweku Nakua E, Agbenyega T, Parry M, Robertson C, et al. An investigation of the protective effect of alpha+-thalassaemia against severe Plasmodium falciparum amongst children in Kumasi, Ghana. International journal of laboratory hematology. 2013.

80. Mockenhaupt FP, May J, Bergqvist Y, Meyer CG, Falusi AG, Bienzle U. Evidence for a reduced effect of chloroquine against Plasmodium falciparum in alpha-thalassaemic children. Tropical medicine & international health : TM & IH. 2001;6(2):102-7.

81. O'Donnell A, Premawardhena A, Arambepola M, Samaranayake R, Allen SJ, Peto TE, et al. Interaction of malaria with a common form of severe thalassemia in an Asian population. Proc Natl Acad Sci U S A. 2009;106(44):18716-21.

82. Tanphaichitr VS, Suvatte V, Mahasandana C, Veerakul G, Pung-amritt P, Tachavanich K, et al. Pyruvate kinase deficiency in an alpha-thalassemia family: first case report in Thailand. The Southeast Asian journal of tropical medicine and public health. 1997;28 Suppl 3:64-8.

83. John DP, W. Markell and Voge's Medical Parasitology. 9th ed: Saunders Elsevier; 2006. 480 p.

84. Moreno-Perez DA, Ruiz JA, Patarroyo MA. Reticulocytes: Plasmodium vivax target cells. Biology of the cell / under the auspices of the European Cell Biology Organization. 2013;105(6):251-60.

APPENDIX A

COA No. 721/2014

IRB No. 432/57

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University

1873 Rama 4 Road, Patumwan, Bangkok 10330, Thailand, Tel 662-256-4455 ext 14, 15

Certificate of Approval

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the following study which is to be carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title : Prevalence of N-Terminal Mutation of Pyruvate Kinase among Vulnerable Malaria-Risk Population in Southeast Asia.

Study Code : -

Principal Investigator : Miss Punchalee Mungkalasut

Affiliation of PI : Department of Biochemistry,
Faculty of Medicine, Chulalongkorn University.

Review Method : Expedited

Continuing Report : At least once annually or submit the final report if finished.

Document Reviewed :

1. Protocol version 2.0 Date 8 October 2014
2. Protocol Synopsis version 2.0 Date 8 October 2014
3. Questionnaire Version 2.0 Date 8 October 2014
4. Information sheet for research participant version 2.0 Date 8 October 2014
5. Informed Consent Form Version 2.0 Date 8 October 2014
6. Informed Consent Form (If the consent can not be read) version 2.0 Date 8 October 2014

7. Principal investigator's CV Version 2.0 Date 8 October 2014

Signature:..... Signature:.....

(Emeritus Professor Tada Sueblinvong MD) (Associate Professor Supeecha Wittayalertpanya)

Chairperson Member and Assistant Secretary, Acting

The Institutional Review Board Secretary The Institutional Review Board

Date of Approval : October 21, 2014

Approval Expire Date : October 20, 2015

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;
2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for the record;
3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;
4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;
5. If the study cannot be finished within the expire date of the approval certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.
6. If the research project is completed, the researcher must be form the Faculty of Medicine, Chulalongkorn University.

* A list of the Institutional Review Board members (names and positions) present at the meeting of Institutional Review Board on the date of approval of this study has been attached. All approved documents will be forwarded to the principal investigator.

