Lack of Association of CD55 Receptor Genetic Variants and Severe Malaria in Ghanaian Children

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ABSTRACT In a recent report, the cellular receptor CD55 was identified as a molecule essential for the invasion of human erythrocytes by Plasmodium falciparum, the causal agent of the most severe form of malaria. As this invasion process represents a critical step during infection with the parasite, it was hypothesized that genetic variants in the gene could affect severe malaria (SM) susceptibility. We performed high-resolution variant discovery of rare and common genetic variants in the human CD55 gene. Association testing of these variants in over 1700 SM cases and unaffected control individuals from the malaria-endemic Ashanti Region in Ghana, West Africa, were performed on the basis of single variants, combined rare variant analyses, and reconstructed haplotypes. A total of 26 genetic variants were detected in coding and regulatory regions of CD55. Five variants were previously unknown. None of the single variants, rare variants, or haplotypes showed evidence for association with SM or P. falciparum density. Here, we present the first comprehensive analysis of variation in the CD55 gene in the context of SM and show that genetic variants present in a Ghanaian study group appear not to influence susceptibility to the disease.

KEYWORDS malaria CD55 DAF high-resolution screen genetic association study

Globally, malaria killed ~438,000 humans in the year 2015 (World Health Organization 2015). The pathogen causing SM is Plasmodium falciparum, a protozoan parasite, which is transmitted to the human host by the mosquito vector Anopheles spec. Humans get infected during a blood meal of the female mosquito, when parasites are injected into the skin together with the insect’s saliva.

The clinical picture of SM is complex and includes a number of life-threatening signs, such as severe malaria anemia (SMA), cerebral malaria (CM), hyperparasitemia, respiratory distress, and prostration. Besides parasitic and environmental determinants, several host factors are known to influence the risk of developing SM and its associated complications. These factors include age and immune state, as well as the genetic make-up of an infected individual. A study by Mackinnon et al. (2005) estimated that 25% of the variation in SM susceptibility could be attributed to the genetic background of the human host. Over the past decades, many candidate and a few genome-wide association studies on SM have repeatedly shown the impact of human genetic variation on disease susceptibility (Kwiatkowski 2005; Jallow et al. 2009; Timmann et al. 2012; Band et al. 2013).

CD55, a surface molecule on human red blood cells (RBCs), was recently identified as an essential receptor for the invasion of human RBCs by malaria parasites (Egan et al. 2015). CD55, also known as decay accelerating factor for complement (DAF), is a glycosylphosphatidylinositol (GPI)-anchored protein that is broadly distributed among hematopoietic and nonhematopoietic cells. Its main function is the regulation of the complement cascade on the cell surface (Lublin and Atkinson 1989). In addition, it carries the antigen determinants for the Cromer blood group system (CROM). In a forward genetic screen, Egan et al. (2015) showed that CD55-knockdown cells were resistant to invasion by laboratory and by clinical P. falciparum isolates, indicating that CD55 is a critical host factor for all P. falciparum strains. Results from invasion assays also revealed that CD55 is not required for the initial attachment of merozoites to the RBCs, but plays an essential role later in the process, possibly during tight junction formation. Regarding naturally occurring genetic variation in CD55, data from the 1000 Genomes Project (www.1000Genomes.org) revealed an
enrichment of two structural CD55 polymorphisms, R52L (CROM3) and A227P (CROM1), in individuals with ancestral exposure to malaria (Egan et al. 2015).

Taken together, there is substantial evidence that human genetic variation affecting CD55 integrity on the surface of RBCs could influence SM phenotypes, rendering CD55 a promising candidate for association studies. Moreover, CD55 has been discussed as a potential drug target (Tham and Kennedy 2015) and, as such, information on the degree of sequence conservation among malaria-exposed populations is crucial. Here, we screened a large case-control study group comprising more than 1700 subjects (May et al. 2007) from the Ashanti Region in Ghana, West Africa, for genetic variation in the CD55 gene and assessed the influence of common and rare coding variants, and the impact of CD55 haplotypes on SM phenotypes.

### MATERIALS AND METHODS

#### SM case-control group

The SM case-control group comprised 831 SM cases and 903 control individuals from the Ashanti Region in Ghana. SM was defined according to World Health Organization (WHO) criteria as described (May et al. 2007), and control subjects were unaffected healthy individuals.

