BRIEF REPORT

Differential Prevalence of Transporter Polymorphisms in Symptomatic and Asymptomatic Falciparum Malaria Infections in Uganda

Stephen Tukwasibwe,[1] Levi Mugenyi,[1] George W. Mbogo,[1] Sheila Nankoberanyi,[1] Catherine Maiteki-Sebuguzi,[1] Moses L. Joloba,[2] Samuel L. Nsobya,[1] Sarah G. Staedke,[3] and Philip J. Rosenthal[4]

[1]Infectious Diseases Research Collaboration, [2]Makerere University, Kampala, Uganda; [3]London School of Hygiene and Tropical Medicine, United Kingdom; and [4]University of California, San Francisco

We explored associations between *Plasmodium falciparum* drug resistance–mediating polymorphisms and clinical presentations in parasitemic children enrolled in a cross-sectional survey in Tororo, Uganda, using a retrospective case-control design. All 243 febrile children (cases) and 243 randomly selected asymptomatic children (controls) were included. In a multivariate analysis adjusting for age, complexity of infection, and parasite density, the prevalence of wild-type genotypes was significantly higher in febrile children compared to asymptomatic children (*pfcrt* K76T: odds ratio [OR] 4.41 [95% confidence interval {CI}, 1.28–15.1]; *pfmdr1* N86Y: OR 4.08 [95% CI, 2.01–8.31], and *pfmdr1* D1246Y: OR 4.90 [95% CI, 1.52–15.8]), suggesting greater virulence for wild-type parasites.

Keywords. fitness; malaria; *Plasmodium*; polymorphism; virulence.

Malaria, particularly infection with *Plasmodium falciparum*, remains one of the most important infectious diseases in the world. A major challenge to the treatment and control of malaria has been resistance to most available antimalarial drugs. In light of this problem, artemisinin-based combination therapy (ACT), including a potent and rapid-acting artemisinin (artesunate, artemether, or dihydroartemisinin) and a longer-acting partner drug (lumefantrine, amodiaquine, piperaquine, or mefloquine), is now the standard of care for the treatment of falciparum malaria. However, resistance to artemisinins, manifest as delayed parasite clearance after therapy, is increasing in parts of Southeast Asia, and resistance has been seen to most artemisinin partner drugs [1]. Altered sensitivity to a number of drugs is mediated in part by polymorphisms in *pfcrt* and *pfmdr1*, genes encoding 2 putative *P. falciparum* transport proteins. The *pfcrt* K76T mutation is the major mediator of resistance to chloroquine and amodiaquine [2]. Polymorphisms in *pfmdr1* impact upon sensitivity to a number of drugs; considering polymorphisms that are common in Africa, the N86Y and D1246Y mutations are associated with decreased sensitivity to chloroquine and amodiaquine, but these same mutations mediate increased sensitivity to lumefantrine, mefloquine, and dihydroartemisinin [1].

Considering the strong and, at times, reciprocal pressures of antimalarial drugs on parasite genetics and the impacts of transporter polymorphisms on sensitivity to important ACT components, it is of interest to determine their effects on parasite fitness and virulence. Considering fitness, valuable insight has come from experiences in areas where widespread chloroquine resistance led to discontinuation of the drug. In Malawi and other areas, discontinuation of chloroquine for the treatment of malaria led to dramatic changes in circulating parasites, with the return of chloroquine-sensitive (*pfcrt* wild type) parasites and also of strong antimalarial efficacy for chloroquine [3]. Clearly, chloroquine-sensitive parasites have a fitness advantage over resistant parasites. Considering polymorphisms in *pfmdr1*, in in vitro competitive growth experiments, wild type parasites had a fitness advantage over those with 3 polymorphisms, only 1 of which (1246Y) is common in Africa [4]. When mixed clinical isolates were followed in culture, modest fitness advantages appeared associated with the mutant 86Y and wild type D1246 alleles [5]. Comparisons of parasites circulating during high and low transmission seasons showed the prevalence of parasites with mutant *pfcrt* 76T and *pfmdr1* 86Y sequences to decrease during the low transmission season, when drug pressure is lowest, implying a fitness advantage for wild type parasites [6]. Overall, although measures of parasite fitness are limited and imperfect, in most cases parasites with wild type sequences in *pfcrt* and *pfmdr1* have appeared to have a fitness advantage over mutant parasites.

