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ORIGINAL ARTICLE

Variation in human genes encoding adhesion and proinflammatory molecules are associated with severe malaria in the Vietnamese

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The genetic basis for susceptibility to malaria has been studied widely in African populations but less is known of the contribution of specific genetic variants in Asian populations. We genotyped 67 single-nucleotide polymorphisms (SNPs) in 1030 severe malaria cases and 2840 controls from Vietnam. After data quality control, genotyping data of 956 cases and 2350 controls were analysed for 65 SNPs (3 gender confirmation, 62 positioned in/near 42 malarial candidate genes). A total of 14 SNPs were monomorphic and 2 (rs8078340 and rs33950507) were not in Hardy–Weinberg equilibrium in controls ($P < 0.01$). In all, 7/46 SNPs in 6 genes (*ICAM1*, *IL1A*, *IL17RC*, *IL13*, *LTA* and *TNF*) were associated with severe malaria, with 3/7 SNPs in the *TNF/LTA* region. Genotype–phenotype correlations between SNPs and clinical parameters revealed that genotypes of rs708567 (*IL17RC*) correlate with parasitemia ($P = 0.028$, $r^2 = 0.0086$), with GG homozygotes having the lowest parasite burden. Additionally, rs708567 GG homozygotes had a decreased risk of severe malaria ($P = 0.007$, OR = 0.78 (95% CI; 0.65–0.93)) and death ($P = 0.028$, OR = 0.58 (95% CI; 0.37–0.93)) than those with AA and AG genotypes. In summary, variants in six genes encoding adhesion and proinflammatory molecules are associated with severe malaria in the Vietnamese. Further replicative studies in independent populations will be necessary to confirm these findings.

INTRODUCTION

Around half of the world's population is at risk of malaria, with 243 million people infected and nearly 1 million deaths in 2008.[1] Vietnam has made great achievements in controlling malaria during the last decade with the number of cases reduced from 187 994 in 1991 to 54 297 in 2010 and the number of deaths reduced from 4646 deaths in 1991 to 21 deaths in 2010.[2] This success is attributable to targeting interventions to high-risk areas and balancing case management with prevention, that is, combining impregnated bed-nets, insecticide spraying and early diagnosis with the availability of effective treatment, namely artemisinin derivatives.[3,4] Despite this success in prevention, malaria still persists in Vietnam. Additionally, a study by Erhart *et al.*[5] demonstrated that the health information system in Vietnam greatly underestimates malaria burden. Malaria cases are generally found in ethnic minority areas of the central region that tend to be impoverished, less accessible to effective health systems and provide the forested areas that support the common mosquito vectors that transmit malaria, *Anopheles dirus* and *A. minimus*.[6] These regions, many of which border Cambodia, are also at risk of acquiring drug-resistant *Plasmodium falciparum*. Recent reports suggest that the effectiveness of artemisinin-based combination therapy and artesunate monotherapy has declined in western Cambodia.[7] Although there have been many successes in malaria control in Vietnam, the emergence of reduced *in vivo* sensitivity of *P. falciparum* to artemisinin derivatives reminds us that challenges still lie ahead.

Malaria control in endemic countries would benefit greatly from an effective vaccine. Recently, a number of pre-erythrocytic-stage vaccines have been developed and tested in clinical trials.[8–11] Even though it is likely that these vaccines, or ones similar, are heading towards licensure, they are not yet able to provide sterilizing and life-long protection against *P. falciparum* infection. Blood-stage vaccines may have a higher potential for protective immunity, but unfortunately have shown less success in clinical studies to date.[12] To develop an efficacious multi-stage malaria vaccine that can provide protection in an endemic setting, it is paramount to have a complete understanding of the molecular mechanisms of protective immunity. Studies of the human genome can teach us about resistance to malaria as populations have evolved different genetic variants to protect against malaria.

The MalariaGEN Consortium (www.malariagen.net) is a global research network that utilizes a genetics approach to identify new mechanisms of protective immunity against malaria, which may lead to novel vaccine development.[13] MalariaGEN has recruited cases of severe malaria and controls from 15 malaria endemic countries for genetic studies. As part of the MalariaGEN consortium, here we report genotyping data from 65 single-nucleotide polymorphisms (SNPs) in 42 malarial candidate genes in 956 severe malaria cases and 2350 controls from Vietnam. Variants in 6 genes (*ICAM1*, *IL1A*, *IL17RC*, *IL13*, *LTA* and *TNF*) were associated with severe malaria in this population.