### Table 1 Characteristics of severe malaria case-control study

| Case-control study | n | Median Age (Range in Months) | Ethnic Group (%) |
|--------------------|---|-------------------------------|-----------------|
| Severe malaria     | 831 | 18 (3–147)                      | Akan 68.3, Northerner 29.5, Ewe 2.2 |
| Controls           | 903 | 27 (4–161)                      | Akan 84.1, Northerner 14.2, Ewe 1.7 |
| Cases stratified by major phenotypes | | | |
| Severe anemia<sup>a</sup> | 506 | 13 (3–132) | Akan 69.7, Northerner 28.2, Ewe 2.1 |
| Cerebral malaria<sup>b</sup> | 166 | 24 (3–147) | Akan 66.9, Northerner 30.7, Ewe 2.4 |

<sup>a</sup> Severe malaria anemia (hemoglobin level < 5 g/dl) with or without additional complications including cerebral malaria.

<sup>b</sup> Cerebral malaria (Blantyre Coma Score [BCS] < 3) with or without additional complications including severe anemia.

#### Table 2 Genetic variants in the CD55 gene detected in 1734 Ghanaian children

| SNP ID, single nucleotide polymorphism identifier; Chr1, chromosome 1; MAF, minor allele frequency; n.a., not applicable. | Position Chr1 (Build 37) | Relative Position<sup>a</sup> | Amino Acid Exchange | Novel | MAF Cases (N = 831) | MAF Controls (N = 903) | Polyphen2 Prediction<sup>b</sup> | SIFT Prediction<sup>c</sup> |
|-------------------------------------------------|------------------------|-----------------------------|-----------------|-------|-------------------|-------------------|------------------|-----------------|
| 1 rs6685886                                      | 207,494,218            | −893C > A                   |                 |       | 0.27              | 0.23              |                  |                 |
| 2 rs28371586                                     | 207,494,913            | −198C > G                   |                 |       | 0.02              | 0.03              |                  |                 |
| 3 rs79066510                                     | 207,495,089            | −21C > G                    |                 |       | 0.02              | 0.03              |                  |                 |
| 4 rs7642430                                      | 207,495,116            | c.6C > G                    |                 |       | 0.02              | 0.03              |                  |                 |
| 5 rs28371587                                     | 207,495,224            | c.100 + 14C > T             |                 |       | 0.02              | 0.02              |                  |                 |
| 6 rs28371588                                     | 207,495,781            | c.155G > T                  |                 |       | 0.04              | 0.04              | Benign           | Tolerated       |
| 7 rs147474393                                    | 207,495,871            | c.245T > G                  |                 |       | 0.008             | 0.01              | Benign           | Tolerated       |
| 8 rs28371603                                     | 207,497,911            | c.294C > T                  |                 |       | 0.005             | 0.007             |                  |                 |
| 9 rs2564979                                      | 207,498,939            | c.479-28T > A               |                 |       | 0.03              | 0.04              |                  |                 |
| 10 rs150286783                                   | 207,500,169            | c.651G > A                  |                 |       |                  | 0.0005            | Tolerated        |                 |
| 11 rs878953964                                   | 207,504,465            | c.677C > T                  |                 |       |                  | 0.0005            | Tolerated        |                 |
| 12 rs60822373                                    | 207,504,467            | c.679G > C                  |                 |       |                  | 0.0005            | Tolerated        |                 |
| 13 rs566298946                                   | 207,504,537            | c.749C > T                  |                 |       |                  | 0.0005            | Tolerated        |                 |
| 14 rs756597715                                   | 207,510,066            | c.882A > G                  |                 |       |                  | 0.0005            | Tolerated        |                 |
| 15 rs797822800                                   | 207,510,087            | c.883A > G                  |                 |       |                  | 0.0005            | Tolerated        |                 |
| 16 rs879028717                                   | 207,510,084            | c.900                        |                 |       |                  | 0.0005            | Tolerated        |                 |
| 17 rs74648822                                    | 207,510,665            | c.980-9A > G                |                 |       |                  | 0.0005            | Tolerated        |                 |
| 18 rs116473488                                   | 207,510,691            | c.997G > A                  |                 |       | 0.02              | 0.02              | Benign           | Tolerated       |
| 19 rs550337224                                   | 207,510,708            | c.1014G > A                 |                 |       | 0.001             | 0.002             |                  |                 |
| 20 rs150172588                                   | 207,510,725            | c.1031C > T                 |                 |       | 0.003             | 0.003             | Possibly damaging| Tolerated       |
| 21 rs28371628                                    | 207,512,734            | c.1081-8G > A               |                 |       | 0.08              | 0.07              |                  |                 |
| 22 rs542054808                                   | 207,513,741            | c.1081 + 979C > T           |                 |       |                  | 0.0005             | Tolerated        |                 |
| 23 rs789071049                                   | 207,513,772            | c.1081 + 1010G > A          |                 |       |                  | 0.0005             | Tolerated        |                 |
| 24 rs138016651                                   | 207,513,861            | c.1081 + 1099C > T          |                 |       |                  | 0.0005             | Tolerated        |                 |
| 25 rs357489214                                   | 207,532,908            | C.1099T > C                 |                 |       |                  | 0.0005             | Tolerated        |                 |
| 26 rs878906671                                   | 207,532,925            | c.1117G > T                 |                 |       |                  | 0.0005             | Tolerated        |                 |

