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Original Article

An Observational Study of the Effect of Hemoglobinopathy, Alpha Thalassemia and Hemoglobin E on *P. Vivax* Parasitemia

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Abstract. ***Background:*** **The protective effect of α-thalassemia, a common hematological disorder in Southeast Asia, against *Plasmodium falciparum* malaria has been well established. However, there is much less understanding of the effect of α-thalassemia against *P. vivax*. Here, we aimed to investigate the proportion of α-thalassemia including the impact of α-thalassemia and HbE on the parasitemia of *P. vivax* in Southeast Asian malaria patients in Thailand.**

Methods: **A total of 210 malaria patients, admitted to the Hospital for Tropical Diseases, Thailand during 2011-2012, consisting of 159 Myanmeses, 13 Karens, 26 Thais, 3 Mons, 3 Laotians, and 6 Cambodians were recruited. *Plasmodium spp.* and parasite densities were determined. Group of deletion mutation (--^{SEA}, -α$^{3.7}$, -α$^{4.2}$deletion) and substitution mutation (HbCS and HbE) were genotyped using multiplex gap-PCR and PCR-RFLP, respectively.**

Results: **In our malaria patients, 17/210 homozygous and 74/210 heterozygous -α$^{3.7}$ deletion were found. Only 3/210 heterozygous -α$^{4.2}$ and 2/210 heterozygous--^{SEA} deletion were detected. HbE is frequently found with 6/210 homozygotes and 35/210 heterozygotes. The most common thalassemia allele frequencies in Myanmar population were -α$^{3.7}$ deletion (0.282), followed by HbE (0.101), HbCS (0.013), -α$^{4.2}$ deletion (0.009), and --^{SEA} deletion (0.003). Only density of *P. vivax* in α-thalassemia trait patients (-α$^{3.7}$/-α$^{3.7}$, --^{SEA}/αα, -α$^{3.7}$/-α$^{4.2}$) but not in silent α-thalassemia (-α$^{3.7}$/αα, -α$^{4.2}$/αα, αα^{CS}/αα) were significantly higher compared with non-α-thalassemia patients (*p*=0.027). HbE did not affect *P. vivax* parasitemia. The density of *P. falciparum* significantly increased in heterozygous HbE patients (*p*=0.046).**

Conclusions: **Alpha-thalassemia trait is associated with high levels of *P. vivax* parasitemia in malaria patients in Southeast Asia.**

Keywords: Silent alpha-thalassemia, Alpha-thalassemia trait, HbE, Malaria, Southeast Asian.

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Introduction. Malaria is the most prevalent parasitic disease worldwide where 214 million patients suffer due to *Plasmodium vivax* and *Plasmodium falciparum* infection, and more than 400,000 people die annually.[1] Both *P. vivax* and *P. falciparum* have been the main causes of malaria on the Thailand-Myanmar border for many years. In Thai villagers, *P. vivax* infection has recently become the largest proportion of cases in patients.[2] The result of selective malaria pressure on recent human genome evolution is presented in the form of high frequencies of genetic disorders of hemoglobin including thalassemias and hemoglobinopathies in populations living in historically malarious regions.[3-6] Such malaria-protective properties have since been demonstrated in glucose 6-phosphate dehydrogenase (G6PD) deficiency,[7] α-thalassemia,[4,8-9] hemoglobin C,[10-11] hemoglobin S[12] and hemoglobin E.[13] The protective effect of thalassemia against *P. falciparum* malaria has been well established.[14] However, the impact of thalassemia on *P. vivax* is not well understood yet. Alpha-thalassemia is caused by the deletion of a number of α-globin genes resulting in an imbalance of α- and β- globin. There are several types of α-thalassemia; silent α-thalassemia, α-thalassemia trait and HbH, which depleted one, two, and three copy of α-globin genes, respectively. The -$\alpha^{3.7}$ and-$\alpha^{4.2}$ deletions are most common forms of silent α-thalassemia in Southeast Asians.[15-16] Clinical symptoms of α-thalassemia traits are mild anemia with hypochromic erythrocytes, whereas heterozygotes are asymptomatic.[17] The meta-analysis demonstrated the protective effect of silent α-thalassemia against *P. falciparum*.[14] A case-control study in Africa and Papua New Guinea (PNG) found that silent α-thalassemia protects against *P. falciparum*.[8-9,18-21] Alpha-thalassemia trait --^{SEA} deletion was commonly found in Thailand and Southeast Asia (SEA).[15-16] The --^{SEA} allele has been identified as the recent balancing selected allele triggered by malaria.[22] However, several studies failed to detect the association of α-thalassemia traits and parasitemia of *P. vivax*.[23] Hemoglobin E (HbE) is the most common β-hemoglobinopathies in Southeast Asia. Several studies have found that HbE confers protection against *P. falciparum*.[13] However, HbE has been found to be more prone to *P. vivax*.[24] This study aimed to investigate the proportion of α-thalassemia and HbE and to clarify the effect of α-globin gene numbers and HbE genotype on the parasitemia in Southeast Asian malaria patients in Thailand.