COA No. 721/2014

IRB No. 432/57

คณะกรรมการจริยธรรมการวิจัยในคน

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

1873 ถ.พระราม 4 เขตปทุมวัน กรุงเทพฯ 10330 โทร. 0-2256-4455 ต่อ 14, 15

เอกสารรับรองโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในคน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ : ความชุกของการกลายพันธุ์ด้าปลายเอ็นของเอนไซมไพรูเวตไคเนสในกลุ่มเสี่ยงต่อการติดเชื้อมาลาเรียในเอเชียตะวันออกเฉียงใต้

เลขที่โครงการวิจัย : -

ผู้วิจัยหลัก : นางสาวปัญญช์ลี มังคลสุต

สังกัดหน่วยงาน : ภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

วิธีทบทวน : แบบเร่งด่วน

รายงานความก้าวหน้า : ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการเสร็จสิ้นก่อน 1 ปี

เอกสารที่ได้รับการทบทวน :

1. โครงร่างการวิจัย version 2.0 Date 8 October 2014
2. โครงการวิจัยฉบับย่อ version 2.0 Date 8 October 2014
3. แบบสอบถาม Version 2.0 Date 8 October 2014
4. เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย version 2.0 Date 8 October 2014
5. เอกสารแสดงความยินยอมเข้าร่วมในโครงการวิจัย Version 2.0 Date 8 October 2014
6. เอกสารแสดงความยินยอมเข้าร่วมในโครงการวิจัย (กรณีผู้ให้ความยินยอมไม่สามารถอ่านหนังสือได้) version 2.0 Date 8 October 2014

7. ประวัติผู้วิจัยหลัก Version 2.0 Date 8 October 2014

ลงนาม .....

(ศาสตราจารย์กิตติคุณแพทย์หญิงธาดา สืบหลินวงศ์)
ประธาน
คณะกรรมการจริยธรรมการวิจัยในคน

ลงนาม.....

(รองศาสตราจารย์สุพีชา วิทยเลิศปัญญา)
กรรมการและผู้ช่วยเลขานุการปฏิบัติหน้าที่แทนเลขานุการ
คณะกรรมการจริยธรรมการวิจัยในคน

วันที่รับรอง : 21 ตุลาคม 2557

วันหมดอายุ : 20 ตุลาคม 2558

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

นักวิจัยทุกท่านที่ผ่านการรับรองจริยธรรมการวิจัยต้องปฏิบัติดังต่อไปนี้

1. ดำเนินการวิจัยตามที่ระบุไว้ในโครงร่างการวิจัยอย่างเคร่งครัด
2. ใช้เอกสารแนะนำอาสาสมัคร ใบยินยอม (และเอกสารเชิญเข้าร่วมวิจัยหรือใบโฆษณาถ้ามี) แบบสัมภาษณ์ และหรือ แบบสอบถาม เฉพาะที่มีตราประทับของคณะกรรมการพิจารณาจริยธรรมเท่านั้น และส่งสำเนาเอกสารดังกล่าวที่ใช้กับผู้เข้าร่วมวิจัยจริงรายแรกมาที่ฝ่ายวิจัย คณะแพทยศาสตร์ เพื่อเก็บไว้เป็นหลักฐาน
3. รายงานเหตุการณ์ไม่พึงประสงค์ร้ายแรงที่เกิดขึ้นหรือการเปลี่ยนแปลงกิจกรรมวิจัยใดๆ ต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ภายใน 5 วันทำการ
4. ส่งรายงานความก้าวหน้าต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ตามเวลาที่กำหนดหรือเมื่อได้รับการร้องขอ
5. หากการวิจัยไม่สามารถดำเนินการเสร็จสิ้นภายในกำหนด ผู้วิจัยต้องยื่นขออนุมัติใหม่ก่อนอย่างน้อย 1 เดือน
6. หากการวิจัยเสร็จสมบูรณ์ผู้วิจัยต้องแจ้งปิดโครงการตามแบบฟอร์มของคณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

* รายชื่อของคณะกรรมการจริยธรรมการวิจัยในคน (ชื่อและตำแหน่ง) ที่อยู่ในที่ประชุมวันที่รับรองโครงการวิจัยได้แนบมาด้วย เอกสารที่รับรองทั้งหมดจะถูกส่งไปยังผู้วิจัยหลัก