<sup>a</sup> Reference sequence NCBI NM_000574.4.

<sup>b</sup> URL: http://genetics.bwh.harvard.edu/pph2.

<sup>c</sup> URL: http://sift.jcvi.org/www/SIFT_chr_coords_submit.html.
Within the SM case group, 507 (61.0%) presented with SMA and 165 (19.9%) had CM (Table 1). The majority of the SM cases were also affected by additional severe manifestations such as prostration, hyperparasitemia, or hyperlactatemia, which were partly overlapping. Parasite density was determined by counting *P. falciparum* asexual blood stages in Giemsa-stained blood smears (May et al. 2007). The median age of cases and control individuals was 18 and 27 months, respectively (ranges 3–147 months in the case group and 4–161 months in the control group). Phenotyping, recruitment of study subjects, and DNA extraction methods have previously been described in detail (May et al. 2007; Schuldt et al. 2011). After genotyping and evaluation of association results for variant rs7542430, additional DNA samples from 2704 study subjects were used for genotyping in order to confirm initial findings. These subjects were cases and controls that were part of the same original study (May et al. 2007).

**Variant discovery and genotyping**

The gene encoding CD55 is located on chromosome 1 and spans ~40 kb of genomic DNA. In order to screen the CD55 locus (chr1:207,494,817–207,534,311, hg19) for genetic variants, DNA from 1734 unrelated Ghanaians was used for high resolution melting analyses (HRM). Therefore, DNA samples were amplified by PCR using primers that capture the 11 CD55 exons, including a small proportion of their flanking regions as well as 140 bp of untranslated sequence upstream of the transcription start site. Oligonucleotides were designed using LightCycler Probe Design Software 2.0 (Roche Applied Science) against reference transcript NCBI NM_000574.4. Sequences of oligonucleotides and PCR conditions for HRM assays are listed in Supplemental Material, Table S1. Previously unknown single nucleotide polymorphisms (SNPs) and singletons were confirmed by resequencing of genomic DNA. Information about all novel variants detected in our study group was submitted to NCBI dbSNP and will be released in Build 148. In addition, two promoter variants, rs28371586 and rs7542430, were genotyped by allele-specific hybridization in a Roche Lightcycler device (Table S2). Consequences of discovered amino acid missense mutations were analyzed by Polyphen2 and SIFT predictions (Ng and Henikoff 2001; Adzhubei et al. 2010).

**Calculation of linkage disequilibrium (LD)**

Haploview was used to generate the LD plot of the CD55 genomic region (Barrett et al. 2005). The LD calculation was based on healthy control individuals and included all variants with a global minor allele frequency (MAF) ≥ 1%.

**Association analyses of genetic variants with MAF ≥ 1%**

Tests for association of seven variants with global MAFs ≥ 1% were done by logistic and linear regression analyses in PLINK v1.9 assuming an additive mode of inheritance (Purcell et al. 2007). In the regression models, ethnic group, age, and gender were used as covariates. The logarithm of the parasite density was used as a quantitative phenotype in a linear regression analysis in PLINK. In order to account for multiple testing, a correction factor of seven was used; hence, a p-value < 0.007 (0.05/7) was considered significant. A factor of seven was applied because the correlation between the seven SNPs was negligible (all pairwise r-values < 0.05 Figure S1), hence the associations tested represented seven independent tests. All variants were tested for fulfilling the Hardy–Weinberg equilibrium (HWE) in PLINK and were found to comply with frequencies under HWE.