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RESULTS

Clinical characteristics of cohort

The baseline clinical and demographic characteristics of the severe malaria patients and population controls are shown in Table 1. A total of 956 adults meeting the clinical definition of severe malaria were recruited. The ethnic majority of malaria patients and controls was Vietnamese Kinh (87% in cases and 93% in controls), with more males in the malaria group than controls (71% in cases and 54% in controls). Our patients were adults with a median (interquartile range) age of 28 (20–40) years and a medium (interquartile range) parasite count of 61 860 (7756–254 200). The patients were rarely severely anaemic (>20% haematocrit) and their mortality rate was 10.4%.

Malaria candidate SNPs are associated with severe malaria in Vietnam

In total, we genotyped 67 SNPs in 1030 severe malaria cases and 2840 population controls. Subjects were removed from the data set if they had >10% of their total genotype data missing. SNPs were removed from the data set if >15% of the subjects genotyped for that SNP had missing genotype data. After the removal of sample and SNP missingness, we analysed data for 65 SNPs genotyped in 956 severe malaria cases and 2350 populations. The 65 SNPs lie in or near 42 genes. Data from three SNPs in the amelogenin gene (*AMELX*) was used for gender confirmation. The remaining 62 SNPs are found near or within 41 malarial candidate genes. Two SNPs (rs33950507, rs8078340) were not in Hardy–Weinberg equilibrium (controls; $P < 0.01$) and 14 SNPs were monomorphic (Supplementary Table 1). This was not unexpected as four SNPs included in our study were of known African origin

and as such were present as the ancestral allele in our population (*HbS* (rs334), *G6PD* + 376 (rs1050829), *G6PD* + 202 (rs1050828) and Duffy (rs1803632)).

Table 2 shows that 7/46 SNPs in six malarial candidate genes are associated with severe malaria in this Vietnamese cohort; rs5498 (*ICAM1*), rs17561 (*IL1A*), rs708567 (*IL17RC*), rs20541 (*IL13*), rs909253 (*LTA* + 252), rs1799964 (*TNF*-1031) and rs1800629 (*TNF*-308). *P*-values representing allelic and genotypic association are shown in Table 2. Data for all 62 SNPs are provided in Supplementary Table 1. All association analysis of the cases and controls was adjusted by ethnic group. We performed an analysis within the Vietnamese Kinh ethnic group alone and found only five out of the seven SNPs above were associated with severe malaria (rs909253 and rs1799964 in *LTA* and *TNF*, respectively, did not remain significantly associated). Owing to the significantly smaller sample sizes of ethnic groups other than Vietnamese Kinh we were unable to perform a sufficiently powered analysis stratified by ethnic group. As the population controls were a mixture of newborn (cord blood controls) and adults (community controls) we used the allele frequencies of the 46 SNPs to calculate the F_{st} of the two groups. A mean and median F_{st} of 0.000595 and 0.000479, respectively, are evidence to suggest both control groups are very similar.

Malaria candidate SNPs are associated with death from severe malaria in Vietnam

Two SNPs in two malarial candidate genes were associated with death from severe malaria (rs7935564 (*TRIM*) and rs708567 (*IL17RC*)) when fatal severe malaria cases were compared with cases that recovered from the disease (Table 3).

Table 1. Baseline characteristics and outcome of severe malaria patients and control subjects