Materials and Methods.

Study subjects and sample collection. The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (Bangkok, Thailand) (COA No. 040/2013 IRB No. 459/55). Malaria patients in this cohort study were referred from many malaria-endemic provinces including borders of Thailand: Tak (Maesod District), Kanchanaburi (Sangkhlaburi District), Phetchaburi (Kaeng Krachan District), Suphanburi (Dan Chang District), Ranong, Sisaket (Kantharalak District) and Chonburi (**Figure 1**). Before enrolment in the study, all patients gave written informed consent. Patients who were slide-positive for *Plasmodium* malaria with no history of antimalarial drug treatment within the preceding 2 weeks, and were admitted to the Hospital for Tropical Diseases in Thailand during 2011-2012, were recruited. G6PD deficiency, an enzymopathy involved in protecting against malaria, which may interfere with interpretation of the effect of α-thalassemia and HbE, was excluded.

Identify Plasmodium spp. infection and parasite density. All blood samples from finger pricks were Giemsa stained for thick and thin blood films. Blood smears were tested every 12 hours from initiation of treatment until they were negative on two consecutive occasions; after that, blood smears were daily tested until patients were discharged. Parasite densities (asexual parasite/microliter of blood) were examined by counting the number per 200 leukocytes (thick film) or per 1,000 erythrocytes (thin film). In interpretation the *Plasmodium spp.*, blood smear films were read under microscope by an independent parasitologist at the Hospital for Tropical Diseases. The species was confirmed by polymerase chain reaction (PCR)-based analysis.

Measurement of G6PD activity. G6PD activity assays were performed prior to treatment and weekly repeated until patients were discharged. Quantitative test for G6PD activity was performed using G6PD kit assay (Trinity Biotech, Bray, County Wicklow, Ireland), which measured

NADPH production at wavelength of 340 nm. All samples were run parallel with positive and negative control. Hemoglobin for calculation of G6PD activity was measured using Hb201 (HemoCue, Sweden). G6PD activity <1.5 IU/g Hb classified as G6PD deficient[25] was excluded from the study. Leftover blood samples were kept at -20°C for molecular typing.

Detection of α-thalassemia. Genomic DNA was extracted from peripheral blood using phenol-chloroform method.[26] Alpha-globin gene variants including α-thalassemia trait (--^{SEA} deletion) and silent α-thalassemia (-$\alpha^{3.7}$, -$\alpha^{4.2}$ deletion) were investigated by multiplex gap-polymerase chain reaction (multiplex gap-PCR).[27] HbCS and HbE were genotyped using PCR-restriction fragment length polymorphism (PCR-RFLP).[28-29]

Statistical analysis. All statistical analyses were performed using the SPSS version 22.0. The main outcomes of interest were parasite densities of *P. falciparum* and *P. vivax* malaria before treatment. Parasite density that was not normally distributed was log-transformed prior to analysis. Parasitemia of α-thalassemia and HbE patients were compared with that of non-thalassemia (HbA) using unpaired T-test. In all statistical analyses, significance levels were set at the 95% confidence interval (CI) (P<0.05).

Results.

Characteristics of the study population. A total of 210 patients (201 males and 9 females) including 159 Myanmeses, 13 Karens, 26 Thais, 3 Mons, 3 Laotians, and 6 Cambodians were recruited for the study. Patients were from Myanmar (N= 159), Tak (Maesod district, N= 127), Kanchanaburi (Sangkhlaburi district, N= 9), Ranong (N= 1), Thailand-Myanmar border (N= 15), Thailand-Cambodia border (N= 1, **Figure 1**) and missing data (N= 6). The average age of all subjects was 28.0±10.0 (range 14-60) years. Eighty-five had *P. falciparum*, while 122 had *P. vivax* infection, two had mixed infection of *P. falciparum*, and *P. vivax* and one had *P. malariae*.

In this study, 17 homozygous and 74 heterozygous -$\alpha^{3.7}$ deletion were found among 210 patients, while only three heterozygous -$\alpha^{4.2}$ and two heterozygous --^{SEA} deletion were detected.

