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FCGR2A FUNCTIONAL GENETIC VARIANT ASSOCIATED WITH SUSCEPTIBILITY TO SEVERE MALARIAL ANEMIA IN GHANAIAI CHILDREN

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ABSTRACT

Background: Severe malarial anemia is a major cause of mortality from malaria. Although of enormous relevance, its pathogenesis is largely unknown. Interestingly, the extent of anemia greatly exceeds the loss of erythrocytes due to direct destruction by the pathogen

Plasmodium falciparum. Immune response against the parasite is partially mediated through the Fc receptor for immunoglobulin (Ig) G IIa (FcγRIIa, CD32). The presence of an arginine instead of a histidine residue at amino acid position 131 (H131R) in the extracellular domain of FcγRIIa reduces the affinity of the receptor for IgG₂ and IgG₃ isotypes but increases the binding activity for C-reactive protein (CRP).

Methods: In Ghana, West Africa, 2504 children with severe malaria and 2027 matched healthy controls were studied for the FcγRIIa^{H131R} polymorphism in order to ascertain its influence on major manifestations of the disease. The study group included patients with partly overlapping symptoms of severe malaria, among them 1591 cases with severe anemia, 562 cases with cerebral malaria and 497 cases with other malaria complications.

Results: Analyses of the genotype distributions indicated that, under a recessive model, FcγRIIa^{131RR} was positively associated with severe malaria collectively (odds ratio [OR], 1.20; 95% confidence interval [CI], 1.05-1.38; p=0.007, p_{corrected}=0.021) and, after stratification for phenotypes, with severe anemia (OR, 1.33; CI, 1.13-1.57; p=0.001, p_{corrected}=0.009) but not with cerebral malaria (OR, 1.04; CI, 0.82-1.33; p=0.733) or other malaria complications (OR, 1.03; CI, 0.78-1.37; p=0.827). No association was found with levels of parasitemia.

Conclusion: The positive association with a CRP-binding variant of FcγRIIa supports evidence for a role of CRP-mediated defense mechanisms in the pathogenesis of severe malarial anemia.

INTRODUCTION

Severe malaria caused by infection with *Plasmodium falciparum* remains one of the major health problems in sub-Saharan Africa.[1] It predominantly affects infants and young children and causes approximately 1 million fatalities each year.[2] Severe falciparum malaria is a complex clinical syndrome comprising a number of life-threatening conditions including severe malarial anemia, cerebral malaria, metabolic acidosis, respiratory distress and other, less frequent complications.[3] Severe anemia is the most frequent malaria complication among infants and young children living in endemic areas with very high transmission levels. Its pathogenesis is considered complex and appears to include intravascular hemolysis, extravascular clearance of parasitized and non-parasitized erythrocytes, and bone-marrow dysfunction. In severe anemia the apparently rapid loss of a large proportion of erythrocytes greatly exceeds the proportion of erythrocytes that are infected by parasites.[4, 5] The mechanisms causing a loss of non-parasitized erythrocytes are still unclear but appear to involve immunological processes.[6, 7]

IgG receptors (FcγR) are crucial mediators of humoral immune responses. FcγRIIa, a low-affinity IgG receptor, links humoral to cellular arms of the immune response by mediating phagocytosis of IgG- and CRP-opsonized structures. The receptor is expressed on various human cell types including macrophages, monocytes and neutrophils. It has been described to bind three of the four IgG subclasses and appears to be the only receptor for the IgG₂ isotype. Its binding capacity is critically influenced by a genetic variant (rs1801274; *FCGR2A* c.497A>G) where the substitution of an adenine (A) by a guanine (G) results in a replacement of a histidine (H) by an arginine (R) residue at amino acid position 131.

