

HOST POLYMORPHISMS AND THE INCIDENCE OF MALARIA IN UGANDAN CHILDREN

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Abstract. Mutations in β -globin, glucose-6-phosphate dehydrogenase, and promoters for tumor necrosis factor- α and inducible nitric oxide synthase (iNOS) were examined for associations with the incidence of symptomatic malaria in a cohort of 307 Ugandan children. After adjustment of incidence rates for age, water source, use of preventative measures, and proximity to mosquito breeding sites, glucose-6-phosphate dehydrogenase A- heterozygous females had a significantly higher incidence of malaria (incidence rate ratio [IRR] = 1.63, P = 0.03) and a trend towards higher parasite densities (37,100 versus 26,200 parasites/ μ L; P = 0.18) compared with wild-type children. Male hemizygotes had trends in the same direction. Heterozygotes for sickle hemoglobin had trends toward a lower incidence of malaria and lower parasite density at presentation. Heterozygotes for the iNOS promoter G954C polymorphism, but not other promoter polymorphisms, had a significantly lower incidence of malaria compared with wild-type children (IRR = 0.69, P = 0.05). Host polymorphisms appear to impact upon the incidence of uncomplicated malaria in Ugandan children.

INTRODUCTION

The malaria parasite has had a substantial influence upon the genetic constitution of its host. Sickle hemoglobin (HbS) heterozygotes and glucose-6-phosphate dehydrogenase (G6PD)-deficient heterozygous (A-) females are protected against severe malaria.¹ Polymorphisms in immune mediators have also been studied, but associations between polymorphisms in promoters of the tumor necrosis factor- α (TNF- α ; G238A, G308A, and G376A) and inducible nitric oxide synthase (iNOS; G954C and C1173T) genes and severe malaria have been inconsistent.^{2–5}

Compared with effects on severe malaria, the effect of host polymorphisms on the overall incidence of malaria has received little attention. This is an important omission because the vast majority of malaria episodes are uncomplicated, and these cases, although not immediately life-threatening, have important public health consequences. To better characterize the impact of key polymorphisms on the incidence of malaria, we examined the effects of polymorphisms in the β -globin, G6PD, TNF- α , and iNOS genes upon the incidence of malaria in a previously described cohort of Ugandan children who were followed for one year.⁶

METHODS

Clinical study. Clinical data and blood samples came from a longitudinal study that took place between July 2000 and August 2001 in Kampala, Uganda, the details of which have been published elsewhere.⁶ In this urban area, malaria is mesoendemic with peaks during two rainy seasons (Ugandan Ministry of Health, unpublished data). Three hundred sixteen healthy children (six months to five years old) were enrolled from the community using convenience sampling and followed for one year using both active and passive case surveillance. All study households were located in the Kawempe district surrounding the study clinic at Mulago Hospital. The clinical study and the subsequent evaluation of host polymorphisms were reviewed and approved by the institutional review boards of the University of California, San Francisco and Makerere University, Kampala.

Assessment of malaria incidence. Upon enrollment, children were randomly assigned to receive sulfadoxine-pyrimethamine (SP), SP plus amodiaquine, or SP plus arte-

unate for treatment of all episodes of malaria that were identified during follow-up.⁶ Parents/guardians were instructed to bring their child to the clinic whenever they needed medical attention and to avoid using any drugs not administered or approved by a study physician. When a child presented with a history of fever (previous 48 hours) or a tympanic temperature $\geq 38.0^{\circ}\text{C}$, a thick blood smear was prepared. Patients were diagnosed with symptomatic malaria if they had 1) a tympanic temperature $\geq 38.0^{\circ}\text{C}$ and any parasitemia, 2) a history of fever and ≥ 500 asexual parasites/ μ L, or 3) severe malaria or danger signs and any parasitemia.⁶ Patients with uncomplicated malaria were treated with assigned regimens; those with severe malaria were treated with quinine. For recurrent episodes, molecular genotyping was performed based on polymorphisms in merozoite surface protein-2 to distinguish recrudescence (treatment failure) from new infections, as previously described.⁷ For the study of associations with genetic polymorphisms, malaria incidence density was based only on new infections and did not include episodes due to recrudescence (treatment failure).⁶ Time at risk for new infection was defined as the duration of study participation minus 14 days after each episode of malaria.

