Minimal Association of Common Red Blood Cell Polymorphisms with Plasmodium falciparum Infection and Uncomplicated Malaria in Papua New Guinean School Children

Enmoore Lin, Livingstone Tavul, Pascal Michon, Jack S. Richards, Elijah Dabod, James G. Beeson, Christopher L. King, Peter A. Zimmerman, and Ivo Mueller*

Papua New Guinea Institute of Medical Research, Papua, New Guinea; Walter and Eliza Hall Institute of Medical Research, Infection and Immunity Division, Royal Parade, Parkville, Australia; Center for Global Health and Diseases, Case Western Reserve University, Cleveland, Ohio

Abstract. Southeast Asian ovalocytosis (SAO), α-thalassemia, and low expression of complement receptor 1 (CR1) have been associated with protection against severe Plasmodium falciparum malaria. In a cohort of children 5–14 years of age the effect of α-thalassemia, SAO (SLC4A1Δ27), CR1 polymorphisms, and Gerbich negativity (GYPCΔex3) on risk of P. falciparum infections and uncomplicated illness were evaluated. The risk of acquiring polymerase chain reaction (PCR)-diagnosed P. falciparum infections was significantly lower for α-thalassemia heterozygotes (hazard ratio [HR]: 0.56) and homozygotes (HR: 0.51) than wild-type children. No such differences were seen in light of microscopy diagnosed infections (P = 0.71) or were α-thalassemia genotypes associated with a reduced risk of uncomplicated P. falciparum malaria. No significant associations between the risk of P. falciparum infection or illness were observed for any of the other red blood cell polymorphisms (P > 0.2). This suggests that these polymorphisms are not associated with significant protection against P. falciparum blood-stage infection or uncomplicated malaria in school-aged children.

INTRODUCTION

A number of red blood cell (RBC) polymorphisms occur at high frequencies in Papua New Guinea (PNG) in geographical areas where malaria is endemic. Throughout the world, epidemiological and clinical studies suggest that these polymorphisms have arisen as a result of positive selection by malaria. Polymorphisms underlying sickle cell anemia [β-globin (HBB1) amino acid substitution glutamate → valine codon 6), α-thalassemia (α-globin (HBA1) deletions), southeast Asian ovalocytosis (SAO; [SLC4A1] 27-bp deletion), and G6PD deficiency have been associated with protection against severe Plasmodium falciparum malaria (reviewed in Reference 1). Additional polymorphisms associated with Gerbich negativity and low levels of complement receptor 1 (CR1, CD35) expression on RBCs may be of additional relevance in PNG. How and low levels of complement receptor 1 (CR1) expres-

prevalence/density or age-specific seroconversion to P. falciparum variant surface antigens. The effect of α-thalassemia on risk of uncomplicated disease in older Melanesian children has not been investigated previously.

Studies in vitro have shown that Gerbich negativity, caused by an exon 3 deletion of the glycophorin C gene (GYPCΔex3), and SAO erythrocytes may be less susceptible to invasion by P. falciparum. However, cross-sectional and case-control studies have so far not found any significant associations between SAO or GYPC genotypes and prevalence or density of P. falciparum infections. To date, no longitudinal studies, which are less prone to bias and confounding factors, have been performed on either polymorphism and no studies investigating the role of GYPCΔex3 and symptomatic malaria have been reported. Although expression of CR1 on the erythrocyte surface appears to be involved in rosetting and polymorphisms in CR1 have been associated with resistance to severe malaria, no studies have yet looked at the relationship between CR1 polymorphisms and uncomplicated malaria.

To address these gaps in our knowledge, we have examined associations of SAO, α-thalassemia, GYPCΔex3, and CR1 expression polymorphisms with susceptibility to P. falciparum infection and uncomplicated malaria episodes = in the context of a prospective longitudinal treatment-reinfection study in children 5–14 years of age in PNG.

METHODS

Study design and human subjects. Details of the study design have been previously reported. Briefly, 206 elementary/primary school students (51.5% female; 5 to 14 years of age) from Madang Province (Mugil and Megiar) participated in a treatment-reinfection study from June to December 2004. After an in-depth baseline assessment, all children were treated with a course of 7-day artesunate monotherapy that successfully removed blood stage infections in all but 12 (5.9%) children. Children were actively followed up every 2 weeks at school and passively at the local health center for a total of 6 months. Only children with fever and light microscopy-confirmed malaria infection were treated according to PNG national treatment guidelines with chloroquine plus sulfadoxine-pyrimethamine.

