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Positively selected G6PD-Mahidol mutation reduces *Plasmodium vivax* density in South-East Asians

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency — the most common known enzymopathy — is associated with neonatal jaundice and hemolytic anemia usually after exposure to certain infections, foods, or medications. Although G6PD-deficient alleles appear to confer a protective effect against malaria, the link with clinical protection from *Plasmodium* infection remains unclear. We investigated the effect of a common G6PD deficiency variant in South-East Asia — the *G6PD*-Mahidol^{487A} variant — on human survival related to *vivax* and *falciparum* malaria. Our results show that strong and recent positive selection has targeted the Mahidol variant over the past 1,500 years. We found that the *G6PD*-Mahidol^{487A} variant reduces *vivax*, but not *falciparum*, parasite density in humans, indicating that *Plasmodium vivax* has been a driving force behind the strong selective advantage conferred by this mutation.

One sentence summary:

Positive selection acts on a hemolytic anemia-causing mutation that affects the proliferation of a blood parasite in humans.

Malaria is a major cause of human mortality worldwide and considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome (1). Host genetic defense mechanisms are likely to have evolved to resist malaria infection in regions where the parasites have been historically prevalent. Among malaria-causing parasites, *Plasmodium falciparum* and *Plasmodium vivax* seem to have exerted strong selective pressure on the cellular phenotype of human erythrocytes, causing increased prevalence of hemoglobinopathies and other inherited blood disorders (1).

Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked essential enzyme that plays a key role in protecting cells from oxidative stress, and is particularly important in red blood cells. G6PD deficiency, affecting over 400 million people worldwide, is associated with several clinical disorders including neonatal jaundice, hemolytic anemia following infection by certain pathogens, and favism (2). The high overall frequency of G6PD-deficient alleles in the population is thought to result from their protective effect against malaria. Evolutionary studies of the *G6PD* locus suggest that local and recent positive selection has targeted the G6PD-deficient allele G6PD^{A-} in Africa (3, 4) and that this process started 2,500-3,800 years before present (YBP) (5). These observations are consistent with signs of recent expansion of *P. falciparum* in Africa (6). However, the clinical link between G6PD deficiency and malaria is less clear. Although a clinical protective effect of G6PD deficiency against human lethal malaria, *P. falciparum*, in Africa has been shown (7), several other reports have not found an association (8, 9).

Most clinical, epidemiological and evolutionary studies of the relationship between G6PD deficiency and malaria protection have focused on *falciparum* malaria, particularly in Africa. The role of G6PD-deficient alleles in the susceptibility, or resistance, to *vivax* malaria has not been

accurately tested and remains anecdotal. Nevertheless, *P. vivax* imposes a considerable burden of disease on the human population and, historically, has been associated with considerable mortality and decreased fertility in human populations (10). Both *P. falciparum* and *P. vivax* coexist in South-East (SE) Asia, with *P. vivax* accounting for over half of malaria cases. Moreover, there is increasing evidence for an ancient origin of *P. vivax* in Asia (11), where its presence apparently predates that of *P. falciparum* (12).

We investigated whether G6PD deficiency increases human survival in SE Asia, through its effects on *vivax* and *falciparum* malaria, in an evolutionary and epidemiological study of the Mahidol^{487A} mutation (MIM #305900.005). This G6PD-deficient variant occurs throughout greater SE Asia, including mainland China and is most common in Myanmar (13). The average allele frequency in Thailand is 12%, but there is distinct local heterogeneity with increased frequency on the western border, particularly in the Mon, Burmese and Karen populations (13, 14). This mutation is classed as a moderate-to-mild G6PD variant with a reduction of 5-32% of wild-type activity levels in healthy individuals (13, 15).

We first used an evolutionary approach to detect the potential molecular signature of positive selection at the G6PD-Mahidol^{487A} mutation. We genotyped 30 SNPs (including the Mahidol⁴⁸⁷ variant, table S1) dispersed along a 2.4-Mb region encompassing *G6PD* (Fig. 1A) in a panel of 384 unrelated individuals, the majority of whom are Karen, living in the Suan Phung district of Thailand (16). After reconstructing the phase of extended haplotypes (16), we implemented the long-range haplotype (LRH) test, which identifies alleles that have undergone recent positive selection, i.e., alleles associated with high levels of extended homozygosity at nearby markers and present at high population frequencies (3). The frequency of the Mahidol^{487A} mutation in our population sample was 24% and showed very high levels of extended