Assessment of host genetic polymorphisms. Blood was collected on filter paper at enrollment and every time an episode of malaria was diagnosed. For genetic analysis, DNA was extracted from filter paper with Chelex (Bio-Rad Laboratories, Hercules, CA). Polymorphisms were detected using nested polymerase chain reaction (PCR) amplification followed by restriction endonuclease digestion (Table 1). For each reaction, 1–2 μ L of Chelex-extracted sample was incubated with *Taq* polymerase (Invitrogen, Carlsbad, CA), 200 μ M dNTPs, and buffer (200 mM Tris, pH 8.4, 500 mM KCl). Primer concentrations and MgCl_2 were optimized for each reaction. After first-round and nested PCRs, 5 μ L of each reaction was digested with restriction endonucleases. Digestion products were subjected to electrophoresis on 2.5% NuSieve agarose (FMC Bioproducts, Rockland, ME) gels and visualized with ethidium bromide. Genotypes were assessed based on comparison of the sizes of reaction products and controls after digestion.

In sub-Saharan Africa, a G6PD deficiency is most commonly caused by two X-linked mutations, A376G and G202A, leading to the G6PD A- genotype.⁸ Since the G202A

TABLE 1
Primers and conditions for polymorphism detection*

Genes	PCR round	Primers	Annealing temperature	Mutation	Restriction enzyme	Reference
Sickle cell and Hb C	First	5'-TCCATCTACATATCCCAAAGC-3' 5'-AGAAAACATCAAGGGTCCCA-3'	54°C	G16A (HbS)	<i>Bse</i> Ri	
	Nested	5'-GTGCCAGAAGAGCCAAGGAC-3' 5'-GCTGGTGGTCTACCCCTTGGGA-3'	61°C	A17T (HbC)	<i>Dde</i> I	
G6PD deficiency (A- allele)	Single	5'-GTGGCTGTTCCGGGATGGCCTTCTG-3' 5'-CTTGAAGAAGGGCTCACTCTGTTTG-3'	56°C	G202A	<i>Nla</i> III	18
iNOS promoter	First	5'-CGGCCATCTTGTCACTTTCTAA-3' 5'-GGGAGATTTTTCTCAGC-3'	54°C			
	Nested	5'-CATATGTATGGGAATACTGTATTTCAGGC-3' 5'-TCTGAACTAGTCACTTGAGG-3'	62°C	G954C	<i>Bsa</i> I	17
	Nested	5'-CTCCATAAGCCAGAGCTCTAA-3' 5'-CCACCACACCCAGCTAATATTT-3'	56°C	C1173T	<i>Fok</i> I	
TNF- α promoter	First	5'-GACCCAAACACAGGCCTCA-3' 5'-TCGAGTTGCTTCTCCCTCTT-3'	58°C			
	Nested	5'-GGCAATAGGTTTGAGGGCCATG-3' 5'-CACACTCCCCATCCTCCCTGATC-3'	58°C	G238A G308A	<i>Alw</i> I <i>Nco</i> I	19
	Nested	5'-TCAACACAGCTTTTCCCTCCAA-3' 5'-ATCTGGAGGAAGCGGTAGTG-3'	60°C	G376A	<i>Tsp</i> 509I	

* PCR = polymerase chain reaction; Hb = hemoglobin; G6PD = glucose-6-phosphate dehydrogenase; iNOS = inducible nitric oxide synthase; TNF- α = tumor necrosis factor- α .

mutation generally occurs only in the background of the A376G mutation, and both mutations are required for a significant deficiency, we assessed only for the G202A mutation.

Statistical methods. Associations between host polymorphisms and malaria incidence were estimated using a multivariate negative binomial regression model, controlling for clustering within households. In previous analysis in this same cohort of children, age, primary source of water, use of malaria preventative measures, and proximity to mosquito breeding sites were identified as independent predictors of malaria incidence.^{9,10} The final model included treatment regimen and all four known predictors of incidence. Geometric mean parasite densities were compared using generalized estimating equations with exchangeable correlation and robust standard errors to control for repeated measures within the same patient. A *P* value < 0.05 was considered statistically significant. Analysis was done using STATA statistical software version 8.0 (StataCorp., College Station, TX).