*Address correspondence to Ivo Mueller, Papua New Guinea Institute of Medical Research, P.O. Box 60, Goroka, EHP 441, Papua, New Guinea. E-mail: ivomueller@fastmail.fm
At every study contact a blood sample was collected for diagnosis of malarial infections by light microscopy (LM) and post-polymerase chain reaction (PCR) ligase detection reaction fluorescent microsphere assay (LDR-FMA). During the follow-up time of the study LM diagnosis found 87.6% of children became reinfected with *P. falciparum* (95.3%, PCR diagnosis), 49.5% with *P. vivax* (82.0%, PCR), 8.3% with *Plasmodium malariae* (29.3%, PCR), and 1.5% with *Plasmodium ovale* (13.6%, PCR). Clinical malaria was defined as a measured fever (axillary temperature ≥ 37.5°C) or history of febrile illness during the 48 hours preceding examination in conjunction with any malaria infection. The cut-off value for uncomplicated disease was set at 5,000 parasites/µL for *P. falciparum* and 1,000 parasites/µL for all other species. Of the uncomplicated malaria episodes, 80 were caused by *P. falciparum* (20 study participants had multiple episodes), five to *P. vivax*, two each to *P. malariae* or *P. ovale*, and one to a *P. falciparum*/P. vivax mixed infection. This study was approved by the Papua New Guinea Medical Research Advisory Council, and institutional review boards of the Walter and Eliza Hall Institute, Australia, and the Veteran’s Affairs Medical Center (Cleveland, OH). Permission for this study was obtained through community-based discussions and written parental consent was obtained for all children who participated in this study.

**Diagnosis of Plasmodium species infections.** Details of blood smear preparation and LM analysis have been provided elsewhere. In short, thick blood films were examined by LM and 100 fields (under a 100x oil-immersion lens) were examined before a smear was classified as being infection negative. Slides were scored as LM-positive for an individual *Plasmodium* species if the species was detected independently by at least two microscopists and subsequent PCR-based analysis confirmed the presence of the species. Densities were converted to the number of parasites per microliter of blood assuming 8,000 white blood cells (WBCs)/µL.

Infection by each of the four human malaria species was assessed in all blood samples collected using a semi-quantitative post-PCR assay. This assay combines PCR amplification of the small subunit (SSU) ribosomal RNA (rRNA) gene (491–500-basepair fragments) using genus-specific primers, followed by a multiplex species-specific ligase detection reaction-fluorescent microsphere assay (LDR-FMA).

**PCR-based genotyping.** Genotyping of common Melanesian 3.7kb (α-37) and 4.2kb (α-42) α-globin (chromosome 16) deletions associated with α-thalassemia was performed as previously described with some modifications. Genotyping of deletion polymorphisms at the band 3 (*SLC4A1Δ27*; chromosome 17) and glycoporphin C (*GYPCΔex3; chromosome 2*) loci, associated with SSO and Gerbich negativity, respectively, were determined by PCR. The PCR-reflection fragment length polymorphism (RFLP) analysis was used to genotype the CR1 exon 22 SNP at nucleotide 3650 and the intron 27 HindIII RFLP, as described in Xiang and others. Enzyme-linked immunosorbent assay (ELISA) assessment of response to *P. falciparum* schizonts. As a marker of the humoral immune response to blood-stage *P. falciparum* antigens, we measured immunoglobulin G (IgG) to *P. falciparum* schizont protein extract by ELISA in pretreatment sera, using established methods.

