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▣ ORIGINAL ARTICLE

The Effect of ABO Blood Groups, Hemoglobinopathy, and Heme Oxygenase-1 Polymorphisms on Malaria Susceptibility and Severity

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Abstract: Malaria is one of the most important public health problems in tropical areas on the globe. Several factors are associated with susceptibility to malaria and disease severity, including innate immunity such as blood group, hemoglobinopathy, and heme oxygenase-1 (HO-1) polymorphisms. This study was carried out to investigate association among ABO blood group, thalassemia types and HO-1 polymorphisms in malaria. The malarial blood samples were collected from patients along the Thai-Myanmar border. Determination of ABO blood group, thalassemia variants, and HO-1 polymorphisms were performed using agglutination test, low pressure liquid chromatography and polymerase chain reaction, respectively. *Plasmodium vivax* was the major infected malaria species in the study samples. Distribution of ABO blood type in the malaria-infected samples was similar to that in healthy subjects, of which blood type O being most prevalent. Association between blood group A and decreased risk of severe malaria was significant. Six thalassemia types (30%) were detected, *i.e.*, hemoglobin E (HbE), β-thalassemia, α-thalassemia 1, α-thalassemia 2, HbE with α-thalassemia 2, and β-thalassemia with α-thalassemia 2. Malaria infected samples without thalassemia showed significantly higher risk to severe malaria. The prevalence of HO-1 polymorphisms, S/S, S/L and L/L were 25, 62, and 13%, respectively. Further study with larger sample size is required to confirm the impact of these 3 host genetic factors in malaria patients.

Key words: Malaria, ABO blood group, thalassemia, heme oxygenase

INTRODUCTION

The widespread of malaria infection is a major public health problem in tropical and sub-tropical areas especially in Africa and Southeast Asia including Thailand. Several host factors such as innate immunity [1], hemoglobinopathy [2], and heme oxygenase (HO) polymorphisms [3,4] were investigated for the possible relationship with susceptibility to malaria and disease severity. Different geographic distributions and different ethnic populations are associated with different genetic variants to protect malaria [5]. The *Plasmodium* parasites are directly related to host red blood cells and therefore pathology of red blood cells could be expected to influence malaria infection and severity.

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The possible association between patient ABO blood groups and malaria development and severity has been suggested since 1957. The frequencies of blood group antigens were determined in Nigerian children with severe falciparum malaria [6,7]. The relationship between ABO blood group and severity of malaria disease was also investigated in other populations with the focus on *Plasmodium falciparum* infection [8-13]. Significant association between ABO blood group and severe *P. falciparum* infection was demonstrated in different populations, *i.e.*, in patients with blood group A in Zimbabwe [8], Gabon [9], and Ethiopia [10], in patients with blood group AB in Sri Lanka [11], Mali [12] and Ethiopia [10], and in patients with blood group B in India [13]. Falciparum malaria patients with blood group O was shown to be significantly associated with protection against cerebral malaria, whereas those with blood groups A and B was significantly associated with increased risk of developing cerebral malaria in Indian population [14]. The association between ABO blood group and anemia induced by *Plasmodium vivax* infection was investigated in Brazilian patients and results suggested increased susceptibility to anemia

in *P. vivax* patients with blood group O [15].

Thalassemia is a hemoglobinopathy caused by the alterations of globin chain synthesis that is classified into 2 forms based on the abnormality of globin chains, *i.e.*, α and β-thalassemia. Thalassemia is widely distributed and commonly found in malaria endemic areas [16]. Previous studies demonstrated protective effect against malaria of thalassemic red cells including hemoglobin E [17], α-thalassemia [2,18], and β-thalassemia [19,20].

The association between human heme oxygenase-1 (HO-1) polymorphisms and malaria infection was described in a few studies in different populations, *i.e.*, Gambian [21], Malawian [22], Angolan [23], and Burmese [3] populations. HO-1 is an inducible enzyme responsible for the breakdown of heme, mainly from hemoglobin into carbon monoxide (CO), biliverdin, and iron [24]. The HO-1 gene promoter contains a polymorphic (GT)n repeat which influences the expression level of HO-1 enzyme. HO-1 is beneficial for malaria parasite for growth and survival within red blood cells, for iron supply from the hemoglobin degradation process. The malaria parasite lacks HO-1 and is unable to cleave heme to release iron. In malaria infected thalassemic red cells particularly those with Hb E, the most common types in Thai population [17,25], the property of red blood cells is changed from normal which might affect iron supply for malaria parasite [26], and as a consequence the level of HO-1.