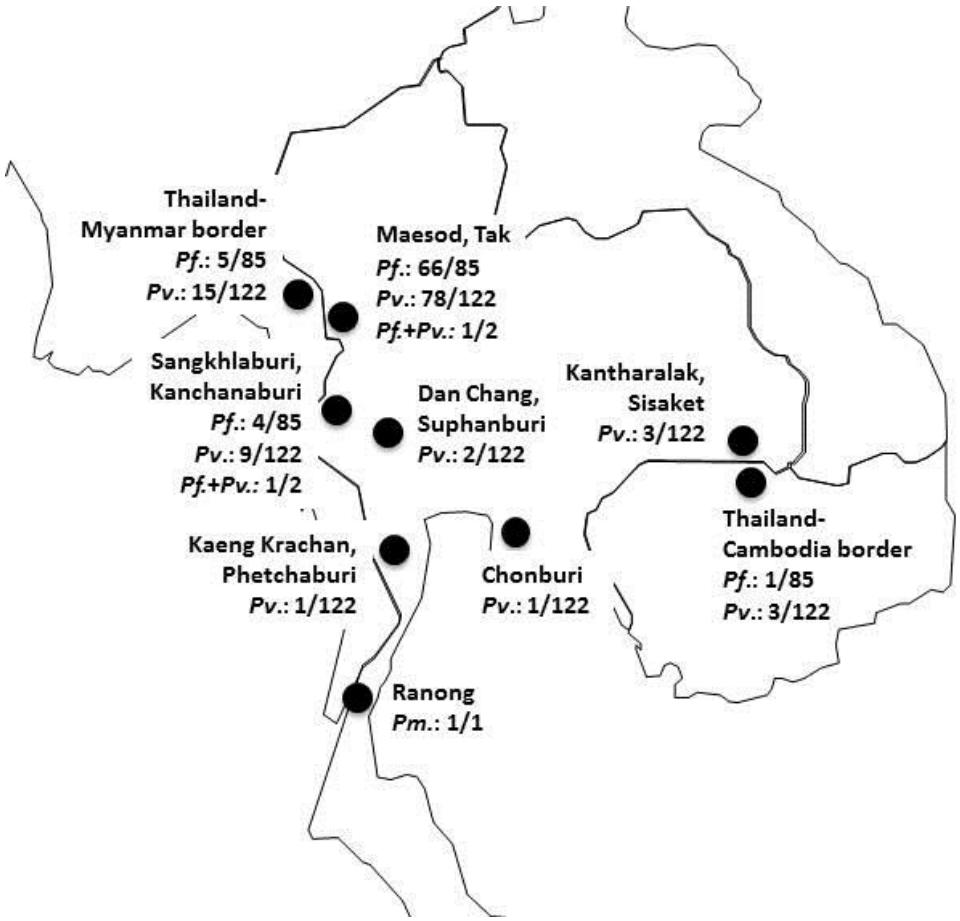

Figure 1 Distribution of malaria patients cohort along Thailand and borders during 2011-2012 (Missing geographic data in 19 cases) (*Pf., Pv., Pm.*, and *Pf.+Pv.* represent *P. falciparum*, *P. vivax*, *P. malariae*, and mixed infection of *P. falciparum* and *P. vivax*, respectively.)

HbE was also highly prevalent, with six homozygotes and 35 heterozygotes. For HbCS, five heterozygous were detected (**Table 1**). The Myanmese was the major ethnic group in this study accounting for 75% of all patients. Among these, the proportion of α- thalassemia was 48.4% (77/159), including 45.9% (73/159) of $-\alpha^{3.7}$ deletion, 1.8% (3/159) $-\alpha^{4.2}$ deletion, 0.6% (1/159) $--^{SEA}$ deletion, and 2.5% (4/159) $\alpha\alpha^{CS}$ whereas HbE was 20.8% (29/159). Allele frequencies were calculated for the major population. The most common was $-\alpha^{3.7}$ deletion (0.282), followed by HbE (0.101), HbCS (0.013), $-\alpha^{4.2}$ deletion (0.009), and $--^{SEA}$ deletion (0.003) (**Table 1**). Thalassemia and hemoglobinopathies were not found in 3 patients with *P. malariae* and mixed infection patients.