APPENDIX B

Equipments and reagents for research

Equipments

1. Refrigerated centrifuge (Hettich, Eppendorf, MSE, Heraeus)
2. Hemocue® Hemoglobin Photometer
3. Spectrophotometer Model UV-1800 (Shimadzu)
4. Nano drop 1000 Spectrophotometer (Thermo Scientific)
5. Veriti® 96-well thermal cycler (Applied Biosystems)
6. Water bath YCW-010 (GEMMYCO)
7. Heat block (Major science, Biosan)
8. Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).
9. Horizontal electrophoresis (MY-RUN, BIO-RAD)
10. Vertical electrophoresis (BIO-RAD)
11. 2 decimal balance (DENVER INTRUMENT, Precisa)
12. 4 decimal balance (OHAUS, Precisa)
13. Hot Plate Stirrer (IKA)
14. pH meter (ESDO, Schott)
15. Vertical Autoclave (Hirayama)
16. Hot Air Oven (Memmert)
17. 0.2 ml μltra Amp PCR products PDR tubes (Sorenson, Bioscience)
18. 0.65 ml, 1.7 ml, 2.0 ml Safesealmicrocentrifuge tube (Sorenson, Bioscience)
19. 15 ml and 50 ml Centrifuge tube (CORNING)
20. 3 ml Pasteur pipette 10 ml
21. Safety Cap Plain Tube 4 ml (VACUETTE)
22. Disposable Syringe 10 ml
23. Gel/PCR DNA Fragments Extraction Kit Cat No.YDF300 Lot.No.PG-913-09362 (RBC Bioscience).

Reagents

1. 0.9% Normal saline solution (Klean & Kare)
2. Tris-Hydroxymethyl (Fisher Scientific, J. T. BAKER),
3. Hydrocholic (J.T. BAKER)
4. Magnesium choridehexahydrate (BIO BASIC INC)
5. Triton X-100 (SIGMA)
6. Sodium chloride (UNIVAR),
7. Sucrose Molecular Grade (USB)
8. Sodium hydroxide (MERCK)
9. Ethylenediaminetetraacetic acid disodium salt (Plusone®Amersham Biosciences)
10. Sodium dodecyl sulphate (GE Healthcare)
11. Proteinase K (Amresco)
12. Saturated phenol (Amresco)
13. Chloroform (RCL labscan)
14. Isoamylalcohol (Amresco)
15. Isopropanol (MERCK)
16. Ethanol (MERCK)
17. PCR buffer (Invitrogen, RBC Bioscience)
18. Taq DNA Polymerase (Invitrogen, RBC Bioscience)
19. Platinum Taq DNA Polymerase (Invitrogen)
20. 10 mM dNTP mix PCR Grade (Invitrogen, RBC Bioscience)
21. 50 mM $MgCl_2$ (Invitrogen, RBC Bioscience)
22. 1 M Betaine (Sigma)
23. Primer (Bio Basic Inc.) (Biolabs)
24. Restriction enzyme (Fermentas) (*Hin*dIII, *Xba*I, *Hha*I, *Afl*II, *Nde*I, *BstE*II, *Pst*I, *Mbo*II, *Tru1*I, *Mnl*I, *Aci*I)
25. Reaction buffer (Fermentas) (Buffer-R, Buffer-Tango, Buffer-O, Buffer-B)
26. 29% Acrylamide + 1% N, N' methylenebisacrylamide (Bio-RAD)
27. (10X) TBE buffer (Bio-RAD)
28. 10% Ammonium persulfate (APS) (Pharmacia Biotech)

29. TEMED (Bio-RAD)
30. Agarose (Thermo scientific)
31. Novel juice (Genedirex)
32. 1 kb Plus DNA ladder (invitrogen)
33. 100 bp Sharp DNA marker (RBC Bioscience)

VITA

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Education 2009 – 2012 Bachelor's degree in Genetics, Faculty of Science, Chulalongkorn University, Bangkok, Thailand