The aim of the study was to investigate the association of ABO blood group, thalassemia variants, and HO-1 polymorphisms in malaria infected blood samples collected from areas along the Thai-Myanmar border.

MATERIALS AND METHODS

Study subjects and sample collection

A total of 100 blood samples (5 ml each) were collected from malaria patients who attended malaria clinics and general hospitals in malaria endemic areas along the Thai-Myanmar (Kanchanaburi and Ranong Provinces). The inclusion criteria were: patients infected with all species of malaria; both males and females; and age 18-65 years. Patients who had received previous treatment within 28 days were excluded from the study. The study protocol was approved by the Ethics Committee of Thammasat University (COA No. 012/2558). Giemsa-stained thin and thick blood smears were prepared and examined under the light microscope for the presence of malaria parasites.

ABO blood group typing

ABO blood group was determined in all malaria infected blood samples by standard agglutination test using agglutinating A, B, and AB monoclonal anti-sera obtained from The Thai Red Cross Society (Bangkok, Thailand).

Analysis on thalassemic variants and other hematologic parameters

Osmotic hemolysis of malaria infected red blood cells was screened by osmotic fragility test (OF test). Hemoglobin typing was performed using automated low pressure liquid chromatography (LPLC) (Automated analyzer, Hb Gold, Cumbria, UK). The common α-thalassemia variants α-thalassemia-1 (SEA and Thai), α-thalassemia-2 (3.7 and 4.2), and Hb constant spring were analyzed using multiplex polymerase chain reaction (PCR). The hematological parameters including hemoglobin, hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) were analyzed using hematology analyzer (DxH, Coulter, Miami, USA).

Analysis on severity of anemia and malaria disease

The severity of anemic status of malaria infected blood samples was classified as mild, moderate and severe anemia according to the criteria of the World Health Organization [27]. Non-anemic condition was defined as hemoglobin level of >13.0 and >12.0 mg/dl for males and females, respectively. Mild degree of anemia was defined as hemoglobin levels of 11.0-12.9 and >11-11.9 mg/dl for samples collected from males and females, respectively. Moderate and severe anemic conditions in samples from both genders were defined as hemoglobin levels of 8.0-10.9 and <8.0 g/dl, respectively. Hyperparasitemia was defined as patients with >2% parasitemia or parasite count >100,000/μl in low intensity transmission areas [28]. Malaria disease severity of all blood samples was classified based on the 2 laboratory criteria, *i.e.*, anemia (hemoglobin and hematocrit levels) and hyperparasitemia (blood parasitemia count). The risk of patients to develop severe malaria based on analysis of the infected red blood cells was defined as: "high risk" if blood parasitemia was >100,000/μl and/or hemoglobin <8.0 and/or hematocrit <20% [27,28].

Preparation of genomic DNA

Genomic DNA of all blood samples was prepared using a QIAamp DNA extraction mini-kit (QIAGEN, Valencia, California, USA) according to manufacturer's instruction and used as

a template for polymerase chain reaction (PCR) amplification.

Analysis on HO-1 polymorphisms

The 5′-flanking region of the HO-1 gene containing the (GT) n repeat was amplified by PCR using the forward primer ACGCCTGGGGTGCATCAAGTC and the reverse primer GTGGGGTGGAGAGGAGCAGTCATA [29]. The gene was amplified in a thermocycler (T100™ Thermal cycler, BioRad) under the following cycling conditions: 5 min denaturation at 94˚C followed by 30 cycles of 45 sec at 94˚C, 30 sec at 65˚C and 45 sec at 72˚C, and a final extension step at 72˚C for 2 min. The PCR products were subjected to high solution electrophoresis and the fragment sizes were compared with (GT) 27 repeat DNA. The alleles consisting of less than (GT) 27 repeat were classified as S (short) alleles, and those consisting of (GT) 27 repeat or longer were classified as L (long) alleles.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 12.0 SPSS Inc., Chicago, Illinois, USA). The frequencies of malaria species, ABO blood group, and types of thalassemia are summarized as percentage (%). Levels of parasitemia, hemoglobin, hematocrit, MCV and MCH are summarized as median and range for data not conforming to normal distribution. Differences in qualitative and quantitative data between groups were analyzed using chi-square test and Kruskal-Wallis Test, respectively. Pair-wise comparison of the statistically significant Kruskal-Wallis test was performed using Mann-Whitney test. Statistical significance level was set at $\alpha = 0.05$ for all tests.