RESULTS

Of the 316 patients enrolled in the clinical study, 234 (74%) were located and all provided informed consent for this study; the remaining samples from individuals not located were delinked from patient identifiers. Of the enrolled children, 307 had at least six weeks of follow-up and were included in this analysis. The incidence of malaria varied widely in our study population. No episodes of malaria were recorded in 123 of the 307 children (40%), while in the remaining 184 children, 519 new episodes of symptomatic malaria were recorded (range = 1–9 per child). The cumulative period of observation covered 93% of potential follow-up time and 282 children (92%) completed the full one-year of follow-up. The PCR-based methods of polymorphism detection were successful for all evaluated mutations in more than 98% of the cases. Treatment group was included as a potential covariate in our analysis, but had no impact upon incidence density for any of the studied polymorphisms.

G6PD A- heterozygous females, hemizygous males, and homozygous females comprised 12% (36 of 303), 8% (25 of 303), and < 1% (1 of 303) of our study population, respectively. Crude annual malaria incidence rates were 1.88 for G6PD wild-type, 2.09 for G6PD A- male hemizygotes, and 1.95 for female heterozygotes. After controlling for previously identified predictors of malaria incidence (age, water source, use of preventative measures, and proximity to mosquito breeding sites), a significantly higher malaria incidence rate ratio for heterozygous G6PD A- females compared with wild type individuals was noted (Table 2). In addition, heterozygous females had a trend toward higher geometric mean parasite densities upon malaria diagnosis compared with those lacking the G6PD A-mutations (37,100 parasites/ μ L versus 26,200 parasites/ μ L; *P* = 0.18). G6PD A- hemizygous males showed similar trends, with a higher incidence of malaria and higher parasite density upon diagnosis.

There were 48 (16%) HbS heterozygotes and one HbS homozygote in the cohort. The crude annual malaria incidence rates were 2.04 for wild type and 1.49 for HbS heterozygotes. With adjustment for known predictors of malaria incidence, HbS heterozygotes had a non-significant trend toward a lower malaria incidence density ratio and a lower parasite density upon diagnosis of malaria, compared with wild-type children (Table 2). Only two children were heterozygous for hemoglobin C (A17T β -globin mutation).

There were 55 (18%) iNOS G954C heterozygotes and no homozygotes in the cohort. The crude annual malaria incidence rates were 1.97 for wild-type and 1.73 for heterozygous children. With adjustment for known predictors of malaria incidence, G954C heterozygotes had a significantly lower malaria incidence rate ratio than wild-type children (Table 2). Parasite densities did not differ between the two groups.

The TNF- α polymorphisms (G238A, G308A, and G376A) and the iNOS polymorphism C1173T did not show significant associations with either incidence or parasite density (Table 2). Of note, all TNF -376 heterozygotes and homozygotes were heterozygous or homozygous at the TNF -238 locus.

TABLE 2
Impact of host polymorphisms upon the incidence of uncomplicated malaria*

Host polymorphism	Number of patients	Adjusted incidence rate ratio†	95% CI	P	Number of malaria episodes	Mean parasite density‡	P
G6PD deficiency							
Heterozygous female	36	1.63	1.0–2.6	0.03	88	37,100	0.18
Homo/hemizygotes	26	1.29	0.8–2.0	0.24	60	29,400	0.65
Wild-type	241	Reference	–	–	504	26,200	Reference
Sickle cell anemia§							
Heterozygotes	48	0.72	0.5–1.1	0.14	81	21,700	0.19
Wild-type	256	Reference	–	–	575	29,000	Reference
Nitric oxide synthase							
Heterozygote –954	55	0.69	0.50–1.0	0.05	104	26,700	0.78
Wild-type –954	252	Reference	–	–	552	28,200	Reference
Heterozygote –1173	25	1.33	0.8–2.3	0.29	50	26,600	0.88
Wild-type –1173	282	Reference	–	–	606	28,100	Reference
TNF-α promoter							
Heterozygote –238	57	1.03	0.7–1.6	0.88	118	24,800	0.58
Homozygote –238	8	1.03	0.5–2.0	0.94	22	32,500	0.67
Wild-type –238	239	Reference	–	–	515	28,600	Reference
Heterozygote –308	48	0.88	0.6–1.3	0.55	93	30,500	0.60
Wild-type –308	254	Reference	–	–	561	27,600	Reference
Heterozygote –376	27	1.04	0.7–1.6	.86	60	21,000	0.34
Wild-type –376	274	Reference	–	–	590	28,700	Reference

* CI = confidence interval. For definitions of other abbreviations, see Table 1.