Considering virulence, chloroquine resistant *P. falciparum* is fully capable of causing frequent and severe malaria, and it is not clear if parasites with resistant or sensitive genotypes differ

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Correspondence: Philip J. Rosenthal, MD, Department of Medicine, San Francisco General Hospital, Bldg 30, Rm 421/402/405, 1001 Potrero Ave, Box 0811, San Francisco, CA 94110 (philip.rosenthal@ucsf.edu).

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in their abilities to cause disease. Studies from India [7] and Mali [8], but not Sudan [9] or Gabon [10] showed associations between presence of the *pfcrt* 76T mutation and severe malaria, but when seen these associations may have been due to clinical progression after chloroquine treatment failure, rather than variations in parasite virulence. To further explore associations between *P. falciparum* resistance-mediating polymorphisms and parasite fitness or virulence, we studied differences in genotypes between parasites causing symptomatic and asymptomatic infections in Ugandan children.

METHODS

In this retrospective case-control study, we evaluated samples collected in a cross-sectional survey conducted from December 2010 to June 2011 in Tororo District, a rural area with very high malaria transmission intensity. The survey was a component of the PRIME trial [11], which is registered at Clinicaltrials.gov (NCT01024426). Briefly, children under 15 years of age were recruited from randomly selected households in 20 geographical clusters. One child under 5 years and 1 child aged 5–15 years were eligible for enrollment from each household. Upon informed consent and enrollment, children underwent a brief clinical examination, including measurement of temperature with an electronic tympanic thermometer, and collection of a finger-prick blood sample for a thick blood smear and storage on filter paper for molecular studies. Thick blood smears were stained with 2% Giemsa for 30 minutes and read by experienced laboratory technologists. Parasite densities were calculated by counting the number of asexual parasites per 200 leukocytes (or per 500 leukocytes, if the count was <10 asexual parasites per 200 leukocytes), assuming a leukocyte count of 8 000/μL. A blood smear was considered negative when the examination of 100 high-power fields did not reveal asexual parasites. For quality control, all slides were read by a second microscopist, and a third reviewer settled any discrepant readings. The PRIME trial was approved by the Uganda National Council of Science and Technology, Makerere University School of Medicine Research and Ethics Committee, the London School of Hygiene and Tropical Medicine Institutional Review Board, and the University of California, San Francisco Committee on Human Research.

The analysis for this study was restricted to samples collected from children with positive blood smears. All samples from parasitemic children with a documented fever (temperature ≥38.0°C tympanic) were selected as cases, and an equal number of samples from randomly selected children with asymptomatic parasitemia (lacking history of fever in the past 48 hours or fever documented by elevated temperature) were selected as controls. For all study samples, polymorphisms at the *pfcrt* K76T, *pfmdr1* N86Y, and *pfmdr1* D1246Y alleles were assessed by polymerase chain reaction followed by restriction fragment length polymorphism analysis, as previously described [12]. Complexity of infection (COI) was assessed based on polymorphisms in the merozoite surface protein-2 gene, as previously reported [13]. Data were entered and verified using GraphPad Prism 5 software and were analyzed using STATA (version 12.0, College Station, TX). Multivariate analysis was conducted with generalized estimating equations using a binomial family, exchangeable correlation coefficients, robust standard errors, and adjustment for samples from the same cluster. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate the strengths of associations.