RESULTS

A total of 100 blood samples were collected from 62 males and 38 females (27 Thais and 73 Burmeses, aged 18-60 years) with mono- or mixed infection with *P. falciparum* and/or *P. vivax*. Of the 100 malaria infected blood samples collected, mono-infection with either *P. falciparum* or *P. vivax* accounted for 35% and 63%, respectively; mixed infection of both species account-

Table 1. The distribution of ABO blood group and malaria species infected in 100 blood samples

Plasmodium species	O (%)	A (%)	B (%)	AB (%)	Total (%)
Plasmodium falciparum	9	11	12	3	35
Plasmodium vivax	27	13	19	4	63
Mixed infections with *P. falciparum* and *P. vivax*	1	1	0	0	2
Total	37	25	31	7	100

Table 2. The distribution of ABO blood group and risk of developing severe malaria based on parasitemia and the anemic status of patients (levels of hemoglobin and hematocrit)

Variables	Distribution in ABO group			
	O	A	B	AB
Parasitemia (median and range, /μl blood)				
Plasmodium falciparum	15,776 (461-80,300)	7,200 (800-41,429)	9,703 (355-70,095)	1,608 (1,600-2,300)
Plasmodium vivax	4,705 (64-74,667)	3,909 (50-12,000)	2,583 (150-42,988)	12,075 (1,563-12,808)
Mixed infections with *P. falciparum* and *P. vivax*	2,920 (2,920-2,920)	22,720 (22,720-22,720)	-	-
Hemoglobin (%)				
Non-anemia (male ≥13, female ≥12 g/dl)	14	13	15	4
Mild anemia (male 11-12.9, female 11-11.9 g/dl)	13	8	8	0
Moderate anemia (8.0-10.9 g/dl for both genders)	6	5	3	2
Severe anemia (<8.0 g/dl for both genders)	4	0	5	0
Hematocrit (%)				
≥20	37	26	28	6
<20	0	0	3	0
Risk of developing severe malaria (%)				
Low risk	30	25	25	7
High risk (parasitemia (≥70,000/μl) and/or hemoglobin (<8.0) and/or hematocrit <20%	7	0[a]	6	0

[a]Statistically significant difference from patients with other blood groups ($P = 0.026$).

ed for 2% of the samples. The median (range) of parasitemia in all samples was 4,205 (50-80,300)/μl, while those with mono-infection with *P. falciparum*, *P. vivax* and mixed infection of both species were 7,764 (355-80,300), 3,618 (50-74,667), and 12,820 (2,920-22,720)/μl, respectively.

Association between ABO blood group and occurrence of malaria infection and severity

The highest frequency of ABO blood group in the samples was O (37%), followed by B (31%), A (25%), and AB (7%). No significant association between ABO blood group and susceptibility of malaria infection was found (Table 1). The distribution of all types of malaria infections was not significantly different in all blood groups, although proportion of samples with blood group O with *P. vivax* infection was about 3 times higher than that with *P. falciparum* infection. The association between the ABO blood group and severity of malaria infection was further determined based on parasitemia, and anemic status (hemoglobin and hematocrit levels). Based on these criteria, proportion of patients with high risk of developing severe malaria was significantly higher in blood group A compared with other blood groups ($P=0.026$) (Table 2).

Association between prevalence of thalassemic variants and occurrence of malaria infection

The prevalence of thalassemia in the samples was 30%. Among 6 common types, *i.e.*, Hb E, β-thalassemia, α-thalassemia-1, α-thalassemia-2, Hb E/α-thalassemia-2, and β-thalassemia/α-thalassemia-2, Hb E and α-thalassemia-2 accounted for the majority of all types (14% and 10%, respectively) (Table 3). Only 1 sample with α-thalassemia-1 was SEA deletion. All samples with α-thalassemia-2 were 3.7 kb gene deletion.

The association between thalassemia variants and the infected malaria species, parasitemia, hemoglobin, hematocrit, MCV, and MCH were investigated. Results suggested significant effects of these red blood cell parameters of the thalassemia in the malaria infected samples. Significant difference in hematocrit, but not hemoglobin level was found between malaria infected samples without thalassemia and those with all types of thalassemia (Table 3). The MCV of malaria infected samples with HbE, α-thalassemia 2 and β/α-thalassemia 2 were significantly different from those malaria infected samples with or without thalassemia. In addition, the MCH of malaria infected samples with HbE, β-thalassemia and β/α-thalassemia 2 were significantly different from those malaria infected samples with or without thalassemia.