While FcγRIIa^{131H} binds IgG₂ efficiently, the structural variant FcγRIIa^{131R} was reported to hardly interact with IgG₂ and to have a decreased affinity for IgG₃. [8] Instead, FcγRIIa^{131R} was found to be the major receptor for CRP on monocytes and neutrophils. [9] Thus, FcγRIIa^{131R} not only results in a loss of function for IgG₂ and IgG₃ binding, but also includes a gain of function regarding the ability to bind CRP, an opsonizing serum protein of the innate immune response. [10] In contrast to the antigen-specific recognition by antibodies, binding of

CRP is based on pattern recognition of altered self and foreign molecules.[11] In infants, very high levels of CRP production have been measured during clinical malaria episodes.[12] Opsonization by CRP causes phagocytosis and an inflammatory response by an induction of cytokine synthesis. These activities are mediated by the ability of CRP to bind to FcγR and to activate complement.

FCGR genetic variants have been reported to influence human susceptibility to a variety of infectious and autoimmune diseases.[13] The effect of FcγRIIa^{H131R} on malaria has been addressed in several studies but the data are difficult to interpret. The H allele was found associated with protection against severe malaria in Sudanese adults [14] and FcγRIIa^{131HH} with protection against both severe and mild malaria in Indian adults. In contrast, the same variant was associated with susceptibility to severe malaria in Gambian children [15, 16], and FcγRIIa^{131RR} was reported to protect against high parasite densities in Kenyan children.[17, 18]

With the objective to investigate the association of the FcγRIIa^{H131R} variants with the various forms of severe falciparum malaria, we studied more than 1500 children with severe malarial anemia and more than 500 cases of cerebral malaria.

METHODS

Patients and Controls

The study was carried out in an area hyperendemic for falciparum malaria of the Ashanti Region of Ghana, West Africa. Patients were recruited at Komfo Anokye Teaching Hospital, a tertiary medical referral center in Kumasi, between 2001 and 2005 in parallel with the “Severe Malaria in African Children” study. All pediatric patients 6 months to 10 years of age were screened for malaria parasitemia using Giemsa-stained blood films. In malaria patients, the level of consciousness according to the Blantyre Coma Score (BCS) [1], blood glucose, lactate (YSI Life Sciences), and hemoglobin concentrations (Sysmex KX-21N) were determined at admission. Patients positive for asexual *P. falciparum* parasitemia with either a BCS of <3, hemoglobin concentration of <5 g/dL or lactate of >5 mmol/L [1] were enrolled

after consent had been obtained from accompanying parents or guardians. Parasite densities were determined per 200 leukocytes and calculated assuming a leukocyte count of 8,000 per μl blood.[19] In the analysis patients with a hemoglobin level of $< 5\text{g/dl}$, a BCS of < 3 , or any other than the two complications were classified as severely malarial anemic ($n=1591$), cerebral ($n=562$), and other ($n=497$), respectively. Within a total of 2504 cases, 146 patients were affected by both conditions, severe anemia and cerebral malaria. A detailed description of the participants, ethnicities and clinical diagnoses has been reported elsewhere.[20] To serve as a control group, apparently healthy children were recruited through community surveys.

The study was approved by the Committee for Research, Publications and Ethics of the School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. All procedures were explained to parents or guardians of the participating children in the local language, and written or thumb-printed consent was obtained.

Genotyping

In order to extract DNA for subsequent genotyping, blood samples were drawn from all participants. 0.5-1 ml blood was collected into citrate, and, before subjected to DNA extraction, the granulocyte fraction obtained from density gradient centrifugation was preserved in 4-M urea. DNA was extracted according to the suppliers' instructions (Nucleo-Mag 96 Blood; Macherey-Nagel, Düren, Germany). Prior to genotyping whole-genome amplification (WGA) using 10 ng DNA of each sample was conducted according to manufacturer's instructions (GenomiPhi HY DNA Amplification Kit, GE Healthcare, Braunschweig, Germany).