**Statistical analyses.** The associations between different genetic traits and genotypes with prevalence of infections were assessed using χ² and Fisher’s exact tests; difference in density of infection at baseline was assessed using analyses of variance. Cox regression was used to test for differences in time-to-first reinfection as detected by LDR-FMA or LM. In these analyses, children were considered at risk start for the first day after completion of the artesunate treatment until they either completed the study or were censored at the time of first (re-)treatment with antimalarial drugs. Poisson regression was used to determine differences in the incidence of uncomplicated malaria during follow-up with children removed from time-at-risk for 4 weeks after each antimalarial treatment. Further details on statistical approaches used are given elsewhere. To control for possible confounding effects of differences in acquired immunity, we included immune status (defined as low, medium, high depending on the tercile of total anti-schizont extract IgG responses) as a covariate in analyses of the association of RBC polymorphism with risk of *P. falciparum* infection or disease. Although small in size, the study had a power of 80% to find protective effects > 50% for all genotypes except *GYPCΔex3* homozygotes. All statistical analyses were performed using STATA 8 statistical analysis software (Stata Corporation, College Station, TX).

**RESULTS**

Genotype and allele frequencies in Table 1 are provided for α-globin (wild-type [wt], α−37, α−42), band 3 (wt, *SLC4A1Δ27*, GYP Wyoming, [wt, *GYPCΔex3*], CR1 3650 SNP (A/G), and intron 27 HindIII RFLP (high [H], low [L]). Results showed that 27 children (13.1%) were heterozygous for *SLC4A1Δ27*, 46 children (22.3%) were heterozygous, and 8 (3.9%) homozygous for *GYPCΔex3*. Only 31 children (15.0%) were homozygous wild-type for α-globin (αα/αα), 93 (45.2%) were heterozygous (αα/α−) and 82 (39.8%) homozygous (α−/α−). The 4.2 deletion accounted for 92.6% of all mutated alleles precluding allele-specific analyses. Thus, all analyses were combined for both alleles. The CR1 alleles distributed into three haplotypes (3650A-HindIII H [0.354], 3650G-HindIII L [0.627], 3650G-HindIII H [0.019]), showing significant linkage disequilibrium (D' = 1, r² = 0.9191, X² = 188.4, degrees of freedom [df] = 2, P < 0.001). Consequently, associations between CR1 genotype and *P. falciparum* infection and disease were performed only for the SNP at exon 22. While no significant associations were observed between α-thalassemia, *GYPCΔex3*, and *CR1ex22* (Fisher exact tests, P ≥ 0.15), there was a significant excess of heterozygotes for *CR1ex22* in SAO children (37.4% versus 63.0%; P < 0.01). Genotype frequencies did not differ by age and sex (P ≥ 0.4). Wild-type and mutant alleles at all loci were present in Hardy-Weinberg equilibrium.

Before treatment, 80 (38.6%) children had microscopically detectable *P. falciparum* infections (trophozoites and/or gametocytes), 17 (8.3%) *P. vivax*, 4 (1.9%) *P. malariae*, and 3 mixed *P. falciparum/P. vivax* infections. There were statistically significant differences either in prevalence (P > 0.17) or geometric mean density (P > 0.2) of *P. falciparum* infections by light microscopy among children with different genotypes (data not shown). Prevalence of *P. falciparum* infection was higher when assessed post-PCR LDR-FMA assay with 67.5% of children infected with *P. falciparum*, 34.0% with *P. vivax*, 6.8% with *P. malariae*, and 1.0% with *P. ovale*; with 26.4% children infected with more than one species. Only *GYPCΔex3* homozygosity was associated with a decreased risk of PCR detectable
Allele and genotype frequencies for common red blood cell (RBC) polymorphisms in Papua New Guinea

<table>
<thead>
<tr>
<th>Allele/Genotype</th>
<th>Counts</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$HBA1$ wt/wt</td>
<td>31</td>
<td>15.0</td>
</tr>
<tr>
<td>$HBA1$ wt/D</td>
<td>27</td>
<td>13.1</td>
</tr>
<tr>
<td>$HBA1$ D/D</td>
<td>8</td>
<td>3.9</td>
</tr>
<tr>
<td>$HBA1$ α−3.7/α−3.7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ α−4.2/α−4.2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ wt/α</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>$HBA1$ α/α</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>$HBA1$ wt−4.2</td>
<td>25</td>
<td>12.1</td>
</tr>
<tr>
<td>$HBA1$ α−4.2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ α−3.7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ α−4.2/α−3.7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ wt/−4.2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ α−4.2/−4.2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ α−3.7/−3.7</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>$HBA1$ α−4.2/−3.7</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