Parameter	Sample size	No. (%) or median (IQR)	Min–max
Severe malaria cases			
Genotyped Sex (%)—male/female	956	684 (71.5)/272 (28.5)	
Ethnicity (%)			
Kinh	956	828 (86.61)	
S'tieng	956	48 (5.02)	
Others	956	43 (4.50)	
Unknown	956	37 (3.87)	
Age (years), median (IQR)	942	28 (20–40)	0.66–79
Fever within past 48 h (%)	475	473 (99.6)	
Administered antimalarials prior admission (%)	248	43 (17.3)	
GCS, median (IQR)	849	11 (8–15)	3–15
BCS, median (IQR)	43	5 (3–5)	1–5
Convulsions after admission (%)	837	69 (8.2)	
Parasite count (μl⁻¹), median (IQR)	828	61 860 (7756–254 200)	0–3534 000
Haematocrit (%), median (IQR)	894	30.6 (24.5–36.0)	6–60
Haemoglobin (g dl⁻¹), median (1QR)	121	10.2 (8.1–11.7)	2.5–14.9
Blood transfusion received (%)	698	213 (30.5)	
Respiratory rate (min⁻¹), median (IQR)	832	28 (24–32)	14–68
Lactate (mmol l⁻¹), median (IQR)	747	3.3 (2–5.05)	0.022–28
Glucose (mmmol l⁻¹), median (IQR)	711	5.5 (4.1–7.3)	0.5–31.3
Administered glucose (%)	941	214 (23)	
Mortality (%)	914	96 (10.4)	
Control subjects			
Genotyped sex (%)—male/female	2221	1195 (53.80)/1026 (46.20)	
Ethnicity (%)			
Kinh	2351	2194 (93.32)	
S'tieng	2351	96 (4.08)	
Others	2351	52 (2.21)	
Unknown	2351	9 (0.38)	
Age (years), median (IQR)			
Cord blood control	1811	0	0
Community control	534	23 (10–34)	0.66–73

Abbreviations: BCS, Blantyre coma score; GCS, Glasgow coma score; IQR, interquartile range.

Table 2. SNPs associated with severe malaria in Vietnam adjusted by ethnic group

rs number	Gene	Chr	1	2	Case allele Freq 1	Control allele Freq 1	P (add)	OR (95% CI)	P (gen)	HWE control P
rs5498[a]	ICAM1	19	A	G	0.80	0.78	0.039	0.87 (0.76–0.99)	0.008	0.53
rs17561	IL1A	2	G	T	0.94	0.95	0.30	1.14 (0.90–1.44)	0.005	0.02
rs708567	IL17RC	3	A	G	0.14	0.11	0.004	0.79 (0.67–0.93)	0.016	0.37
rs20541[b]	IL13	5	C	T	0.62	0.58	0.007	0.86 (0.77–0.96)	0.023	0.90
rs909253[c]	LTA	6	C	T	0.46	0.49	0.035	1.12 (1.01–1.25)	0.079	0.36
rs1799964[d]	TNF	6	C	T	0.25	0.23	0.023	0.86 (0.76–0.98)	0.072	0.87
rs1800629[e]	TNF	6	A	G	0.06	0.07	0.054	1.24 (0.99–1.54)	0.027	0.58

Abbreviations: Add, the additive model; Freq, frequency; Gen, general; HWE, Hardy–Weinberg equilibrium; OR (95% CI), odds ratio (95% confidence interval).
[a]ICAM1 codon469. [b]IL13 46457. [c]LTA Nco1. [d]TNFa-1031. [e]TNF-308.

Table 3. SNPs associated with death from severe malaria in Vietnam adjusted by ethnic group

rs number	Gene	Chr	1	2	Fatal cases Allele freq 1	Recovered cases Allele freq 1	P (add)	OR (95% CI)	P (gen)	HWE Control P
rs7935564	TRIM	11	A	G	0.125	0.188	0.049	1.55 (0.98–2.44)	0.138	0.25
rs708567	IL17RC	3	A	G	0.176	0.136	0.073	0.68 (0.45–1.02)	0.047	0.13

Abbreviations: Add, the additive model; Freq, frequency; Gen, general; HWE, Hardy–Weinberg equilibrium; OR (95% CI), odds ratio (95% confidence interval).
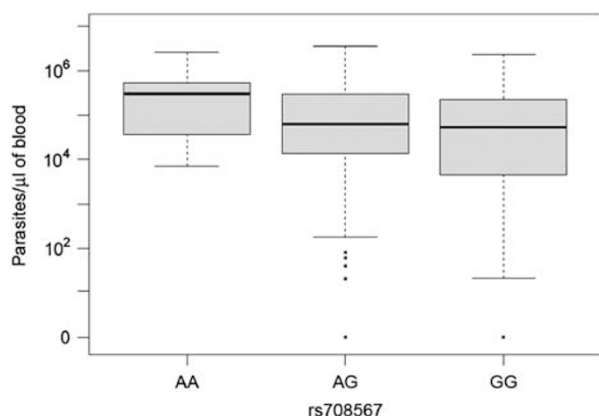
Figure 1. Parasite count by rs708567 genotype. Parasite counts of 832 severe malaria patients were grouped by genotype of IL17RC SNP rs708567. To test for genotype–phenotype correlations we compared the mean parasitemia from patients with the AA, AG and GG genotypes of rs708567 by anova (linear model; $P = 0.028$, $r^2 = 0.0086$). Data for parasites per μl of blood are plotted with the median and interquartile range represented by the horizontal bar and light grey box.