The non-synonymous substitution of an A by a G in the *FCGR2A* gene (rs1801274) results in the amino acid substitution at position 131 (H131R) in the second extracellular Ig-like domain of the Fc γ RIIa protein. In this study, the single-nucleotide polymorphism rs1801274 was analyzed by dynamic allele-specific hybridization with fluorescence resonance energy transfer (FRET) in a LightTyper device (Roche Diagnostics, Mannheim,

Germany). A fragment of 558 bp of the human *FCGR2A* gene, which includes the single base polymorphism at position 497 in the coding sequence (c.497A>G), was amplified by PCR. The reaction mixture contained 1x PCR Buffer BD, 1x Solution S, 1 U FIREPol DNA polymerase I, 1.5 mM MgCl₂ (Solis BioDyne, Estonia), 200 µM of each dNTP, 1 µM PCR Primer FCGR2A_for, 0.2 µM of PCR Primer FCGR2A_rev, 0.2 µM FCGR2A_anchor, 0.2 µM FCGR2A_sensor, 2 µl of the WGA sample (1:200 dilution), and water to a final volume of 10 µl. The following reaction conditions were used: 94°C for 3 min, 55 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 30 s followed by one cycle of 72°C for 5 min. Primer and sensor/anchor sequences were 5′-CTTATCATATTTTGTCTTTCAGAATG-3′ (FCGR2A_for), 5′-CAGCCTGACTACCTATTACCTGG-3′ (FCGR2A_rev), 5′-TGCTTGTGGGATGGAGAAGGTGGG-Fluorescein (FCGR2A_anchor), 5′-CY5-TCCAAACGGGAGAATTTCTGGGA-Phosphate-3′ (FCGR2A_sensor). To confirm the validity of the typing method, PCR products of 23 DNA samples were sequenced using primers FCGR2A_ex4-F (5′-TTCTGCTTTTAGAAGTCCCAYAG-3′) and FCGR2A_ex4-R (5′-CAATACTTAGCCAGGCTTCCAC-3′). DNA re-sequencing confirmed the results obtained by FRET genotyping in all cases.

Statistical analyses

Genotype frequencies were tested for deviation from Hardy-Weinberg Equilibrium (HWE) using Pearson's χ^2 test. The distribution of genotypes among cases and controls was evaluated by logistic regression analysis. ORs and 95% CI were calculated using STATA v9.2 software (Stata, StataCorp LP, Texas, USA). In order to reduce the effect of confounding factors, corresponding controls for each case group were selected randomly from the whole group using age, gender and ethnicity as matching criteria. In the same study group as presented here, carriers of the sickle cell hemoglobin (HbS) were found to be protected from all major forms of severe malaria, whereas the protection for carriers of the hemoglobin C (HbC) variant was restricted to cerebral malaria.[20] That study also revealed a lower risk to develop severe malarial anemia in patients with α^+ -thalassemia. Hence, HbS

genotypes were used as a covariate for all comparisons, and genotypes underlying α^+ -thalassemia and HbC were used exclusively in calculations including severe malarial anemia and cerebral malaria phenotypes, respectively. According to the Bonferroni correction for multiple comparisons [21], p-values were adjusted as follows: The working hypothesis was that severe malaria may be associated with *FCGR2A* variants at any mode of inheritance (additive, recessive or dominant) which required a correction factor of 3 for multiple testing. Then, three phenotypes of severe malaria (severe anemia, cerebral malaria, other manifestations) were tested assuming any mode of inheritance, which required a correction factor of 9. Statistical power to detect a genetic effect in our study group was estimated using CaTS power calculator for association studies.[22] For this purpose a disease prevalence of 2% for severe malaria and a required significance level of 0.005 (correction factor 9) were applied.

RESULTS

The study group consisted of 2504 children with severe falciparum malaria and 2027 apparently healthy controls matched for age, gender and ethnicity (Table 1).

Table 1. Demographic Data of the Study Group

	Severe Malaria				Controls
	All severe malaria cases	Severe Anemia ^a	Cerebral Malaria ^b	Others ^c	
n	2504	1591	562	497	2027
Median age (months, range)	20 (7-117)	16 (7-114)	25 (7-117)	27 (7-117)	30 (7-117)
Male-female ratio	54:46	54:46	54:46	55:45	53:47
Ethnic group, %					
Akan	67.3	69.2	63.7	64.8	69.2
Northerners ^d	29.8	27.8	34.0	31.6	29.3
Ewe	2.9	3.0	2.3	3.6	1.5

^a Severe malarial anemia with or without additional complications including cerebral malaria.