### Table 3

| No. | SNP ID | Major/Minor Allele | MAF (AFU/mm³) | MAF (AFU/mm³) | MAF (AFU/mm³) |
|-----|--------|-------------------|--------------|--------------|--------------|
|     |        |                   |              |              |              |
| 1   | rs6658986 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |
| 2   | rs28371586 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |
| 3   | rs28371586 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |
| 4   | rs28371586 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |
| 5   | rs28371586 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |
| 6   | rs28371586 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |
| 7   | rs28371586 | GA | 0.27 ± 0.23 | 0.27 ± 0.23 |

Severe malaria, severe malaria anemia, cerebral malaria, and parasite density results of logistic regression analyses (linear regression for parasite density) are presented, assuming an additive mode of inheritance adjusted for gender, age, and ethnicity. No., number; SNP ID, single nucleotide polymorphism identifier; Maj, major; Min, minor; MAF, minor allele frequency; Aff, affected individuals; Unaff, unaffected individuals; OR, odds ratio.
Power for detecting effects of variants in the case-control study was estimated with CATS (Skol et al. 2006). For power estimations, a disease prevalence of 2% for SM and \( p < 0.007 \) were assumed. Power calculations for single association tests resulted in a power of 99, 95, and 53% to detect a genotype–phenotype association in the SM, SMA, and CM study groups, respectively, when assuming a genotype relative risk of at least two and a MAF \( \geq 5\% \).

### Haplotype-based association testing

Full-length haplotype analyses were done for the case-control group with the HaploStats package v1.4.4 (Schaid et al. 2002) in R (version 3.1.0: http://www.r-project.org), including reconstructed haplotypes with estimated global frequencies \( \geq 1\% \). In addition, subhaplotypes were evaluated in sliding window analyses capturing a minimum of two and a maximum of six alleles. SM, as well as the major manifestations SMA and CM, and parasite density (logarithmic scale) were used as phenotypes, and the three covariates ethnic group, age, and gender were included in the model. Empirical p-values were computed using default values.

### Association analyses of rare variants

Four different methods integrated in variant tools were used to assess the overall genetic burden due to rare variants (global MAF < 1\%) (San Lucas et al. 2012). These included: (i) the combined and multivariate collapsing (CMC) method described by Li and Leal (2008), (ii) the weighted sum statistic (WSS) described by Madsen and Browning (2009), (iii) the variable-threshold (VT) model described by Price et al. (2010), and (iv) the \( C(\alpha) \) test described by Neale et al. (2011). Variants were pooled on the basis of their MAF and their cumulative effect was tested in univariate and multivariate analyses, in which age, gender, and ethnic group were included as covariates. Tests for association of rare variant carrier status in \( CD55 \) were done for SM, SMA, CM, or hyperparasitemia, defined as \( > 200,000 \) asexual parasites/\( \mu l \) blood.

### Ethics statement

Ethical clearance was granted 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 informed consent was obtained.

### Data availability

The \( CD55 \) mRNA reference sequence for design of HRM reactions was accessible under NCBI NM_000574.4. Oligonucleotides and conditions for HRM reactions and for SNP genotyping are found in Table S1 and Table S2. Genomic locations and frequencies of the SNPs present in this Ghanaian study group are shown in Table 2. Information on previously unknown genetic variants was submitted to NCBI dbSNP and assigned SNP IDs are included in Table 2.

### RESULTS

Variant discovery of \( CD55 \) was conducted in 1734 unrelated individuals from the Ashanti Region in Ghana. The study group comprised 831 SM cases and 903 apparently healthy control individuals. The genomic region screened included coding and regulatory regions of \( CD55 \) as well as the intron–exon boundaries. As a result, a total of 26 SNPs were found to be present in the study group. Of these, five variants were previously unknown (Table 2).
Table 5 CD55 rare variant analyses including eight SNPs with MAF ≤ 1%

|                      | Severe Malaria | Severe Malaria Anemia | Cerebral Malaria | Hyperparasitemia (> 200,000/µl) |
|----------------------|---------------|-----------------------|------------------|-------------------------------|
|                      | N\_cases = 831, N\_controls = 903 | N\_cases = 507, N\_controls = 903 | N\_cases = 165, N\_controls = 903 | N\_cases = 220, N\_controls = 547 |
| p-Value              | p-Value       | p-Value               | p-Value          | p-Value                      |
| Univariate tests     |               |                       |                  |                              |
| CMC                  | 0.952         | 0.979                 | 0.890            | 0.507                        |
| WSS                  | 0.954         | 0.976                 | 0.835            | 0.451                        |
| C(α)                 | 0.400         | 0.412                 | 0.777            | 0.641                        |
| Multivariate tests   |               |                       |                  |                              |
| CMC                  | 0.935         | 0.963                 | 0.904            | 0.423                        |
| WSS                  | 0.972         | 0.970                 | 0.907            | 0.491                        |
| VT test (13 variants)| 0.966         | 0.982                 | 0.829            | 0.896                        |

CMC, combined and multivariate collapsing; WSS, weighted sum statistic; VT, variable-thresholds methods.