homozygosity: 63% of Mahidol^{487A}-bearing haplotypes showed complete haplotypic conservation over the entire 2.4-Mb region (Fig. 1, A and B). The high level of homozygosity surrounding Mahidol^{487A} was highly significant given its frequency ($P < 10^{-4}$), when compared to the empirical distribution of allelic homozygosity vs. frequency for all X-linked HapMap Phase II SNPs in Han Chinese (17), after matching for SNP density (Fig. 1C). However, the power of the LRH test can be challenged by uncertainties related to phase reconstruction and specific population histories (18). To circumvent this, we compared the observed allelic homozygosity associated with Mahidol^{487A} in males only (whose haplotypic phase is known) with simulations of a ~1-Mb recombining X-linked region for a population experiencing different demographic regimes (16). Consistently, we found a highly significant signal of positive selection at Mahidol^{487A}, independently of the demographic scenario considered (Fig. 1D; $P < 10^{-4}$ for the constant population size, the bottleneck and the expansion models). The low microsatellite diversity associated with Mahidol^{487A} (Fig. 1E) confirmed the results based on the LRH test. Together, our evolutionary analyses show that the Mahidol^{487A} mutation is under recent and strong positive selection in the Karen population, indicating that this mutation has conferred a strong selective advantage in human survival.

We estimated the age of G6PD-Mahidol^{487A} and the selection coefficient that would be consistent with such a strong positive selection. We obtained similar estimates using two different methods (19, 20), which showed that the frequency of the Mahidol^{487A} mutation started to increase at ~1,500 YBP, with a selection intensity of ~0.23 (Table 1). The selection coefficients of Mahidol^{487A} are among the strongest detected so far in the human genome, including HIV-protective haplotypes (~0.30) (21), malaria-protective G6PD^{A-} (~0.2) (5, 20) and β -globin variants (~0.26 and ~0.08) (22, 23), as well as lactase persistence (~0.1) (24).

We investigated the nature of the selective advantage conferred by the G6PD-Mahidol^{487A} mutation by testing its influence on the outcome of infection with either *P. falciparum* or *P. vivax*. To this end, we conducted a community-based longitudinal study in the Suan Phung district of Thailand, which has a total population of 5,368. From the 3,484 participants of the malaria epidemiology study (25), we obtained genotypes at the G6PD-Mahidol⁴⁸⁷ position from 925 individuals (16). Between 1998 and 2005, there were 1,090 *P. falciparum* clinical episodes made by 460 of these individuals, and 524 *P. vivax* clinical episodes made by 262 of these individuals. Reliable parasite density data was available for 823 observations of *P. falciparum* parasite density in 400 individuals, and for 417 observations of *P. vivax* parasite density in 227 individuals. Details on the sample selection procedures are provided in Materials and Methods (16) and summarized in Fig. S1.

To test for a genetic association between G6PD-Mahidol^{487A} and the number of *Plasmodium* species clinical cases or parasite density, we performed a family-based association test (FBAT), which corrects for spurious association due to population stratification (16). Seventy-seven families were informative for the association with the number of *Plasmodium* species clinical cases, 44 of which were informative for the association with *P. falciparum* density and 35 for the association with *P. vivax* density (i.e., some families were infected by different *Plasmodium* species at different times) (16). We found that Mahidol^{487A} had no effect on the number of cases of clinical malaria due to either *P. vivax* or *P. falciparum*, reported for each individual during the seven-year observation period (16). This is likely due to the high heterogeneity of exposure to infection in this area of low transmission intensity, where virtually all infections by either parasite species lead to symptomatic episodes (25). Our analyses also revealed that Mahidol^{487A} was not significantly associated with *P. falciparum* density, using both

annual values of parasite density (16) or mean density values across all years under any genetic model (table S2). By contrast, Mahidol^{487A} was significantly associated with reduced *P. vivax* density using both annual values of parasite density (χ^2 test, $P=0.029$) and mean density values across years (χ^2 test; dominant model $P=0.011$, additive model $P=0.016$, recessive model $P=0.048$; table S2) taking into account age and environmental covariates that affect parasite density (16, 26). A permutation test with 100,000 iterations confirmed that these results were significant (χ^2 test, $P=0.017$).