^b Cerebral malaria with or without additional complications including severe anemia.

^c Severe malaria complications not including severe anemia or cerebral malaria (hyperlactatemia, 100%; prostration, 83%; hyperparasitemia, 56%; acidosis, 54%; respiratory distress, 27%).

^d The group of Northerners comprises members of several ethnicities of Northern Ghana

Genotyping for *FCGR2A* variants showed that the allele frequencies deviate from HWE exclusively in the group of severe-anemia cases ($\chi^2=6.92$, $p=0.009$), but not in the control group and the other subgroups investigated (Supplementary Table 1).

FCGR2A genotype frequencies in patients and controls were compared by multiple logistic regression and adjusted for age, gender and ethnicity. Evaluating the entire case group collectively, $\text{Fc}\gamma\text{RIIa}^{131\text{RR}}$ was found more frequently among cases than among controls (Table 2). In contrast, both $\text{Fc}\gamma\text{RIIa}^{131\text{HR}}$ and $\text{Fc}\gamma\text{RIIa}^{131\text{HH}}$ showed the opposite distribution, suggesting a recessive influence of the $\text{Fc}\gamma\text{RIIa}^{131\text{R}}$ allele. Accordingly, statistical calculations were made under the assumption of a recessive mode of inheritance.

The case group was stratified for the phenotypes of cerebral malaria ($n=562$) and severe anemia ($n=1591$), whereby patients with both forms were assigned to each of the two subgroups and those with neither of the two forms were combined in a third subgroup ($n=497$). The latter included patients with partly overlapping hyperlactatemia (100%), prostration (83%), hyperparasitemia (56%), acidosis (54%), and respiratory distress (27%).

A significant positive association of $\text{Fc}\gamma\text{RIIa}^{131\text{RR}}$ was observed in the subgroup of patients with severe anemia, whereas the frequencies of $\text{Fc}\gamma\text{RIIa}^{131\text{HR}}$ and $\text{Fc}\gamma\text{RIIa}^{131\text{HH}}$ were similar in the two other subgroups with odds ratios (OR) being close to 1 (Table 2). The skewed distribution of genotypes in the severe-anemia subgroup may explain the deviation of allele frequencies from HWE observed in this subgroup.

Given the disease-associated allele frequency of 0.6 in the controls and the estimated OR of 1.33 in the severe anemia subgroup, the statistical power to detect a recessive effect in the phenotypic subgroups is 68% for severe anemia, 20% for cerebral malaria, and 17% in other severe malaria cases. However, when combining all non-anemic severe malaria cases ($n=913$) and using the complete control group ($n=2027$), the power achieved is 56%. Analyzing the genotype distribution among these cases and all available controls revealed no evidence for an effect of $\text{Fc}\gamma\text{RIIa}^{131\text{RR}}$ on the susceptibility to non-anemic severe malaria in our study group (OR, 1.07; CI, 0.91-1.26; $p=0.395$).

When comparing parasite densities (log-transformed number of asexual parasites per microliter blood) in the genetic subgroups, no significant differences were observed between patients with FcγRIIa^{131RR} (median, 4.83; 95% confidence interval [CI], 4.76-4.90), FcγRIIa^{131HR} (median, 4.95; CI, 4.87-5.02), and FcγRIIa^{131HH} (median, 4.77; CI, 4.63-4.90).

Studying in the same case/control group the association of α^+ -thalassemia, we had previously noted a difference between patients with severe anemia alone and those with severe anemia plus additional complications.[20] We had speculated that the two groups might differ in the speed with which anemia develops. Regarding the positive association of severe anemia with FcγRIIa^{131RR}, we found a discrete but not significant difference between the two subgroups (anemia-alone subgroup, OR, 1.30; CI, 0.91-1.87; p=0.152; n=261; anemia plus other complications, OR, 1.27; CI, 1.08-1.49; p=0.004, n=1330).