IL17RC genotypes are associated with parasitemia in severe malaria patients

Genotype–phenotype correlations between our SNP genotype data and clinical parameters of the severe malaria patients were tested. We compared the mean parasite count/μl of blood from 832 patients with the AA, AG and GG genotypes of rs708567 by anova (linear model) and found that genotypes of this SNP correlated with parasitemia ($P = 0.028$, $r^2 = 0.0086$; Figure 1). rs708567 in IL17RC was associated with severe malaria, and the recessive model shows a protective effect of GG homozygotes (that is, the risk of severe malaria is lower in GG homozygotes; $P = 0.007$, OR = 0.78 (95% CI; 0.65–0.93)). In addition, rs708567 in IL17RC was associated with death from severe malaria (genotypic $P = 0.047$, Table 3), and the recessive model also shows a protective effect of GG homozygotes (that is, the risk of death from severe malaria is lower in GG homozygotes; $P = 0.028$,

OR = 0.58 (95% CI; 0.37–0.93)). Interestingly, the parasite burden was lowest in GG homozygotes (Figure 1).

DISCUSSION

As malaria is a complex disease it is expected that the overall disease risk of an individual will be determined by modest contributions of multiple genes. By genotyping SNPs in a number of known malarial candidate genes we found disease associations with genes involved in a variety of critical events in malaria pathogenesis. Genes encoding molecules involved in adhesion (ICAM1) as well as the proinflammatory response (TNF, LTA, IL1A) were associated with severe malaria and as expected, all variants modestly contributed to the overall disease risk.

Cytoadherence is a critical event in malaria pathogenesis and is thought to be an immune evasion strategy. Parasitised erythrocytes are sequestered in small blood vessels[14] allowing the parasites to remain within the vascular compartment and avoid circulation through the spleen. Sequestration occurs through binding of parasitized erythrocytes to a range of receptors, one of these being intercellular adhesion molecule 1 (ICAM1; CD54). ICAM1, a cell surface glycoprotein, functions as an endothelial and immune cell adhesion receptor for integrin-expressing leukocytes. Genetic associations of ICAM1 variants with severe malaria have often been contradictory. Early studies in Kenya[15] and Gabon[16] identified associations between rs5491 (ICAM-1Kilifi) and malaria; however, these initial observations were not replicated,[17–19] including a large, well-powered study combining three populations (Gambia, Kenya and Malawi) with familial and case–control association analysis.[20] In the Vietnamese cohort rs5491 was excluded owing to high genotyping failure rate; however, we found an association with rs5498 and severe malaria. rs5498, a non-synonomous SNP in the exon 6 region encoding Ig-like domain 5 of the extracellular portion of ICAM1, is also associated with an increased risk of severe malaria in Nigeria[21] and India.[22]

Many reports on the human immunological response to malaria exist; however, it is still unclear which of these provide protective immunity against malaria, and what is their underlying mechanism. Studying the genetic interaction between malaria and the immune system may provide further opportunities to uncover

these mechanisms, therefore variants of proinflammatory cytokine genes have been assessed in multiple populations. The *TNF* gene region has been a candidate for malaria as a promoter polymorphism (*TNF*-308) was first found associated with cerebral malaria in 1994;[23] however, subsequent studies have not always replicated this finding. A recent large study involving >10 000 individuals from three African populations sought to clarify these inconsistencies, and found some evidence of *TNF* haplotypes associated with severe malaria in the Gambia, but not in the closely related Kenya and Malawi populations.[24] The *TNF* SNPs associated with severe malaria in the Gambia (*TNF*-308, *TNF*-1031) were also associated in the Vietnamese Kinh, along with *LTA* + 252. Explanations for inconsistencies in data across different populations with sufficiently powered sample sizes, are the haplotypic diversity between populations, as well as phenotypic differences in patients. The issue of haplotypic diversity between populations is particularly relevant when the causal variant responsible for the disease association is unknown and surrogate markers are being genotyped. This may be the case here as it has been suggested that the causal variants may be located some distance downstream of *TNF* and *LTA*.[25,26]