To verify this association, we performed a population-based association study between Mahidol^{487A} and *Plasmodium* parasite density in the whole population, excluding all individuals used in the FBAT analyses (16). Mahidol^{487A} significantly reduced *P. vivax* parasite density (the most significant P -value was obtained for the dominant model, χ^2 test, $P=0.006$), while no association was observed with *P. falciparum* parasite density for any genetic model (table S2). When considering both the family-based and the population-based datasets, *P. vivax* parasite density decreased with age (χ^2 test, $P<0.001$), indicative of the acquisition of anti-parasite immunity (Fig. 2A). Mean *P. vivax* density was reduced by 30% in non-Mah/Mah and 61% in Mah/Mah females compared to non-Mah/non-Mah females; parasite density was reduced 40% in Mah/Y males compared to non-Mah/Y males (Fig. 2B). Mahidol^{487A} accounted for 3.3% of the observed variation in *P. vivax* density. Although increasing age was again associated with decreasing *P. falciparum* density (χ^2 test, $P<0.001$) (Fig. 2C), there was no significant effect of Mahidol^{487A} on *falciparum* density (Fig. 2D).

A Mahidol^{487A} gene dose effect on *P. vivax* density was observed, whereby parasite density tends to be lower in Mah/Mah females with respect to both non-Mah/Mah females and

Mah/Y males (Fig. 2B). Two main factors can explain the observed tendency. Firstly, G6PD activity in heterozygous non-Mah/Mah females depends on the random inactivation of the X-chromosome: each heterozygous female will harbour two populations of red blood cells (expressing Mahidol^{487A} or Mahidol^{487G}) and their relative abundance is unpredictable. Indeed, when measuring G6PD activity in Karen individuals using the fluorescence spot test (16), we observed that the non-Mah/Mah genotype can express either normal or deficient phenotypes (table S3). Secondly, the Mahidol^{487A} mutation markedly increases protein thermo-instability with consequences for enzyme activity (15). Hence, in *P. vivax* infection, which induces high fever, G6PD deficiency may be more pronounced than in non-infected individuals contributing to the reduction in parasite density. Because genotype/phenotype correlations for G6PD can be complex, we analyzed our data using G6PD phenotypes rather than genotypes. We confirmed that both age and G6PD-deficient phenotype significantly decreased *P. vivax* density (χ^2 test, $P < 0.001$ and $P = 0.039$, respectively) (16).

Although the precise mechanism underlying this protective effect is unknown, it is likely to be related to the effects of G6PD deficiency on red cell physiology, particularly by increasing oxidative stress. Young red cells (i.e., reticulocytes) contain more anti-oxidant enzymes than mature red cell populations (27). The preference shown by *P. vivax* for reticulocytes suggests that *P. vivax* is more sensitive to oxidative stress than *P. falciparum*, which is known to have no red-cell preference (28). Under these conditions, reduced G6PD activity would have a greater effect on *P. vivax*.

The historical expansion of malaria has been linked to that of agriculture, which generated breeding grounds for mosquitoes and increased human population density (29), thereby

facilitating human-mosquito contact and the conditions for stable malaria transmission. In East-Asia, farming is mainly associated with the development of rice culture in China ~8,000 YBP. Although there is evidence of rice cultivation in SE Asia dating back to 4,200 YBP, it developed mainly over the last 2,000 years (30). The Karen people, belonging to the Sino-Tibetan language group, are thought to descend from Tibetan people who entered Myanmar ~1,500 YBP (31). Interestingly, the estimated age of Mahidol^{487A} at ~1,500 YBP coincides with the proposed arrival of the Karens into the region and with the time at which rice started to be extensively cultured. This supports a link between the selective advantage conferred by the G6PD-Mahidol^{487A} mutation and its protective effect against *vivax* malaria.

In conclusion, we showed that the G6PD-Mahidol^{487A} mutation has been under strong positive selection for the last 1,500 years and that it reduces *P. vivax* parasite density in humans. These findings provide evidence that *vivax* malaria has been a driving force behind the selective advantage conferred by the Mahidol^{487A} mutation, supporting the notion that *P. vivax* historically had a considerable impact on human health (10), at least in SE Asia. Indeed, *P. vivax* infection is not only responsible for clinical malarial attacks, but has also been implicated in causing low birth weight (32) and malnutrition in children (33), both of which strongly impact upon childhood survival. The significant health burden imposed by *P. vivax* has been seriously underestimated and increased global efforts to combat malaria should encompass *P. vivax* as well as *P. falciparum* malaria.

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Supporting Online Material

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Materials and Methods

Fig. S1

Tables S1, S2, S3

References

Tables

Table 1 Joint estimates of the age and the selection coefficient of the Mahidol^{487A} mutation.