Table 1. Characteristics of Study Children and Available Assay Results

	Febrile (Cases; N = 243)	Asymptomatic (Controls; N = 243)	*P* Value[a]
Mean age in years (SD)	4.2 (3.0)	6.2 (3.9)	<.001
Children <5 y (%)	165 (67.9)	122 (50.2)	<.001
Mean COI (SD)	3.3 (1.7)	2.6 (1.4)	<.001
Geometric mean parasite density (95% CI)	4742 (3615–6219)	1120 (911–1378)	<.001
pfcrt K76T			
Genotyping[b] results available (%)	240 (98.8)	214 (88.1)	<.001
Genotyping[b] and COI results available (%)	211 (86.8)	184 (75.7)	.002
pfmdr1 N86Y			
Genotyping[b] results available (%)	243 (100.0)	229 (94.2)	<.001
Genotyping[b] and COI results available (%)	214 (88.1)	194 (79.8)	.013
pfmdr1 D1246Y			
Genotyping[b] results available (%)	207 (85.2)	231 (95.1)	<.001
Genotyping[b] and COI results available (%)	182 (74.9)	195 (80.3)	<.001

Abbreviation: COI, complexity of infection.

[a] *P* values comparing mean age and COI were based on a 2-sample *t* test and comparing parasite density on a 2-sample Wilcoxon rank-sum (Mann–Whitney) test. *P* values for categorical variables were based on the χ^2 test.

[b] Genotyping consisted of characterization of the indicated polymorphism.

Table 2. Associations Between Parasite Genotypes and Clinical Presentations

			Unadjusted Results		Adjusted Results[a]	
Polymorphism	Febrile Subjects (%)	Asymptomatic Subjects (%)	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
K76T						
Mutant	219 (91.3)	211 (98.6)	Reference	. . .	Reference	. . .
Mixed	21 (8.8)	3 (1.4)	6.20 (1.97–19.5)	.002	4.41 (1.28–15.1)	.02
N86Y						
Mutant	40 (16.5)	64 (27.9)	Reference	. . .	Reference	. . .
Mixed	88 (36.7)	118 (51.5)	1.16 (.55–2.44)	.69	1.02 (.43–2.40)	.97
Wild type	115 (47.9)	47 (20.5)	3.67 (1.87–7.23)	<.001	4.08 (2.01–8.31)	<.001
D1246Y						
Mutant	72 (34.8)	86 (37.2)	Reference	. . .	Reference	. . .
Mixed	111 (53.6)	138 (59.7)	0.97 (.58–1.62)	.91	0.91 (.52–1.59)	.73
Wild type	24 (11.6)	7 (3.0)	4.13 (1.81–9.43)	.001	4.90 (1.52–15.8)	.008

Abbreviations: CI, confidence interval; OR, odds ratio.

[a] Adjusted for age, complexity of infection, parasite density, and clustering.

RESULTS AND DISCUSSION

Of 8798 children enrolled in the cross-sectional survey, 4391 (49.9%) were under 5 years of age and 5331 (60.6%) had a positive blood smear. Of these parasitemic children, 243 (4.6%) had a documented fever and were selected as cases and 3149 (59.1%) were asymptomatic; the remaining 1939 (36.4%) reported fever in the past 48 hours, but were afebrile on exam. A total of 243 samples were randomly selected from the asymptomatic group to act as controls. Children with documented fever were younger than the asymptomatic children (Table 1). Both mean COI and geometric mean parasite density were higher in the febrile children. Of the selected samples, genotyping results were available for 454 (93.4%) for *pfcrt* K76T, 472 (97.1%) for *pfmdr1* N86Y, and 438 (90.1%) for *pfmdr1* D1246Y, and these results were included in the unadjusted analysis. The adjusted analysis included only those samples for which both genotyping and COI data were available, including 395 (81.3%) for *pfcrt* K76T, 408 (84.0%) for *pfmdr1* N86Y, and 377 (77.6%) for *pfmdr1* D1246Y.

For the *pfcrt* K76T polymorphism, no samples had a pure wild-type genotype, consistent with the known high prevalence of the mutant genotype in Uganda, but the mixed genotype was more common in children with fever than in those who were asymptomatic (Table 2). Similarly, for both *pfmdr1* genotypes, the prevalence of wild-type genotypes was greater in febrile compared to asymptomatic children. The association between wild-type genotypes (or for K76T, the mixed genotype) and fever was significant for all 3 polymorphisms in both an unadjusted analysis and in a multivariate analysis, including age, COI, parasite density, and adjustment for clustering (Table 2).