When infections were diagnosed by LDR-FMA, the risk of acquiring new $P. falciparum$ infections was significantly lower for α−thalassemia heterozygotes (hazard ratio [HR]: 0.56) and homozygotes (HR: 0.51, Table 2) than wild-type children (likelihood ratio [LR] $X^2 = 7.7$, df = 2, $P = 0.02$). However, this association was not seen when infections were diagnosed by LM (LR $X^2 = 0.7$, df = 2, $P = 0.71$). No significant association between risk of $P. falciparum$ infection and genotype was observed for any of the other RBC polymorphisms ($P > 0.2$). Similarly, there were no significant differences in incidence of uncomplicated $P. falciparum$ malaria between genotypes for any of the traits studied (Table 2, $P > 0.2$). The size of the study precluded a thorough analysis of associations between multiple polymorphisms and risk of infection or uncomplicated malaria.

Finally, we evaluated the humoral immune response to $P. falciparum$ blood-stage antigens by measuring serum IgG levels against a $P. falciparum$ schizont protein extract among samples collected at baseline. In this analysis α+α− children had significantly lower levels of $P. falciparum$-specific IgG compared with wt/α− and wt/wt children (median optical density 1.28, inter-quartile range [IQR] 0.88–1.46 versus 1.38, IQR 1.20–1.58, respectively; $P = 0.01$). No significant differences in immune status were observed for associations with the other RBC polymorphisms evaluated. We then adjusted for $P. falciparum$-specific antibody levels in analyses examining associations between genotypes and risk of infection or uncomplicated malaria described previously. Following this adjustment, we observed no significant change in the strength of associations between LDR-FMA-diagnosed $P. falciparum$ and GYPCex13 (baseline) or α−thalassemia (re-infection) (data not shown).

**DISCUSSION**

In a longitudinal study of school-age children living in a region highly endemic for malaria along the north coast of PNG near Madang, we found only marginal and inconsistent associations between the common RBC polymorphisms and the prevalence of $P. falciparum$ infection (measured by LM or LDR-FMA) or uncomplicated disease.

Although children with α−thalassemia were observed to experience a reduced risk of PCR-diagnosed $P. falciparum$ infections during the post-treatment, reinfection phase of the study, we found no association between α−thalassemia and LM-diagnosed infections or uncomplicated $P. falciparum$ malaria. The lack of a protection against uncomplicated $P. falciparum$ malaria is in line with both earlier studies in Africa and observations in children > 5 years of age in Vanuatu, but contradicts a more recent study from Tanzania that did find a significant decreased risk in children > 5 years of age.

Given the lack of protection against disease and LM-diagnosed infections, the finding of a significant reduction in risk of PCR-diagnosed infection is surprising. A possible
So efficiently. Even if these effects were also present in vivo, the high number of new infections and the expected multiplicity of phenotypic variants among these infections, SAO children are likely to be infected with a sufficient number of variants that efficiently infect RBCs to result in comparable numbers of infections and clinical cases as seen in non-SAO children.

Previous studies in PNG have found that α-thalassemia, SAO, and CR1 genotypes were only associated with protection against severe, but not uncomplicated malaria. The absence of protection against P. falciparum infection or complicated disease in our study is thus consistent with these reports. The mechanisms of protection, however, remain unclear. The CRIex22 SNP and α-thalassemia could protect against severe malaria from a reduction in CR1-mediated rosetting. Both polymorphisms have been associated with reduced levels of RBC CR1 expression. Redistribution of sequestered infected RBCs away from the brain may be responsible for the protection of SAO against cerebral malaria. Plasmodium falciparum-infected SAO RBCs have an increased ability to bind to CD36, an endothelial cell receptor that is largely absent in the brain endothelium, and this might redirect sequestration to non-vital organs where CD36 is expressed.

As with the other erythrocyte polymorphisms, our findings that GYPDCex3 does not influence risk of P. falciparum infection or uncomplicated malaria, does not rule out that GYPDCex3 may be associated with protection against severe malaria in younger, less immune children. Studies on associations of GYPDCex3 with severe malaria are thus needed to determine if GYPDCex3 has been selected for P. falciparum or other factors.

