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Hemoglobin E protects against acute *Plasmodium vivax* infections in a Kachin population at the China-Myanmar border

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Summary

Objectives—Hemoglobin E (HbE, β^{26} Glu-Lys) is the most prevalent hemoglobinopathy in Southeast Asia. This study aimed to determine whether HbE protects against clinical *Plasmodium vivax* malaria in Southeast Asia.

Methods—In a case-control study performed in villages along the China-Myanmar border, we determined the prevalence of HbE in 247 villagers who had acute *P. vivax* infections and in 157 control healthy villagers.

Results—HbE in *P. vivax* patients (17.4%) was significantly less prevalent than in the healthy villager population (36.3%). Moreover, there was a complete lack of HbEE homozygotes in the *vivax* patients as compared to 9.5% prevalence in the healthy villagers. Using the HbAA group as the reference, both the HbEA heterozygotes and HbEE homozygotes had significantly lower odds of presenting with acute *P. vivax* infections. Furthermore, HbEA heterozygotes also had significantly lower *P. vivax* asexual parasite densities. HbEA did not affect the proportion of *P. vivax* patients with gametocytemia nor the gametocyte densities.

Conclusions—HbE offers significant protection against the occurrence and parasite density of acute *P. vivax* infections and provides a renewed perspective on *P. vivax* malaria as a potentially strong driving force behind the high frequencies of HbE in the Kachin population.

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Keywords

hemoglobinopathy; hemoglobin E; *Plasmodium vivax*; malaria hypothesis; protection

Introduction

Malaria has been a strong selection force responsible for the extremely high frequencies of certain red blood cell (RBC) disorders such as the hemoglobinopathies and glucose-6-phosphate dehydrogenase (G6PD) deficiency in human populations from malaria endemic regions (1, 2). This “malaria hypothesis”, originally proposed by Haldane to explain that the highly prevalent thalassemias in the tropical and subtropical populations might confer protection against malaria (3), has been extensively investigated, and supported by strong epidemiological, clinical, genetic and in vitro evidence (4, 5). Hemoglobin is an $\alpha_2\beta_2$ tetramer, and major hemoglobinopathies are caused by either reduced production of the normal α -globin (α -thalassemias) or β -globin (β -thalassemias), or the production of structural variant forms of the β -globin such as hemoglobin S (HbS), HbC and HbE. HbE, resulting from an E26K substitution in the β -globin gene, is an extremely common hemoglobin variant in Southeast Asia (6). The prevalence of HbE varies among populations; at the junction of Thailand, Laos and Cambodia, 50–60% of the human population are HbE carriers (7). In some ethnic groups residing along the China-Myanmar border, HbE prevalence is also high and almost reaches 40% (8, 9). Whereas there is particularly strong evidence to support that the HbS variant and α -thalassemias offer protection against severe malaria, evidence about HbE’s protection against malaria is less convincing (1, 10).

In Thailand, one cross-sectional survey found higher HbE prevalence in an area of higher malaria endemicity (11), but another study did not find selective advantages of HbE against *P. falciparum* infection (12). A later, case-control study revealed the protective effect of HbE only after taking into account of fava bean consumption as a source of oxidative stress (13). This seems to agree with impaired antioxidant defense in HbE RBCs (14). A cross-sectional study conducted in northeast India reported that both HbE homozygotes (HbEE) and heterozygotes (HbAE) were associated with lower prevalence of *P. falciparum* infection than normal (HbAA) (15). However, a more recent case-control study from Bangladesh showed the opposite, where HbEE was associated with increased *P. falciparum* incidence (16). In two hospital-based studies in Myanmar and Thailand, comparison of HbE prevalence in severe and uncomplicated *P. falciparum* patients produced conflicting findings on the protection of HbE against severe malaria (17, 18). Similarly, small cohort studies also did not find protective advantages of HbE in terms of malaria incidence, clinical presentation, and cerebral malaria (19, 20). In vitro studies aiming to provide a mechanistic basis for HbE’s protection against *P. falciparum* malaria also yielded equivocal findings. Whereas some studies showed significantly reduced invasion of HbAE RBCs and reduced growth of the parasites in HbEE RBCs (21–23), others did not find such inhibitory effects (24, 25). Nevertheless, population genetic studies suggested a single origin of the HbE variant in the Thai population and provided an estimate for a recent emergence of the HbE mutation (<5000 years) (26), a time frame coinciding with the estimated period of expansion of *P.*

falciparum (27). Altogether, convincing evidence linking malaria to the high HbE prevalence in Southeast Asia is still lacking.