Association of α-globin gene dosage, HbE, and parasitemia. To assess the effect of α-globin gene presence and HbE genotype on the parasitemia of *P. vivax* and *P. falciparum*, the number of parasites in the blood of α-thalassemia and HbE genotypes were compared with that of non-thalassemia (HbA). The results found that *P. vivax* density in patients with α-thalassemia trait ($-\alpha^{3.7}/-\alpha^{3.7}$, $--^{SEA}/\alpha\alpha$, $-\alpha^{3.7}/-\alpha^{4.2}$) was 4.21±0.32 $\log_{10}$ value/μl, which was significantly higher than HbA patients (3.89±0.71 $\log_{10}$ value/μl) (p=0.027) (**Table 2**). Whereas, *P. vivax* parasitemia was not significantly different in patients who depleted only one α-globin gene or had silent α-thalassemia ($-\alpha^{3.7}/\alpha\alpha$, $-\alpha^{4.2}/\alpha\alpha$, $\alpha\alpha^{CS}/\alpha\alpha$) (3.94±0.66 $\log_{10}$ value/μl) (p=0.707) (**Table 2**). Nevertheless, HbH patient ($-\alpha^{3.7}/--^{SEA}$) had low level of *P. vivax* parasitemia compared with HbA (2.08 $\log_{10}$ value/μl). However, there was no significant effect of the number of alpha globin gene deletions on *P. falciparum* parasitemia.

However, significant increases of *P. falciparum* density in heterozygous HbE patients was detected (4.45±0.66 $\log_{10}$ value/μl) (p=0.046) (**Table 2**). On the other hand, *P. falciparum* parasitemia was reduced in homozygous HbE patient (3.00 $\log_{10}$ value/μl) (**Table 2**). Nevertheless, this study could not find the effect of HbE on *P. vivax* parasitemia.

Discussion. Our study is an association study between α-thalassemia and *P. vivax* density in Southeast Asia. The proportion of *P. vivax* infection in this study was higher than *P. falciparum* infection with a ratio of 1.4:1, which corresponds to the WHO World Malaria Report in 2015[1] which reported that *P. vivax* (54%) was detected more frequently than *P. falciparum* (38%) in Thailand. The distribution of *P. vivax* in Thailand is predominantly along the western region; Tak Province or the Thailand-Myanmar border (**Figure 1**), which had the highest malaria incidence.[30] Since all patients in the study, who were referred to the Hospital for Tropical Diseases after malaria infection, were immigrant laborers,

Table 1. Proportion of thalassemia and hemoglobinopathies in malaria patients, divided by *Plasmodium spp.* infection and ethnicity.

Mutation	Genotype	*Plasmodium spp.*(N)		Ethnic group(N)						Total number
		Plasmodium falciparum	*Plasmodium vivax*	Myanmese	Karen	Thai	Mon	Laotian	Cambodian	
HbA[a]	αα /αα	45	68	84	9	16	2	0	5	116
$-\alpha^{3.7}$	$-\alpha^{3.7}/\alpha\alpha$	31	43	57	4	8	1	3	1	74
	$-\alpha^{3.7}/-\alpha^{3.7}$	9	8	16	0	1	0	0	0	17
	Allele frequency			0.282	0.154	0.192	0.250	0.500	0.083	
$-\alpha^{4.2}$	$-\alpha^{4.2}/\alpha\alpha$	1	2	3	0	0	0	0	0	3
	Allele frequency			0.009	0.000	0.000	0.000	0.000	0.000	
$--^{SEA}$	$--^{SEA}/\alpha\alpha$	0	2	1	0	1	0	0	0	2
	Allele frequency			0.003	0.000	0.019	0.000	0.000	0.000	
HbCS	$\alpha\alpha^{CS}/\alpha\alpha$	3	2	4	0	1	0	0	0	5
	Allele frequency			0.013	0.000	0.019	0.000	0.000	0.000	
HbE	β^E/β	17	18	26	0	6	0	2	1	35
	β^E/β^E	1	5	3	0	2	0	0	1	6
	Allele frequency			0.101	0.000	0.192	0.000	0.333	0.250	

HbA[a] 2 cases were mixed infection of *P. falciparum* and *P. vivax* and 1 had *P. malariae*.

Table 2. Association between α-globin gene dosage, HbE genotype and number of *Plasmodium vivax* and *Plasmodium falciparum* parasitemia.