The associations between malaria infected samples with or without thalassemia and anemic status (non-anemia, mild anemia, moderate anemia, and severe anemia) were investigated and results are summarized in Table 4. Significant association between malaria infected samples with and without thalassemia was found. β-thalassemia and Hb E/α-thalassemia 2

Table 3. The prevalence of thalassemia in 100 malaria infected samples

Thalassemia	No. (%)	Malaria Type			Hb (g/dL)	Hct (%)	MCV (fl)	MCH (pg)
		PF	PV	Mixed				
Malaria with thalassemia	30	9	20	1	11.5 (5.1-14.3)	34.3 (15.9-44.1)	71.0 (53.0-91.0)	23.2 (15.2-30.2)
Hb E	14	3	10	1	11.8 (7.8-14.3)	35.5 (21.9-43.0)	73.5 (63.0-83.0)[b]	24.1 (20.5-27.4)[f]
β-thalassemia	1	1	0	0	6.0 (6.0-6.0)	18.0 (18.0-18.0)	58.0 (58.0-58.0)	18.7 (18.7-18.7)[g]
α-thalassemia 1	1	1	0	0	12.7 (12.7-12.7)	41.3 (41.3-41.3)	68.7 (68.7-68.7)	21.2 (21.2-21.2)
α-thalassemia 2	10	3	7	0	12.0 (5.1-14.3)	35.5 (15.9-44.1)	73.5 (53.0-91.0)[c]	23.8 (15.2-30.2)
Hb E/α-thalassemia 2	1	0	1	0	7.6 (7.6-7.6)	24.0 (24.0-24.0)	62.0 (62.0-62.0)	19.7 (19.7-19.7)
β/α-thalassemia 2	3	1	2	0	10.9 (9.8-11.1)	33.6 (28.3-34.3)	67.0 (60.0-67.0)[d]	21.3 (19.6-22.7)[h]
Malaria without thalassemia	70	26	43	1	12.4 (3.4-17.7)	37.7 (10.7-55.1)[a]	84.5 (50.0-101.0)[e]	27.80 (20.8-32.3)[i]

The hematological parameters are presented as median (range) values.
PF, *P. falciparum*; PV, *P. vivax*; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume (MCV); MCH, mean corpuscular hemoglobin.
[a]Statistically significant difference from samples with thalassemia ($P=0.006$).
[b]Statistically significant difference from samples with other types of thalassemia and without thalassemia ($P=0.002$).
[c]Statistically significant difference from samples with other types of thalassemia and without thalassemia ($P=0.045$).
[d]Statistically significant difference from samples with other types of thalassemia and without thalassemia ($P=0.007$).
[e]Statistically significant difference from samples with thalassemia ($P<0.001$).
[f]Statistically significant difference from samples with other types of thalassemia and without thalassemia ($P=0.001$).
[g]Statistically significant difference from samples with other types of thalassemia and without thalassemia ($P=0.043$).
[h]Statistically significant difference from samples with other types of thalassemia and without thalassemia ($P=0.006$).
[i]Statistically significant difference from patients with thalassemia ($P<0.001$).

Table 4. The association of thalassemia and anemic status in malaria infected samples

Thalassemia	Anemic status			
	Non-anemia (%)	Mild anemia (%)	Moderate anemia (%)	Severe anemia (%)
Malaria with thalassemia[a]	10	7	7	6
Hb E	6	2	4	2
β-thalassemia[b]	0	0	0	1
α-thalassemia 1	0	1	0	0
α-thalassemia 2	4	3	1	2
Hb E/α-thalassemia 2[c]	0	0	0	1
β/α-thalassemia 2	0	1	2	0
Malaria without thalassemia	36	20	11	3[d]

[a]Statistically significant difference from patients with thalassemia (P = 0.016).
[b]Statistically significant difference from patients with other types of thalassemia and without thalassemia (P = 0.023).
[c]Statistically significant difference from patients with other types of thalassemia and without thalassemia (P = 0.023).
[d]Statistically significant difference from patients with other types of thalassemia (P = 0.019).

were significantly associated with the anemic status. In malaria infected samples without thalassemia, the proportion of severe anemia was significantly lower than that with thalassemia.