† Adjusted for distance to swamp or stream, use of preventive measures, water source, age, and treatment regimen.

‡ Geometric mean parasite density (parasites/ μ L) at the time of diagnosis of symptomatic malaria.

§ Data were excluded for two children who were heterozygous for hemoglobin C, and one child who was homozygous for hemoglobin S.

DISCUSSION

In our longitudinal study of children in Kampala, significant associations were seen between G6PD and iNOS polymorphisms and malarial incidence. Relationships between host polymorphisms and malarial incidence were complex, and larger studies will be required to both confirm these associations and to appreciate less dramatic associations, such as the protective trend seen with sickle cell heterozygosity and malarial incidence. Our study benefited from data previously collected from our cohort regarding other predictors of malaria incidence.^{9,10} Adjustment for these other data in a multivariate model allowed for improved determination of the causal association of host polymorphisms with incidence. In summary, it appears that in addition to effects on severe malaria, host polymorphisms impact upon rates of uncomplicated malaria.

G6PD A– heterozygous females and hemizygous males had a higher incidence of uncomplicated malaria and higher parasite densities when presenting with malaria compared with wild type children. Our results might be seen as surprising, since G6PD A– females (and in some studies hemizygous males) were protected against severe malaria.¹¹ However, our results are consistent with two recent longitudinal studies in Gabon, in which G6PD A– females had significantly higher incidences of uncomplicated malaria.^{12,13} Some other studies have reported a protective effect of G6PD deficiency in uncomplicated malaria, but these studies were case-control or cross-sectional in design, and thus not best suited for this analysis.⁸ In addition, older studies often relied on phenotypic G6PD assessment, a method known to be inaccurate, particularly in heterozygotes.⁸

Our findings, in addition to two prior studies, now suggest that G6PD-deficient individuals have an increased incidence of malaria.^{12,13} How can we reconcile the findings that G6PD deficiency protects against severe malaria but is associated

with an increased overall incidence of malaria? The protection afforded by G6PD deficiency against severe malaria has been attributed to a diminished ability of parasites to survive additional oxidative stress in deficient erythrocytes, a finding that has been confirmed *in vitro*, although differences in growth between parasites in wild-type and G6PD deficient erythrocytes have been modest.¹⁴ We hypothesize that increased malarial incidence renders G6PD-deficient individuals more immune, and thus better able to control malaria once it has progressed to clinical illness. Similarly, it was hypothesized that α -thalassemia was selected in Vanuatu despite an increased incidence of malaria, as increased incidence protected against severe malaria.¹⁵ Our hypothesis agrees with prior suggestions that a G6PD deficiency has been maintained in humans due to the selective pressure of malaria, but suggests that it has been selected, at least in part, due to an increased predilection for clinical malaria in heterozygotes.

We also found that G6PD A– individuals had a trend towards higher parasitemias when they presented with malaria, although this finding differed from two other studies, and so must be interpreted with caution.^{12,13} Nonetheless, it offers support for the hypothesis that higher rates of malaria in G6PD deficient children lead to higher levels of immunity, thus requiring greater parasitemias for the expression of clinical illness.

We also identified a lower incidence of malaria in heterozygotes for the iNOS G954C mutation (which led to higher iNOS activity in some studies) compared with wild-type children.^{16,17} A study in Gabon found that the –954C allele was correlated with both higher baseline iNOS activity and protection, with a delay in the development of clinical malaria after prior treatment.¹⁷ Other studies in Tanzania and Gabon found no association between G954C and cerebral malaria or the incidence of uncomplicated malaria, although the latter study did not include younger children most likely to benefit from protection afforded by higher levels of nitric oxide. Our

result suggests a protective role for this mutation against malaria in Ugandan children.

Significant associations were not identified between malarial incidence and the other polymorphisms studied, although trends toward protection in sickle hemoglobin heterozygotes were consistent with other studies showing protection against severe malaria and trends toward protection against malarial incidence.¹ Differences in the associations seen in this cohort and others may relate to variations in the impact of these polymorphisms upon severe compared with uncomplicated malaria, or may relate to the interplay of other polymorphisms not studied in this cohort. Additional longitudinal studies with larger sample sizes will be needed to more fully evaluate associations between these host polymorphisms and the incidence of malaria.

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