Our results indicate that, in a region of very high malaria transmission intensity, children harboring *P. falciparum* with wild-type genotypes were more likely to be febrile than those with parasites containing mutations associated with resistance to chloroquine and amodiaquine. These results suggest that wild-type parasites are more capable of causing clinical illness than those with resistance-mediating mutations in *pfcrt* and *pfmdr1*. Thus, resistance-mediating polymorphisms may come with some cost in decreased parasite virulence. This decreased virulence is likely due to a loss of fitness of mutant erythrocytic parasites, but it is not clear if the mutant parasites also have decreased fitness at other parasite stages (in particular, mosquito stages). If mutant parasites are less readily transmitted than wild type, this might have important implications for the spread of resistance to different drugs. Similar results were seen in a recent study from Benin [14]. This study compared parasites collected from symptomatic and asymptomatic children, but it was different in design from our study, as it was a case-control study comparing ill children presenting to clinics with matched community controls. In Benin, the prevalence of the *pfcrt* 76T mutation was very high, and was unchanged between symptomatic and asymptomatic children. However, the *pfmdr1* 86Y mutation was more prevalent in asymptomatic compared to symptomatic children, which is consistent with our results. The *pfmdr1* 1246Y mutation is uncommon in West Africa, and was not studied. Of interest, parasites harboring the *pfcrt* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y mutations have decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to other important antimalarials, including artemisinins, lumefantrine, and mefloquine. Thus, the wild-type parasites that we found to be more likely to cause symptomatic malaria are less susceptible to key drugs than mutant parasites. In other words, the polymorphisms that appear to increase the virulence of malaria parasites also increase the likelihood that parasites will be less sensitive to key antimalarials, including both components

of the Ugandan national regimen, artemether-lumefantrine, possibly resulting in inadequate treatment. These results are concerning, as they suggest that the same parasite polymorphisms that favor survival despite treatment with artemether-lumefantrine also favor progression of infection to clinical illness. Consideration might be given for adding different treatment regimens with varied selective pressures. Artesunate-amodiaquine is an alternative regimen that is currently little used in Uganda, and selects for the opposite polymorphisms selected by artemether-lumefantrine. Increased use of artesunate-amodiaquine might counter selection toward more virulent malaria parasites. However, it is important to note that the studied *pfmdr1* polymorphisms have only modest effects on drug sensitivity, and recent trials have shown continued excellent antimalarial efficacy for artemether-lumefantrine in Uganda and other parts of Africa.

One limitation of this study was that, due to technical factors, parasite polymorphism results were unavailable in up to 15% and COI data unavailable in up to 25% of subjects in different groups. Thus, results may not have been representative of those of the entire sample set, potentially introducing bias. In addition, our genotyping method was not highly sensitive, and some samples categorized as pure wild type or mutant may have included minority populations that were missed. Another important limitation was that, due to the cross-sectional design of the study, reliable information on prior treatment histories of study subjects was not available. Prior treatment with artemether-lumefantrine leads to increased prevalence of *pfmdr1* wild-type genotypes in parasites, causing subsequent infections within 2 months after treatment [15]. We cannot exclude the possibility that symptomatic parasitemic children were more likely than asymptomatic parasitemic children to have had recent prior episodes of malaria that were treated with artemether-lumefantrine, and thus that prior treatment rather than inherent differences in parasite virulence explained the association between wild-type genotypes and symptomatic malaria.

In summary, wild-type sequences at 3 polymorphic *P. falciparum* alleles were associated with symptomatic falciparum malaria. For all 3 alleles, the wild-type sequences are also associated with decreased sensitivity to both components of artemether-lumefantrine, the current national malaria regimen in Uganda, and these alleles are selected by treatment with artemether-lumefantrine. Additional study is needed in other populations to determine if the identified relationships are seen in settings with lower malaria transmission intensity and to rule out the influence of prior malaria therapy on parasite genotypes. Nonetheless, it is remarkable that the odds of experiencing symptomatic malaria were over 4-times higher in children infected with parasites harboring wild-type sequences at any of the 3 studied alleles than in those with mutant parasites.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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