It is noteworthy that studies addressing the HbE's protection against malaria focused on *P. falciparum*, since this parasite is responsible for the majority of malaria deaths and thus is supposed to exert the strongest selection. Considering the recent recognition of *P. vivax* as the cause of severe diseases (28, 29) and the overlapping geographical distribution of *P. vivax* with HbE, we speculate that *P. vivax* may also be a strong selection force leading to the high frequencies of HbE in Southeast Asia. In this aspect, only a single community survey in northeast India supported the protection of HbE against asymptomatic *P. falciparum* and *P. vivax* infections (15). Another report from Sri Lanka showed, however, that subjects with the clinical condition of HbE/ β thalassemia were at a greater risk of *P. vivax* malaria (30). To address the question of whether HbE protects against vivax malaria, we conducted a case-control study to document acute *P. vivax* malaria incidences and HbE prevalence in Kachin villages at the China-Myanmar border, where HbE in the study group is highly prevalent (8, 9) and *P. vivax* malaria recently has become the predominant parasite species (31).

Materials and methods

Study subjects and sample collection

This case-control study was conducted in nine villages near Laiza Township of northeast Kachin State, Myanmar, where HbE is prevalent and *P. vivax* malaria is endemic (8, 9, 31). All residents in these villages are of the Kachin (Jingpo) ethnicity. The study was approved by the Institutional Review Boards of Kunming Medical University and Kachin Bureau of Health. Prior to sample collection, written informed consent (assent for minors) was obtained from all participants. Acute *P. vivax* malaria in the study villages was monitored in 2013–2017. As control, 157 unrelated healthy villagers (63 females and 94 males, aged 5 – 73 years) were randomly recruited as the control. The control subjects resided in the same villages, had no clinical malaria in the previous 12 months, and were not carrying malaria infection by microscopy at the time of recruitment. For each control participant and malaria patient, 0.5 ml of peripheral blood were collected in an EDTA-tube. Both thin and thick blood smears were prepared, stained with Giemsa, and examined by two experienced microscopists using a light microscope at 100 $\times$ under an oil immersion lens. Parasite densities (both asexual stages and gametocytes) were estimated by counting the number of parasites in thick smears against 500 white blood cells, assuming a white blood cell count of 6000/ μ l of blood (32). Control villagers and *P. vivax* patients were further confirmed by nested PCR targeting the 18S rRNA gene (33).

DNA extraction and HbE genotyping

Genomic DNA was extracted from 200 μ l of whole blood with the TaKaRa Genomic DNA Kit (TaKaRa Biotechnology) according to the manufacturer's instructions. DNA was eluted in 50 μ l of elution buffer and used for identification of *P. vivax* infection and HbE genotyping. The HbE mutation was determined using semi-nested PCR with pfu DNA polymerase and primary primers F1 (GGACAGGTACGGCTGTCATC) and R1

(TCAAGGGTCCCATAGACTCACC) and semi-nested primers F1 and R2 (GTGAGCCAGGCCATCACTA) (34). The amplified DNA fragment was analyzed on a 1.5% agarose gel and sequenced by GENEWIZ® (Suzhou, China).

Statistical analysis

Statistical comparison was done using the GraphPad Prism software version 6.0 and SPSS for windows version 10, and significance level was set at $P < 0.05$. Differences in asexual stage and gametocyte densities were compared using the Mann-Whitney U test. Gametocyte and HbE carriage rates were compared using the χ^2 test. A logistic regression model was used to estimate the odd ratios (ORs) and 95% confidence intervals (CIs) for contracting vivax malaria using the HbAA group as the reference. Further, adjusted odds ratios (AORs) were determined to include age and sex as potential confounding factors in a multivariate regression analysis.

Results

HbE protects against *P. vivax* infection in the Kachin population

In the 157 control healthy villagers, 57 (36.3%) were HbE carriers, including 15 (9.6%) HbEE and 42 (26.8%) HbEA subjects (Table 1). During 2013–2017, a total of 247 cases of acute, uncomplicated *P. vivax* infections (93 females and 154 males, aged 5 – 75 years) were recorded. Genotyping of the 247 *P. vivax* patients identified 17.4% (43/247) as HbE carriers, which was significantly lower than the prevalence of HbE in the village populations ($P = 0.0001$, χ^2 test). Moreover, all the HbE carriers in the vivax patients were HbAE (Table 1).