One generation is considered equivalent to 25 years. ML stands for Maximum-Likelihood and CI for Confidence Interval.

	Age (generations)	95% CI	Selection coefficient	95% CI
ML deterministic method (19)	60.8	53.7-73.6	0.235	0.20-0.30
Bayesian method (20)	64.4	38-94	0.228	0.16-0.40

Figure legends

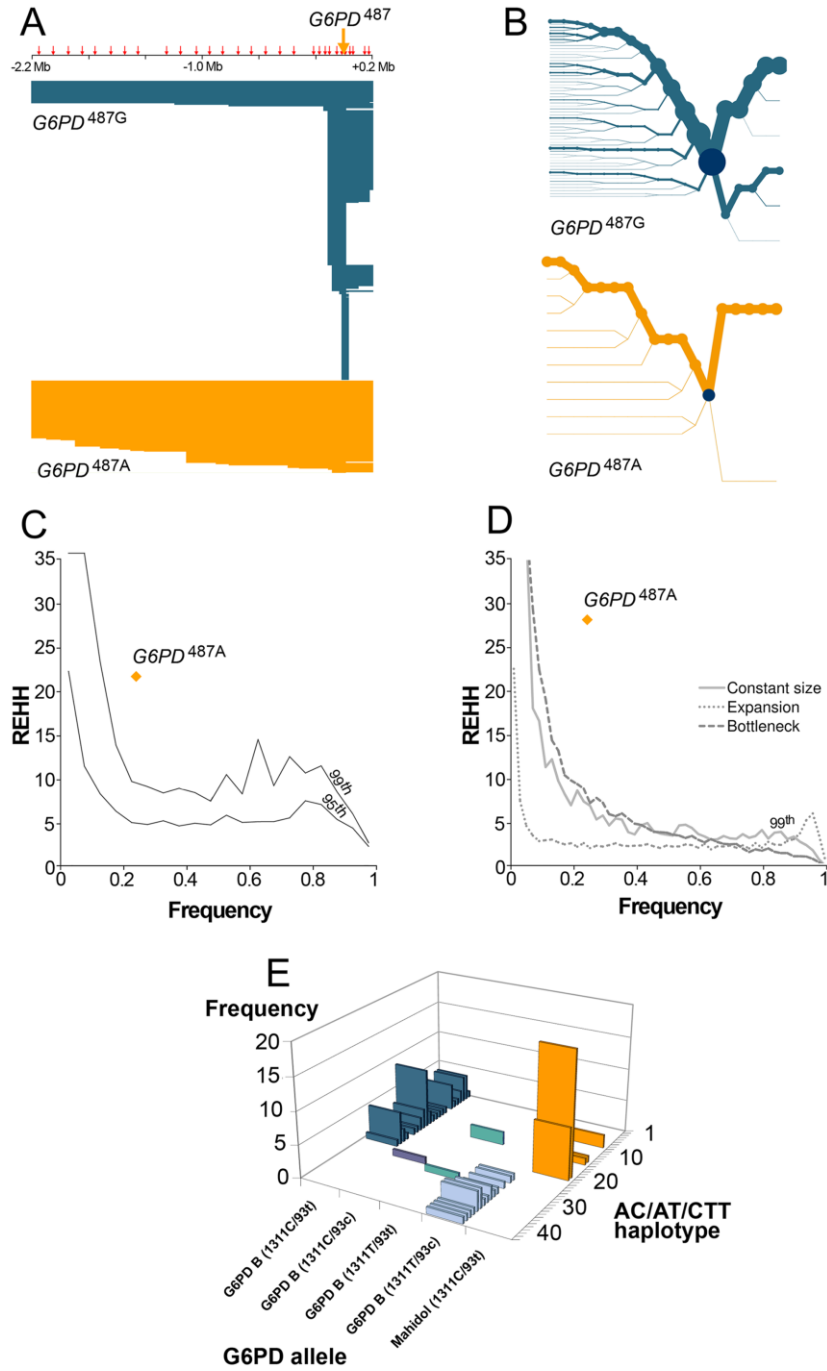


Fig. 1 Positive selection at the *G6PD*-Mahidol^{487A} mutation

(A) Comparison of extent of homozygosity flanking the *G6PD*^{487A} (Mahidol) and the *G6PD*^{487G}