Association between heme oxygenase-1 promotor genotypes and malaria disease severity

The number of (GT)n repeat of the HO-1 gene in the malaria infected samples ranged from 13 to 39. The size of HO-1 gene was categorized as short (S: 13-26 repeats), and long (L: 27-39 repeats). The allele frequencies of (GT) n allele in each sample was further classified into 3 genotypes, *i.e.*, S/S, S/L, and L/L (Table 5). There was no association between HO-1 promotor genotype and the risk of development of severe malaria.

Relationship between ABO blood group, thalassemia status, HO-1 genotypes and malaria severity

The relationships between the 3 host genetic factors in malaria infected samples were investigated. Results showed no significant association between ABO blood group and thalassemia type (P = 0.149), ABO blood group and HO-1 polymorphisms (P = 0.890), as well as thalassemia type and HO-1 polymorphisms (P = 0.811).

DISCUSSION

The ABO blood group distribution found in the samples

Table 5. Distribution of HO-1 promoter genotypes (S and L) including allele (S/S, S/L, and L/L) frequencies and malaria severity in 100 malaria infected samples

	Number	Malaria species			Malaria severity	
		P. falciparum	*P. vivax*	Mixed infection	UM	RSM
Alleles, n (%)						
S	112 (56)					
L	88 (44)					
Genotypes, (%)						
S/S	25	8	16	1	22	3
S/L	62	25	37	0	53	9
L/L	13	2	10	1	12	1

UM, uncomplicated malaria; RSM, risk to severe malaria.

analyzed in the present study was similar to that previously reported in other studies in Thailand [30-32]. The most prevalent blood group was O (37%). The most prevalent blood group in blood samples infected with *P. falciparum* and *P. vivax* were B and O, respectively. It was noted however that the proportion of samples infected with *P. vivax* with blood group O was about 3 times of *P. falciparum*. This observation may suggest increased susceptibility to *P. vivax* infection compared with *P. falciparum* infection in patients with blood group O. Blood samples with A antigen on red blood cells (blood group A and AB) were not found in samples identified as high risk of developing severe malaria. It is possible that patients with blood group A and AB are more likely protected from severe malaria.

The association between thalassemia and malaria disease was investigated in several studies and the protective effect against *P. falciparum* malaria by different types of thalassemic red cells was demonstrated. These include hemoglobin E [17], α-thalassemia [2,18], and β-thalassemia [19,20]. In Southeast Asia, the major type of thalassemia is Hb E [33]. In the present study, the most prevalent thalassemia variants found in 100 malaria infected, both *P. falciparum* and *P. vivax* infected samples, were Hb E and α-thalassemia-2. Different types of thalassemia had different degree of impact on the anemic status of the malaria infected samples. The β-thalassemia and Hb E/α-thalassemia 2 were significantly associated with severe anemia and the risk to development of severe malaria.

The association between HO-1 promotor genotypes and malaria infection was investigated in various populations. Elevated HO-1 expression was associated with severe malaria in Gambian children [21] and with cerebral malaria in Malawian [22], Burmese [3] and Angolan children [23]. The association

between HO-1 polymorphism and malaria disease severity was initially proposed by Shibahara et al. [24,34]. Later studies provided supporting evidence on the association between (GT)n repeat polymorphisms of HO-1 and malaria severity in Karen ethnic minority group in Myanmar [3], and Thai, Burmese and Karen patients in Thailand [4]. The proportion of Karen patients with short (GT) n alleles was found to be significantly higher in cerebral malaria than uncomplicated malaria [3]. Significant difference in HO-1 genotypes was found among 3 ethnics patients that may explain difference in pathogenicity/severity of malaria infection in various ethnics. Nevertheless, the relationship between ABO blood group, thalassemia type, HO-1 polymorphisms and malaria disease severity were not found in the present study. This could be explained by the observed low prevalence of S/S genotype and high proportion of samples with *P. vivax* infection with a typical characteristic of low parasitemia compared with *P. falciparum* infection.

In conclusion, malaria infected red blood cells with β-thalassemia and Hb E/α-thalassemia 2 are more likely to develop severe malaria. Those with blood group A and AB are more likely to protect patients from the risk of developing severe malaria. Further study with increasing sample size should be performed to confirm the impacts of these 3 host genetic factors, *i.e.*, ABO blood groups, thalassemia types, and HO-1 polymorphisms, in malaria patients.

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