The association of the glycoporphin C exon 3 deletion with ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea

Sheral S. Patel, Rajeev K. Mehlotra, William Kastens, Charles S. Mgone, James W. Kazura, and Peter A. Zimmerman

Erythrocyte polymorphisms, including ovalocytosis, have been associated with protection against malaria. This study in the Wosera, a malaria holoendemic region of Papua New Guinea, examined the genetic basis of ovalocytosis and its influence on susceptibility to malaria infection. Whereas previous studies showed significant associations between Southeast Asian ovalocytosis (caused by a 27-bp base pair deletion in the anion exchanger 1 protein gene) and protection from cerebral malaria, this mutation was observed in only 1 of 1019 individuals in the Wosera. Polymerase chain reaction strategies were developed to genotype individuals for the glycoporphin C exon 3 deletion associated with Melanesian Gerbich negativity (GPCΔex3). This polymorphism was commonly observed in the study population (GPCΔex3 frequency = 0.465, n = 742). Although GPCΔex3 was significantly associated with increased ovalocytosis, it was not associated with differences in either Plasmodium falciparum or P vivax infection measured over the 7-month study period. Future case-control studies will determine if GPCΔex3 reduces susceptibility to malaria morbidity. (Blood. 2001;98:3489-3491)

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Study design

Study population and malaria

The study was conducted in the Wosera region of Papua New Guinea, where all 4 human Plasmodium species are transmitted year-round. Blood samples were collected monthly from permanent residents (median age, 17 years; range, 1-86 years) of 6 villages within the Wosera from July 1998 to January 1999. The human investigations institutional review boards of Case Western Reserve University, University Hospitals of Cleveland, and the Papua New Guinea Medical Research Advisory Committee approved all protocols.

Malaria and red blood cell morphology

Thick and thin films stained with 4% Giemsa were prepared at the time of blood collection (Figure 1A-D). Malaria parasites were identified by light microscopy. Parasite densities were recorded as the number of parasites per 200 leukocytes (average 8000 leukocytes per microliter).

Thin smears were examined by light microscopy for the proportion of ovalocytes (erythrocytes with length:width ratio more than 1:1) without knowledge of genotypic results. Because previous studies have shown that elliptocytes are rare in the Wosera, the distinction between ovalocytes and elliptocytes (RBC with length:width more than 2:1) was not made. Blood smears from North Americans were prepared using a modified Wright stain (Diff-Quick Stain Set, Dade-Behring, Newark, DE). One reader reviewed all of the blood smears. Two additional observers independently reviewed a subset of smears.

Genotyping for AE1 and GPC polymorphisms

Blood was collected in ethylenediaminetetraacetic acid vacutainer tubes and stored at −70°C until DNA extraction was performed with the QIAmp96 DNA blood kit (Qiagen, Valencia, CA). Genotyping of band 3 (PCR) genotyping strategies for GPC are described in Figure 1E-G.

Statistical analysis

Categorical variables were analyzed by the χ2 test and continuous variables by the Wilcoxon or Kruskal-Wallis test. Statistical Analysis Systems version 8.1 software package (Cary, NC) was used.
were performed as previously described.12 (E) To assess the presence of exons 2 and 3, FACTS

To identify the GPC\textit{ex3} allele and differentiate between wt/wt and wt/

\textit{ex3} homozygotes, this \textit{ex3} genotype in the Wosera

are needed to delineate all 3 genotypes. All PCR amplifications were performed as previously described.12 (E) To assess the presence of exons 2 and 3, primers (GPC\textit{up} 5\textsuperscript{-}TCAAAACACACCTCTGAGGGAGAG-3\textsuperscript{3} and GPC\textit{dn} 5\textsuperscript{-}GGAAACTGCCGTGACTTCAGA-3\textsuperscript{3}) annealing to conserved sequence around exons 2 and 3 were used (thermocycling program: 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds \cite{40}). PCR products were subjected to electrophoresis on a 4% 5:1 GTG NuSieve:LE agarose gel (FMC Bioproducts, Rockland, ME) gel. A 264 bp band (exon 2) is detected in wt/wt, wt/\textDelta, and \textDelta/\textDelta. A 240 bp band (exon 3) is detected in only wt/wt and wt/\textDelta. The heteroduplex product is created by hybridization between complementary strands of exon 2 and 3 amplicons. (F) To identify individuals with the \textDelta allele, primers annealing to a 51-bp intron 2 insert and downstream intron 3 polymorphism (GenBank M24627)\cite{5} (GPC\textit{up} 5\textsuperscript{-}GGAAACTGCCGTGACTTCAGA-3\textsuperscript{3} and 1678de

\textit{ex3} homozygotes. This \textit{ex3} genotype and ovalocytosis

\textit{ex3} homozygotes, where the disadvantage of lethality in the homozygous form is outweighed by the selective advantage against severe malaria for heterozygotes.2

GPC genotype in the Wosera

GPC genotyping was performed on 742 individuals (Figure 1E-G). The first reaction, screening for the presence or absence of the wild-type (wt) GPC allele where exons 2 and 3 are both present (Figure 1E), identified homozygous GPC\textit{wt} individuals (lane 3). The second (Figure 1F) and third reactions (Figure 1G) amplified GPC sequence within the wt or GPC\textit{ex3} alleles, respectively, and allowed homozygous wt individuals (Figure 1F-G, lanes 1 and 4) to be distinguished from heterozygous individuals (Figure 1F-G, lane 2). Allele frequencies for GPC wt and GPC\textit{ex3} were 0.535 and 0.465, respectively. Genotyping showed 211 (28.4%) of 742 individuals as homozygous wt, 372 (50.1%) of 742 as heterozygous, and 159 (21.4%) of 742 as GPC\textit{ex3} homozygotes. This distribution is in Hardy-Weinberg equilibrium, indicating that GPC\textit{ex3} does not confer a selective disadvantage. This is in contrast to AE1\textDelta27, a balanced polymorphism, where the disadvantage of lethality in the homozygous form is outweighed by the selective advantage against severe malaria for heterozygotes.2