Assuming that all people in the study villages (HbEE, HbAE and HbAA) were equally exposed to *P. vivax* infections, both HbAE and HbEE would be significantly less likely to be infected with *P. vivax*. Using the HbAA group as the reference, HbAE offered significant protection against acute *P. vivax* infection (OR = 0.507, $P = 0.006$, 95% CI: 0.308 – 0.817). Moreover, HbEE offered even stronger protection against *P. vivax* infection with almost one hundred times lower the odds than the HbAA (OR = 0.01, $P = 0.004$, 95% CI: 0.001 – 0.268). When age and sex were taken into account as potential confounding factors, both HbAE (AOR = 0.488, $P = 0.005$, 95% CI: 0.297 – 0.802) and HbEE (AOR = 0.013, $P = 0.004$, 95% CI: 0.001 – 0.239) still offered significant protection against acute vivax malaria.

HbAE is associated with lower asexual parasitemia of *P. vivax* infection

We next tested whether HbE protects against the severity of *P. vivax* infection using asexual-stage parasite density as a proxy for disease severity (Table 2, Fig. 1). The median parasite density (358/μl of blood) in HbAE *P. vivax* patients was significantly lower than that (825 parasites/μl of blood) in HbAA *P. vivax* patients ($P < 0.05$, Mann-Whitney test).

In the 247 *P. vivax* cases, gametocytes were present in 98 (40%) individuals (Table 2). The frequencies of gametocytemia in HbAA vivax patients (41.67%, 85/204) and HbAE vivax patients (30.2%, 13/43) were not significantly different ($P < 0.05$, χ^2 test). Besides, gametocyte densities were also similar between the HbAA and HbAE vivax patients (Table

2, Fig. 2). Collectively, these data suggest that the HbAE genotype did not affect the prevalence nor the density of gametocytes in *P. vivax* patients.

Discussion

Malaria is the strongest known evolutionary force of selection in the recent history of human genome (35). Analysis of various hemoglobinopathies indicates that hemoglobin mutations confer protection against malaria, but differ in the degree of protection against uncomplicated malaria and asymptomatic infections (4, 5, 36). As a result, the mutant alleles have risen to high frequencies in malaria-exposed populations despite the potential fatal consequences for homozygotes (37). In comparison to other hemoglobinopathies, the heterozygous state HbAE is asymptomatic causing minimal morphological changes of the RBCs, while HbEE displays mild anemia. Geographical difference in the prevalence of the resistant hemoglobin alleles (HbS in Africa, HbC in west Africa, and HbE in Southeast Asia) suggests different demographic histories of the malaria parasites in these regions, and co-evolution of the human genomes from interactions with the different parasites (25, 38, 39). Here we evaluated the *P. vivax* malaria, the distribution of which overlaps significantly with HbE, as a selection force for the evolution of HbE in an ethnic Kachin population along the China-Myanmar border. The significant lower HbE prevalence and lack of HbEE in vivax patients as compared to their much higher prevalence in the control villager population suggest that HbE protects against *P. vivax* infection.

Our finding on the protective effect of HbE against acute vivax malaria is consistent with the report from a cross-sectional study in northeast India on HbE's protection against asymptomatic *P. vivax* infection (15). This stands in stark contrast to the recent study in Bangladesh, where HbEE was even found to be associated with the increased passive cases of malaria (16). However, the selected small number of people (142) from a large survey population to match the age, sex and ethnicity of the acute malaria patients may not be representative of true HbE prevalence in the population (16). Another report showed that subjects with HbE/ β thalassemia were more prone to malaria infection, especially *P. vivax* (30). HbE/ β thalassemia is a totally different clinical condition from that of HbE alone, and patients with HbE/ β thalassemia often suffer from severe damage to the spleen (6). It is possible that impaired immune responses as the result of spleen damage might be responsible for the increased susceptibility to malaria infection. Our results indicating a protective role of HbE against *P. vivax* malaria provide a fresh view of *P. vivax* as a potentially strong selective force for the high prevalence of HbE in endemic populations in Southeast Asia (26). Our data is in parallel to the recent recognition of *P. vivax* as the driving force for the evolution of the Mahidol variant of G6PD deficiency in the Thai-Myanmar border (40).