The overall lack of associations between genotypes and risk of P. falciparum infection and uncomplicated malaria might partly be caused by the significant level of acquired, clinical immunity among these children that may have masked any protective effects of specific genotypes against uncomplicated disease. However, with the exception of α-thalassemia no differences in immune status as measured by total IgG against schizont protein extract were observed between genotypes or did an earlier study in a neighboring population find an

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### Table 2

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Gene Locus</th>
<th>Genotype</th>
<th>LDR-FMA1 (MTR = 55 days)</th>
<th>LMT (MTR = 99 days)</th>
<th>Clinical episode IRR (IR = 1.17/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HR</td>
<td>CI&lt;sup&gt;**&lt;/sup&gt;</td>
<td>HR</td>
</tr>
<tr>
<td>α&lt;sup&gt;-&lt;/sup&gt;Thalassaemia</td>
<td>HBA1</td>
<td>wt/wt</td>
<td>0.56&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>[0.36, 0.87]</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wt/α</td>
<td>0.51&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>[0.32, 0.81]</td>
<td>1.12</td>
</tr>
<tr>
<td>SAO</td>
<td>SLC4A1</td>
<td>α&lt;sup&gt;-&lt;/sup&gt;/α&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1.15</td>
<td>[0.75, 1.76]</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>GYPC</td>
<td>wt/wt</td>
<td>0.86</td>
<td>[0.60, 1.24]</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δex3/Δex3</td>
<td>1.8</td>
<td>[0.87, 3.69]</td>
<td>1.11</td>
</tr>
<tr>
<td>CR1 expression</td>
<td>CRI</td>
<td>3650G/G</td>
<td>1.22</td>
<td>[0.89, 1.68]</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3650A/G</td>
<td>0.91</td>
<td>[0.58, 1.43]</td>
<td>0.88</td>
</tr>
</tbody>
</table>

* Gene locus, allele names, genotype designations, and phenotypes as described in Table 1.
† LDR-FMA = post-PCR ligase reaction-fluorescent microsphere assay for diagnosis of 4 Plasmodium parasites of humans.
‡ LM = light microscopy, or conventional blood smear, light microscopy diagnosis of four Plasmodium parasites of humans.
¶ MTR = median time to re-infection (days), IR = incidence rate (/child/yr) for entire cohort.
§ Clinical Episode = febrile episode with concurrent parasitaemia.
‡‡ LDR-FMA = post-PCR ligase detection reaction-fluorescent microsphere assay for diagnosis of 4 Plasmodium parasites of humans.
CI = 95% confidence interval.
** CI = 95% confidence interval.
effect of any of these polymorphisms on anti-VSA antibodies is and thus unlikely that differences in immune status could have directly compensated for potential effects of SAO or GYPCAex4 polymorphisms. Nevertheless, the relatively high degree of immunity in all children is likely to have reduced possible effects against uncomplicated \textit{P. falciparum} and studies in younger children with a low degree of immunity will be required to conclusively rule out an effect of these RBC polymorphisms on risk of \textit{P. falciparum} infection and uncomplicated malaria.

Using rigorous epidemiological methodology with active follow-up and molecular-based methods for detection of parasitemia, this study did not observe consistent significant associations between common PNG RBC polymorphisms and protection from \textit{P. falciparum} blood-stage infection or uncomplicated malaria. The mechanisms involved in their protection against severe disease are yet to be fully understood.

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Authors’ addresses: Enmoore Lin, Livingstone Tavul, Pascal Michon, Elijah Dabod, and Ivo Mueller, Papua New Guinea Institute of Medical Research, Papua, New Guinea, E-mails: enmoorelin@yahoo.com, lterbu1a1@gmail.com, michon.pascal@gmail.com, suparat@hotmail.com, and ivomueller@fastmail.fm. Jack S. Richards and James G. Beeson, Walter and Eliza Hall Institute of Medical Research, Infection and Immunity Division, Royal Parade, Parkville, Australia, E-mails: richards@wehi.edu.au and beeson@wehi.edu.au. Christopher L. King and Peter A. Zimmerman, Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH, E-mails: christopher.king@case.edu and peter.zimmerman@case.edu.

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