Interleukin 1 (IL1) exists in two forms, IL1α and IL1β, and both have a prominent role in the acute phase response by inducing expression of multiple cytokines and inflammatory molecules, leading to activation of T cells and monocytes and upregulation of adhesion molecules. Variants in this gene region have been associated with various diseases[27–30] and specifically to malaria.[31,32] Walley *et al.*[31] reported the only association between *IL1A* + 4845 G-T (rs17561) and mild malaria. In the Vietnamese rs17561 is associated with severe malaria, a variant that causes a non-conservative amino-acid change (Ala 114 Ser) of the IL1α protein. Different case definitions or demographics of the malarial populations could explain why rs17561 is associated with mild malaria in the Gambia and severe malaria in the Vietnamese. In Vietnam, an area of low transmission, malaria is generally a disease of non-immune adults as opposed to areas of high transmission in Africa where it is generally a disease of children.

For our analysis we have classified different clinical phenotypes together as severe malaria, even though the genetic aetiology underlying each subphenotype may be different. In particular, the cerebral form of malaria may have a different genetic determinant compared with hemocratic forms of malaria. When the association analysis was restricted only to the patients affected by the cerebral form of malaria (Glasgow coma score, GCS < 9), we observed a suggestive genotypic association between cerebral malaria onset with rs17561 (*IL1A*; P = 0.039; data not shown). A polymorphism in the *TLR4* gene, rs4986790, also had a suggestive association between cerebral malaria onset (rs4986790, P = 0.044, OR = 2.85 (1.14–7.17)); however, both cerebral malaria association were not significant after the most conservative Bonferroni adjustment.

IL17A and IL17F are inflammatory cytokines expressed by Th17 cells, a unique lineage of CD4+ helper T cells, which promote innate effector mechanisms of inflammation.[33] The IL17 receptor complex composed of IL17RA and IL17RC is essential for the biological activity of IL17,[34] and this complex induces the activation of the NF-κB and MAPK signalling pathways. Appropriate regulation of the IL17 signalling axis has an integral role in host defence against extracellular bacteria and fungi; however, there is limited evidence regarding its role in the host defence against protozoa.[33,35] Previously, rs708567 in *IL17RC* was associated with two subsets of malaria patients within an ethnically diverse epidemiological cohort from Sudan[36] and based on data available for rs708567, Fumagalli *et al.*[37] determined that *IL17RC* is a target of pathogen-driven selection along with 44 other IL genes.[37] For the Vietnamese, having the GG genotype of rs708567 makes one 22% less likely to get severe malaria and affords 42% protection from death if one happens to get it. Interestingly, the GG homozygotes (Ser 111 Leu), which are the most frequent in the Vietnamese, have a lower parasite burden than AA or AG patients. Carrying the A allele of rs708567 is detrimental in terms of malaria; however, it is yet to be determined whether this is due to interference in mediating the IL17 signalling axis or from an IL17RC-specific function.

Using a genome-wide approach to identify disease genes using a human model with natural conditions of infection can potentially reveal genes encoding important protective immune responses that are currently unknown. GWA studies are therefore a crucial step in determining the contribution of all known and unknown genes involved in an unbiased manner. We are currently preparing a GWAS of severe malaria in the Vietnamese Kinh using the case and control cohorts introduced here within the MalariaGEN consortium, who have already published a GWAS of severe malaria in the Gambians.[38] Meta-analysis of multiple GWAS from ethnically diverse populations will ultimately be able to identify all contributing mechanisms necessary for protective immunity against malaria.