A hospital-based study suggested that HbE trait protected against severe *P. falciparum* malaria (18). Likewise, the association of HbAE with significantly reduced *P. vivax* parasite densities in patients also suggests protection of HbEA against severe *P. vivax* malaria. Whereas the mechanism of protection is not clear, the lower parasite density in HbAE patients might be resulted from decreased infection of the reticulocytes, which is similar to the observed refractoriness of the HbAE normocytes to *P. falciparum* invasion in vitro (21).

HbAE reticulocytes may also have increased oxidative stress as in HbAE normocytes to inhibit *P. vivax* growth (14). In addition, infected HbAE and HbEE cells by *P. vivax* may be more susceptible to phagocytosis as *P. falciparum*-infected HbEE and HbAE RBCs (41). With regard to suspected elevation of intracellular oxidative stress in HbE RBCs, we wanted to evaluate whether *P. vivax* would undergo increased gametocytogenesis in HbE cells. However, our results did not show significant difference in *P. vivax* gametocyte carriage nor in gametocyte density between HbAE and HbAA *P. vivax* patients.

Though there are clear, differential geographical distributions of hemoglobinopathy traits, some are sympatric and show complex interactions (9, 42). In the Kachin population along the China-Myanmar border, our preliminary analysis detected low prevalence of β -thalassemia (unpublished), similar to what was found as in a Shan population (42). Yet, our previous study found 23% co-occurrence of the HbE and G6PD deficiency traits (9). Among the carriers of these two traits, two G6PD-deficient males with HbAE were found to be severely anemic, suggesting negative interactions of these two traits, like those between HbE and β -thalassemia (43). In our study area, G6PD is highly prevalent and the predominant variant is the Mahidol type (44). Since the Mahidol variant is protective against higher parasite density in *P. vivax* malaria (40), further investigations are needed to explore the interaction between these two highly prevalent traits in protection against vivax malaria in this malaria-endemic area.

In summary, this case-control study conducted in Kachin villages along the China-Myanmar border identified protection of the HbE trait against both *P. vivax* infection and higher parasite load, suggesting that *P. vivax* malaria might also be a strong driving force behind the high frequencies of HbE in Southeast Asia. However, there are several limitations to this study. One caveat is the rather small numbers of study subjects involved and the study site is restricted to a total of nine villages within a 10-km of radius. The other limitation is that parasitemia could not capture the clinical severity of the disease. Thus, future analysis should consider these points to determine whether findings from this study can be generalized.

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Highlights

- Hemoglobin E (HbE) is highly prevalent in the Kachin population of eastern Myanmar
- HbE was significantly less prevalent in *P. vivax* patients than in healthy villagers
- HbEA heterozygotes had significantly lower *P. vivax* asexual parasite densities
- HbE offers protection against occurrence and severity of acute *P. vivax* infections

Figure 1. Comparison of asexual stage parasite density (A) and gametocyte density (B) between HbAA and HbAE *P. vivax* patients. Mann-Whitney U test.

Table 1Characteristics of the healthy villagers and *P. vivax* patients of a rural Kachin population

Characteristics	Control villagers	Vivax patients	P values
Number of participants (% males)	157 (59.8%)	247 (62.3%)	0.6745 *
Age (years) (median/range)	24.82 (5–73)	27.73 (5–75)	0.1127 **
Asexual parasite density/μl [median (IQR)]	-	825 (213–2475)	
Patients with gametocytes [N (%)]	-	98 (40.0%)	
Gametocyte density/μl [median (IQR)]	-	1059 (550–2475)	
HbE carrier [N (%)]	57 (36.31%)	43 (17.41%)	0.0001 *
HbEA [N (%)]	42 (26.75%)	43 (17.41%)	0.0074 *
HbEE [N (%)]	15 (9.55%)	0	0.0001 *

* χ^2 test;

** Mann-Whitney test.

Table 2Comparison of parasitemia and gametocytemia between HbEA and normal *P. vivax* patients

Parasitemia	Non-HbE patients	HbE carrier patients	P values
Asexual parasite density/μl [median (IQR)]	1128 (220–2530)	358 (193–1953)	0.0412 ^{**}
Patients with gametocytes [N (%)]	85 (41.67%)	13 (30.23%)	0.1749 [*]
Gametocyte density/μl [median (IQR)]	1100 (564–2475)	1018 (481–2104)	0.7699 ^{**}

^{*} χ^2 test;^{**} Mann-Whitney test.