GPC genotype and ovalocytosis

The association between ovalocytosis and GPC genotype was evaluated in 134 individuals who did not carry AE1\textDelta27. The wt individuals (\textit{n} = 32) had the lowest proportion of ovalocytes per 1000 RBCs (median, 238 ± 115.1). Heterozygous individuals (\textit{n} = 52) had a higher proportion of ovalocytes (median, 297 ± 103.8), while homozygotes (\textit{n} = 49) had the highest of all 3 genotypes (median, 312 ± 145.9). In a comparison of all 3 genotypes, the proportion of ovalocytes was significantly associated with GPC\textit{ex3} (Kruskal-Wallis, \textit{P} = .021). Individual comparisons among the 3 genotypic groups showed significant differences by a one-sided Wilcoxon test (wt/wt vs wt/GPC\textit{ex3}, \textit{P} = .0392; wt/wt vs GPC\textit{ex3}/GPC\textit{ex3}, \textit{P} = .0045). These results suggest that GPC\textit{ex3} contributes to ovalocytosis in the Wosera. When erythrocyte morphology was compared between homozygous wt individuals from the Wosera and North Americans, the former had a significantly increased ovalocyte frequency (Wilcoxon, \textit{P} < .0001). This suggests that altered RBC morphology in the Wosera is a heterogenous condition caused by additional unknown mutations in RBC membrane proteins, such as protein 4.1 and spectrin as well as environmental or nutritional factors.
intervals. This analysis showed that the prevalence or density of population over 7 months. Results for genotype and infection status ability to malaria infection more rigorously, we studied a larger To examine the relationship between GPC genotype and susceptibility to malaria infection was not significantly different for individuals in the 3 GPC genotypic groups at any time (Table 1). These results parallel findings of other RBC polymorphisms, such as AE1Δ27, where genotypic differences are associated with reduced susceptibility to severe malaria morbidity with no effect on susceptibility to infection.3 The relationship of GPCΔ3 to malaria morbidity in young children, the age group most susceptible to the clinical phenotype, requires further study.

Acknowledgments

We thank the residents of the Wosera for participating in this study. We thank Chloe Hill for reviewing blood smears.

Table 1. Glycophorin C genotype and infections over time

<table>
<thead>
<tr>
<th>Month</th>
<th>No.§</th>
<th>Prevalence (%)</th>
<th>Parasitemia (95% CI) Prevalence (95% CI)</th>
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<tr>
<td>July</td>
<td>696</td>
<td>34/200 (17.0)**</td>
<td>2.21 (2.16-2.62) 72/245 (20.9) 2.42 (2.26-2.55)</td>
<td>2.28 (2.42-2.55) 34/151 (22.5) 2.38 (2.24-2.71)</td>
<td>.392 .880</td>
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<td>30/125 (24.0) 2.38 (2.21-2.72) .638 .739</td>
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<tr>
<td>Sept</td>
<td>415</td>
<td>23/121 (19.0)**</td>
<td>2.30 (2.16-2.57) 56/199 (28.1) 2.15 (2.14-2.49)</td>
<td>19/95 (20.0) 2.08 (2.02-2.47) .112 .627</td>
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<tr>
<td>Oct</td>
<td>466</td>
<td>23/136 (16.9)**</td>
<td>2.21 (2.06-2.56) 41/235 (17.5) 2.30 (2.29-2.70)</td>
<td>24/95 (25.3) 2.08 (2.04-2.76) .203 .315</td>
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<td>17/70 (24.3) 2.38 (2.12-2.92) .769 .049</td>
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Glycophorin C genotype and P falciparum

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<th>Month</th>
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<th>Prevalence (%)</th>
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<td>696</td>
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<td>1.91 (1.95-2.32) 63/345 (18.3) 2.15 (2.14-2.49)</td>
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<td>21/121 (19.0)**</td>
<td>2.08 (2.02-2.30) 52/235 (22.1) 2.08 (2.05-2.25)</td>
<td>26/95 (27.4) 2.08 (2.06-2.57) .638 .739</td>
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<tr>
<td>Oct</td>
<td>466</td>
<td>23/136 (17.5)**</td>
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<td>Nov</td>
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<tr>
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<td>2.60 (2.04-2.81) 14/162 (8.6) 2.21 (2.01-2.70)</td>
<td>1.70 (1.4) 1.61 (N/A) .115 .298</td>
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</table>

GPC genotype and infection status

The prevalence of infection with P falciparum or P vivax determined by blood smear has been examined in relation to serologic Ge antigen status in one published study of 266 people.11 This study observed a lower combined smear positive rate for P falciparum and/or P vivax infection in Ge-negative individuals, suggesting that Ge antigen negativity protects against infection.11 To examine the relationship between GPC genotype and susceptibility to malaria infection more rigorously, we studied a larger population over 7 months. Results for genotype and infection status were available for 325 to 696 individuals at each of 7 monthly intervals. This analysis showed that the prevalence or density of P falciparum and P vivax infection was not significantly different for individuals in the 3 GPC genotypic groups at any time (Table 1). These results parallel findings of other RBC polymorphisms, such as AE1Δ27, where genotypic differences are associated with reduced susceptibility to severe malaria morbidity with no effect on susceptibility to infection.3 The relationship of GPCΔ3 to malaria morbidity in young children, the age group most susceptible to the clinical phenotype, requires further study.

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