Table 2. FcγRIIa^{H131R} Associations in Severe Falciparum Malaria Collectively and Stratified for Major Forms of the Disease

FcγRIIa ^{H131R}	Severe Malaria				Severe Anemia ^a				Cerebral Malaria ^b				Others ^c			
	Cases % n=2504	Controls % n=2027	OR ^f (95% CI ^g)	p	Cases % n=1591	Controls % n=1517	OR (95% CI)	p	Cases % n=562	Controls % n=545	OR (95% CI)	p	Cases % n=497	Controls % n=482	OR (95% CI)	p
Additive model																
HH	15.5	16.2	1		15.1	16.8	1		16.2	17.3	1		15.9	15.0	1	
HR	44.3	47.6	1.04 (0.86-1.26)	0.697	43.5	48.3	1.06 (0.85-1.33)	0.605	45.0	44.3	1.10 (0.79-1.56)	0.558	46.5	46.0	1.23 (0.82-1.82)	0.310
RR	40.1	36.2	1.24 (1.02-1.26)	0.032	41.4	34.9	1.39 (1.10-1.76)	0.006	38.8	38.4	1.12 (0.79-1.59)	0.558	37.6	39.0	1.19 (0.80-1.80)	0.381
Recessive model																
HH/HR	59.9	63.8	1		58.6	65.1	1		61.2	62.2	1		62.4	61.6	1	
RR	40.1	36.2	1.20 (1.05-1.38)	0.007^d	41.4	34.9	1.33 (1.13-1.57)	0.001^e	38.8	37.8	1.04 (0.82-1.33)	0.733	37.6	38.4	1.03 (0.78-1.37)	0.827

HbS genotypes were used as covariates in all logistic regression analysis. Genotypes for α⁺-thalassemia and HbC were used exclusively in logistic regressions including severe malarial anemia and cerebral malaria phenotypes, respectively.

^a Severe malarial anemia with or without additional complications including cerebral malaria

^b Cerebral malaria with or without additional complications including severe anemia

^c Severe malaria complications not including severe anemia or cerebral malaria (hyperlactatemia, 100%; prostration, 83%; hyperparasitemia, 56%; acidosis, 54%; respiratory distress, 27%)

^d P value corrected by a factor of 3 for multiple testing (3 genetic models): P_{c3}= .021

^e P value corrected by a factor of 9 for multiple testing (3 manifestations in 3 genetic models): P_{c9}= .009

^f Odds ratio

^g Confidence interval

DISCUSSION

Here we report that FcγRIIa^{131RR} was positively associated with severe falciparum malaria and, more specifically, with severe malarial anemia, one of the most prevalent malaria complications. No evidence was obtained for any influence of the FcγRIIa^{H131R} polymorphism on cerebral malaria or other major malaria complications in the absence of severe anemia. The effect of FcγRIIa^{131RR} on severe malarial anemia appeared to be recessive, as FcγRIIa^{131HH} and FcγRIIa^{131HR} showed similar ORs. These findings are only partly in agreement with previous observations. For example, in the largest study on this subject so far, Cooke and co-workers obtained a strikingly different result.[16] Including approximately 500 case/controls from The Gambia, they found FcγRIIa^{131HH} associated with susceptibility to severe malaria including both patients with severe anemia and cerebral malaria. Similarly, two studies on FcγRIIa polymorphisms in study cohorts from Kenya comprising 182 and 493 participants, respectively, provided evidence for protection against malaria by FcγRIIa^{131RR}, in these cases not against severe disease but against high parasite densities.[17, 18] On the other hand, one study from India with a total of 1871 individuals and a second study from Sudan investigating 256 individuals showed similar findings as ours but in differently defined malaria phenotypes and without discriminating between the various forms of disease.[14, 15] One possible explanation for these discrepancies may be different genetic backgrounds of the ethnically diverse study groups. For instance, linkage disequilibrium has been described between alleles of *FCGR2A* and *FCGR3B*, both of which appear to be involved in similar functions and, therefore, may interfere with each other's evolutionary selection.[23] Additionally, epistatic effects involving variants located in other genes in the *FCGR* gene cluster on chromosome 1q23-24 are likely to exist. A structural polymorphism (V158F) in the extracellular domain of FcγRIIIa has been found to be in linkage disequilibrium with the FcγRIIa^{H131R} polymorphism in Caucasian populations as well as in an Afro-American population.[24] A study performed by van der Pol and colleagues underscores the apparent differences in *FCGR* heterogeneity between ethnic groups, which might explain inconsistent results between different study cohorts.[25]