Genotype	*Plasmodium falciparum*			*Plasmodium vivax*		
	Patient (N)	**Parasitemia (log_{10} value/µl)**	***P*-value**	**Patients (N)**	**Parasitemia (log_{10} value/µl)**	***P*-value**
α-globin dosage						
HbA (αα/αα)	40	4.06±0.89	Reference group for alpha globin gene dosage	62	3.89±0.71	Reference group for alpha globin gene dosage
Silent α-thal ($-\alpha^{3.7}/\alpha\alpha$, $-\alpha^{4.2}/\alpha\alpha$,$\alpha\alpha^{CS}/\alpha\alpha$)	30	4.18±1.06	0.631	42	3.94±0.66	0.707
α-thal trait ($-\alpha^{3.7}/-\alpha^{3.7}$, $-\alpha^{3.7}/\alpha\alpha^{CS}$, $--^{SEA}/\alpha\alpha$, $-\alpha^{3.7}/-\alpha^{4.2}$)	10	3.96±1.14	0.740	10	4.21±0.32	**0.027**
HbH ($-\alpha^{3.7}/--^{SEA}$)	0	-	-	1	2.08	There is not enough statistical evidence.
HbE genotype						
β/β	64	4.02±1.03	Reference group for HbE	94	3.94±0.65	Reference group for HbE
β^E/β	17	4.45±0.66	**0.046**	17	3.83±0.94	0.662
β^E/β^E	1	3.00	There is not enough statistical evidence.	5	3.75±0.55	0.512

the ratio of males was much higher than female malaria patients. Since a more numerous population of men had been working outdoors, it was exposed to a higher chance of malaria infection.

The overall frequencies of α-thalassemia and HbE in Myanmar villagers living in malaria-endemic regions of Myanmar were 37.5% (343/916) and 20.3% (186/916), respectively.[31] Our study is comparable to a previous study and may reflect real prevalence. From our finding and the report of Than[31] support α-thalassemia especially $-\alpha^{3.7}$ deletion and HbE are highly frequent in both malarial and non-malarial infected Myanmar populations. While it is difficult to demonstrate the protective effect of α- thalassemia and HbE when conducting a study only in malaria patients, our findings of high prevalence of thalassemia traits among malaria patients supports the conclusion that malaria infection risk is not reduced in people with α-thalassemia and HbE. In line with this finding, an increased frequency of uncomplicated malaria was found in people with α^+-thalassemia in the Vanuatu study.[23] The high prevalence of α-thalassemia and HbE in Southeast Asia remains unexplained.

In contrast to the Haldane hypothesis, where α-thalassemia is expected to protect from malaria, we observed higher levels of *P. vivax* parasitemia among people with α-thalassemia trait. Similarly, a study in Papua New Guinea also showed higher *P. vivax* parasitemia (but not *P. falciparum*) in α^+-thalassemia heterozygous and homozygous children.[17] In addition, the study in Kenya also showed that α^+-thalassaemia neither protected against symptomatic malaria nor reduced parasitemia.[9] However, α^+-thalassaemia appeared to reduce the rate of severe anemia in *falciparum* malaria and had lower hospitalization.[9] The contrasting effects may be explained by the lack of *P. vivax* in African population, while both *P. vivax* and *P. falciparum* are prevalent in Southeast Asian region.

Despite the dosage effect of *P. vivax* density where two alpha gene deletions have higher levels of parasitemia than one gene deletion, the single case of HbH (3 genes deletion) had an unexpectedly lower rather than higher level of parasitemia. We could not make a meaningful conclusion from this one case as it could have occurred by chance. It was possible that this patient was referred early, so parasitemia was still low. It is hypothesized that people with α-thalassemia have more baseline erythropoiesis, resulting in a high proportion of reticulocytes which is the susceptible stage for *P. vivax* infection.[23] This hypothesis, however, is unlikely as there is no evidence of reticulocytosis in people with α^+-thalassemia heterozygous.[32]

Our results showed increased parasitemia of *P. falciparum* in heterozygous HbE, but also a decrease in one single case of homozygote. Our finding is in line with a previous study in Myanmar population.[33] *In vitro* studies reveal conflicti

ng results. Nagel et al. demonstrated impairment of the growth of *P. falciparum* in homozygous HbE, but an average growth in heterozygous HbE.[34] Whereas, Chotivanich et al. found *in vitro* a reduction in RBC invasion in HbAE heterozygotes, associated with a 4-fold increase in the selectivity index compared the other hemoglobin types studied and in particular the EE homozygotes suggesting that in heterozygote individuals with AE hemoglobin, only a quarter of the RBC population can be invaded by *P. falciparum*, so parasitemia could remain low.[13] Parasitemia of *P. vivax* in HbE patients had been previously observed but did not reach significant difference.[28] The effect of HbE on *P. vivax* parasitemia was not found in this study. Nevertheless, O'Donnell and colleagues showed that HbE patients might be more susceptible for malaria infection, especially *P. vivax* because their malarial antibodies were significantly increased than non-thalassemia children, which reflected in their clinical severity.[20] Although limited by a small number of patients, one strength of our study is that G6PD deficiency was excluded, which has been well known to confer protection against *vivax* malaria.[7]

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