(non-Mahidol) alleles over a 2.4-Mb region. Positions of the 30 genotyped SNPs are tagged by red arrows. **(B)** Haplotype bifurcation plots for the two $G6PD^{487}$ alleles. Line thickness represents the number of haplotypes in our sample. Each successive bifurcation corresponds to evidence for recombination at an increasing distance from the core $G6PD^{487}$ alleles (dark blue dots). **(C)** Relative extended haplotype homozygosity (REHH) of the $G6PD^{487A}$ allele (orange diamond) against an empirical distribution of REHH for the X chromosome in Han Chinese (17) matched for SNP density. **(D)** REHH of the $G6PD^{487A}$ allele (orange diamond) against simulated distributions under different demographic regimes (16). **(E)** Microsatellite diversity associated with different $G6PD$ lineages (16). The Mahidol^{487A} mutation was associated with low microsatellite diversity, in contrast with other $G6PD$ mutations at similar population frequencies.

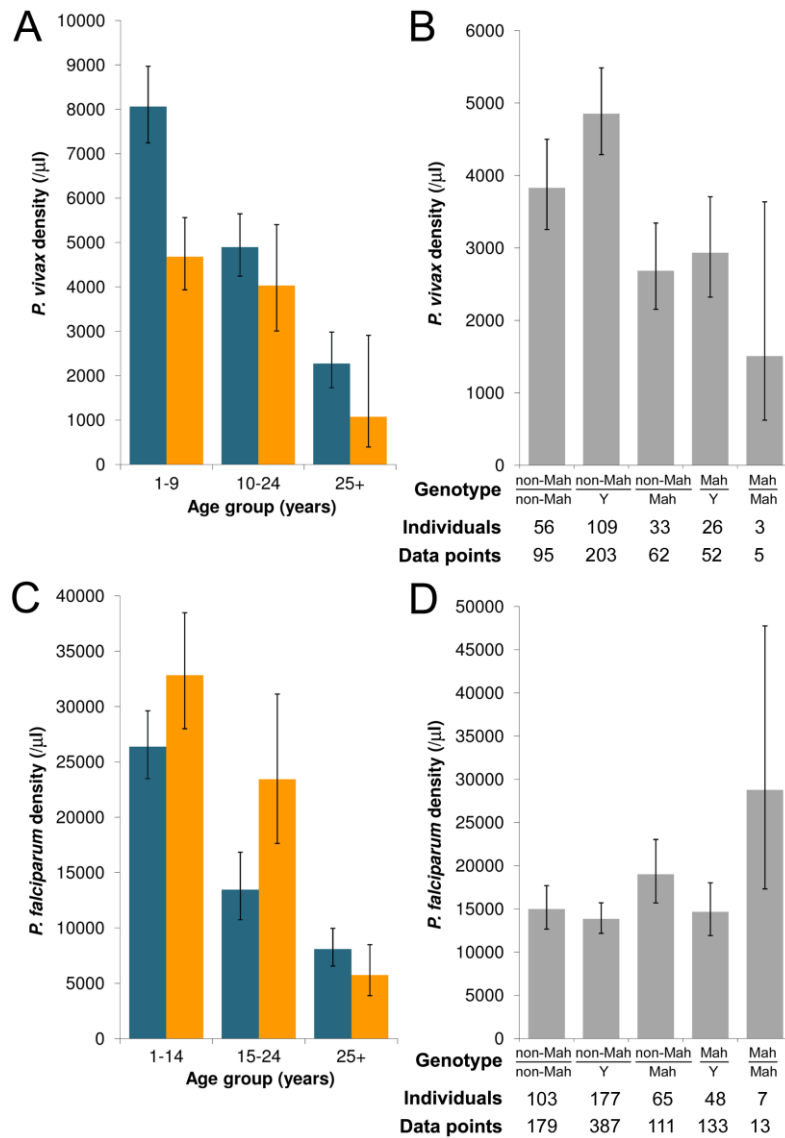


Fig. 2 Effect of G6PD status on parasite densities

(A) *P. vivax* and (C) *P. falciparum* parasite densities (mean \pm SD) according to age group and G6PD-Mahidol⁴⁸⁷ status (blue: non-Mahidol, orange: Mahidol). (B) *P. vivax* and (D) *P. falciparum* parasite densities (mean \pm SD) according to G6PD-Mahidol⁴⁸⁷ genotype status taking into account age (16). Data points refer to the total number of observations of parasite densities obtained for each group of individuals. Note that all data were used, including the FBAT informative families.