Alternatively, it may be argued that several of the previous reports (i) were based on relatively small study groups providing limited statistical power to reveal significant associations with severe malaria, (ii) showed marginal levels of statistical significance and (iii) included stratifications without correcting for multiple testing. As they also yielded largely inconsistent results, some of those studies leave some doubt as to whether the associations reported may have been fortuitous. The study presented here was based on 5 to 20 times more cases and resulted in clearly significant associations or in ORs close to 1 when significance was missing.

Due to the fact that the severe anemia subgroup comprises a nearly three times greater sample size than the other phenotypic subgroups, there is a decline in statistical power to detect the observed effect from 68% in the severe anemia group to 20% and 17% in the two smaller subgroups. A maximum of 56% statistical power can be achieved by analyzing genotype frequencies of all non-anemic severe malaria cases together with the entire control group. Compared to the severe anemia cases and matched controls this analysis provides only marginally less statistical power, and the absence of association of FcγRIIa^{131RR} with non-anemic severe malaria cases supports the evidence for patients homozygous for FcγRIIa^{131R} being specifically more susceptible to severe malarial anemia in our study.

Notably, in a population with steady exposure to falciparum malaria, the allele frequency of 0.6 of the SMA-associated allele appears to be relatively high. It is likely that the increased affinity between FcγRIIa^{131R} and CRP exerts a beneficial role in the defense against bacterial pathogens, as CRP binds to several encapsulated bacteria including *Haemophilus influenza* and *Streptococcus pneumonia*. [26, 27] Both pathogens were found to be among the leading causes of childhood bacterial meningitis in southern Ghana. [28] Thus, the R allele could be subject to counterbalancing positive selection maintaining the malaria associated allele in the population.

The association data reported here appear to indicate that certain humoral defense mechanisms contribute to severe malarial anemia substantially more strongly than to other

forms of severe malaria. However, addressing the question of whether the loss of the FcγRIIa^{131R} function to bind IgG₂ and IgG₃ or the gain of function to bind CRP is involved must be highly speculative because, to our knowledge, neither the role of antibody isotypes nor that of CRP has so far been the subject of functional studies in malaria pathology. If the crucial property of FcγRIIa^{131R} compared to FcγRIIa^{131H} would be the reduced ability to mediate the clearance of IgG₂- and IgG₃-opsonized parasites or parasitized erythrocytes, patients homozygous for FcγRIIa^{131R} would be expected to have higher parasite densities than those with FcγRIIa^{131HH} and FcγRIIa^{131HR}, which was not the case in our study. In addition, it is difficult to envisage that a reduction in parasite clearance would selectively affect severe anemia rather than all forms of complicated malaria because severe anemia has been found not to be associated with higher parasite densities than coma or other malaria complications.[29] Therefore, it appears unlikely that the loss of binding activity of FcγRIIa^{131R} for IgG₂ and IgG₃ causes the increase in risk for severe anemia. Rather, the gain of function of FcγRIIa^{131R} to bind CRP might be relevant. CRP is known to bind to the surface of apoptotic cells [30], and circumstantial evidence suggests that, in severe malarial anemia, nonparasitized erythrocytes acquire surface properties of apoptotic cells.[31] Hence, CRP/FcγRIIa-mediated elimination may contribute to a loss of nonparasitized erythrocytes in severe malarial anemia.

It needs to be stressed that genetic associations as the one presented here provide circumstantial evidence only and do not monitor biological processes directly. They may, however, stimulate further attempts to design experimental and clinical studies to confirm the genetic results at the functional level.

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CONFLICT-OF-INTEREST DISCLOSURE

The authors have no competing interests to declare.

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