* Adjusted for age, gender, and ethnic group.

Nine variants were found to be very rare with MAFs below 1% including 11 singletons. Only three SNPs with MAFs ≥ 1% were found that caused nonsynonymous amino acid exchanges in the receptor protein. These were R52H, L82R, and V333I, with MAFs of 4, 1, and 2% in the control group, respectively. According to Polyphen2 and SIFT predictions all three substitutions were classified as benign. With the exception of V406A, all other missense mutations, all being singletons, were predicted to be probably damaging or damaging (Table 2). Seven variants were found to be more frequent than 1%, and these were initially used in single variant association tests with SM, SMA, CM, or parasite density. The findings are summarized in Table 3.

As a result, the G allele of rs7542430 was found to be associated with a reduction in risk of SM (odds ratio (OR) 0.44, 95% C.I. 0.25–0.79, and p = 0.006). However, when additional subjects of the study were analyzed (N\_cases = 2588 and N\_controls =1850) the association did not hold (OR 0.92, 95% C.I. 0.67–1.26, and p = 0.598).

Reconstruction of haplotypes generated eight full-length haplotypes with a global MAF above 1% (Table 4). Of these, haplotype CD55-1 was the most prominent one with an estimated frequency in the control subjects of 57%. Haplotype CD55-2 was found more frequently in SM cases than in controls (AF 25% in cases and 21% in controls). This difference was statistically significant in the haplotypic-specific score test (p = 0.03, P\_empirical = 0.04). A similar result was found for haplotype CD55-8, which had an estimated AF of 1% in the SM case group and 2% in the control group, and haplotype-specific p-values of 0.01 (Table 4). However, after correction for multiple hypotheses testing, the global test-statistic produced p-values of 0.12 and 0.32, indicating no association of full-length haplotypes with any of the phenotypes.

In order to test for an accumulation of rare variants in either the case or control group, four different algorithms were applied: the CMC method, the WSS, the VT model, and the C(α) test. None of the approaches, which included univariate and multivariate collapsing tests, provided evidence for a joint effect of rare variants on the phenotypes tested (Table 5).

**DISCUSSION**

Following a recent report on CD55 and its essential role for *P. falciparum* survival, it was hypothesized that host genetic variation of CD55 may have an impact on SM disease severity. Here, we analyzed variants in the coding sequence and regulatory regions of CD55 in a large SM case-control study from Ghana. The findings of our study do not support an association between genetic variants and SM in additional chromosomal regions of the human genome that influence CD55 expression. Here, we screened the CD55 coding sequence, intron–exon boundaries, and part of the promoter region, because the main focus was laid on structural...
variation or splice variants of the CD55 receptor, which are likely to influence invasion of RBC by *P. falciparum* merozoites.

Another reason why existing genotype–phenotype associations may remain obscure is lack of power. The power of a study depends on the number of study participants and the MAFs and effect sizes of the alleles tested. For single SNP associations, our study provided enough power for all SNPs > 5% in the SM and SMA study groups. However, the study was underpowered when analyzing SNPs with lower frequencies in the single SNP association tests. Hence, low frequency variants were analyzed using several collapsing approaches that have been developed in order to increase the power for association studies of rare variants.

In addition, there may be true associations between genetic variants and SM in chromosomal regions of the human genome that influence CD55 expression, but that were not covered in this study. Here, we mainly screened the CD55 coding sequence, including intron–exon boundaries and part of the promoter region, because the main focus was laid on structural variation or splice variants of the CD55 receptor, which are likely to influence invasion efficiency. In this regard, exact mechanisms of how CD55 expression is regulated and the genetic variation with potential influence on expression levels on the RBC surface remains to be explored. For instance, a study that evaluated expression quantitative trait loci (eQTLs) in blood cells in samples from over 5300 donors did not identify any variation that functions as eQTLs for CD55 (Westra et al. 2013). One reason may be that the study did not include individuals of African ancestry. In this respect, CD55 expression studies in RBCs originating from African individuals would be helpful to specifically determine eQTLs for CD55 in RBCs.

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