SUBJECTS AND METHODS

Human subjects and study design

The Hospital for Tropical Diseases (HTD) and Oxford University Clinical Research Unit in Vietnam have recruited severe malaria patients into research studies between 1991 and 2009 (Table 4). Severe malaria patients were recruited specifically into randomized controlled clinical trials[39,40] at HTD, a tertiary referral hospital in Ho Chi Minh City (HCMC), or into an epidemiological study conducted at HTD and two provincial hospitals in Binh Phuoc province, Phuoc Long and Dong Xoai district hospitals. Binh Phuoc province is a low highland region in south central Vietnam. It has low seasonal transmission of *P. falciparum* and *P. vivax* mainly in the forested rural areas, with very low entomological inoculation rates (in most

Table 4.	Sample collection of severe malaria cases and controls		
Collection year	Study description	Location	Sample no.
Severe malaria			
1991–1995	Randomized controlled trial[40]	HTD, HCMC	420
1996–2001	Randomized controlled trial[39]	HTD, HCMC	249
2000–2005	Epidemiological study	HTD, HCMC	300
		Phuoc Long and Dong Xoai District Hospitals, Binh Phuoc Province	
2005-ongoing	MalariaGEN collection	HTD, HCMC	132
Controls			
2000–2005	Epidemiological study	Community controls, Phuoc Long and Dong Xoai District Hospitals	600
2000–2005	Epidemiological study	Family controls, Phuoc Long and Dong Xoai District Hospitals	132 (66 Trios)
2003–2006	Birth cohort	Cord blood controls, Hung Vuong hospital, HCMC	1000
2006–2007	Birth cohort	Cord blood controls, Hung Vuong hospital, HCMC	1250

Abbreviations: HTD, Hospital for Tropical Diseases; HCMC, Ho Chi Minh City.

areas <1). A collection of severe malaria patients primarily for genetics studies is currently ongoing at HTD.

Severe malaria cases were defined as those who had asexual forms of *P. falciparum* in their peripheral blood smear and had at least one of the following; impaired consciousness (Glasgow coma score <11 or Blantyre coma score <5), pulmonary oedema, acute renal failure (oliguria and serum creatinine >265 μmol l^{-1}), jaundice (serum bilirubin >51 μmol l^{-1} with parasite count >100 000 μl^{-1} or with serum creatinine >250 μmol l^{-1}), hypoglycaemia (blood glucose <2.2 mmol/l^{-1}), anaemia (haematocrit <20% with parasite count >100 000/μl^{-1}), hyperparasitaemia (parasite count >500 000/μl^{-1}), hyperlactataemia (plasma lactate >4 mmol/l^{-1}), metabolic acidosis (standard base excess >−5 mmol/l^{-1}, base deficit <10 mmol/l^{-1}), pigmented neutrophil count (>4/100) and shock (SBP <80 mm Hg with cool extremities); or had parasitemia ⩾5% but none of the above features. Patients with cerebral malaria were defined as those with a Glasgow coma score <9.

The population control individuals ($N = 2840$) were either cord blood controls or community controls. Cord blood control samples ($N = 2270$) were collected from babies born in 2003 and between 2006 and 2007 at Hung Vuong Obstetric Hospital in HCMC and from babies born in 2003 at Dong Thap Hospital in Dong Thap province. In addition, community controls ($N = 570$) were recruited as part of the epidemiological study, who were individually matched to a subset of the severe malaria cases by age (0–73 years), gender, ethnicity and location. Potential community controls were questioned about any possible history of severe malaria or time spent in hospital. Any candidates who had spent more than 48 h in hospital other than for an operation, injury or known non-malaria diagnosis were excluded.

The samples came from unrelated individuals whose ethnic background was assessed by questionnaire. The treating physician was responsible for obtaining the patient's informed consent and recruiting patients into the study. Before study recruitment, patients were informed of the risks and benefits of being in these studies and patients could refuse to participate. Written informed consent was obtained from each volunteer; however, in the cases where the patient was unable to consent, that is unconscious, the consent of the relatives was acceptable. For cord blood control samples, informed consent was obtained from the mother. Ethical approvals were granted by the scientific and ethical committees at either the HTD HCMC, Hung Vuong Hospital HCMC, Dong Thap Hospital Dong Thap Province and the People's Committee of Ho Chi Minh City, Department of Health. Protocols were also approved by the Oxford Tropical Research Ethics Committee, UK.

DNA extraction and quantification

Genomic DNA from severe malaria patients and community controls was extracted from between 1 and 5 ml of venous blood collected in tubes containing EDTA anticoagulant. DNA was extracted by using either the blood midi kit or maxi kit from Qiagen (Lewes, UK). For controls, genomic DNA was extracted from 10 ml of cord blood using the Qiagen blood maxi kit (Qiagen). DNA was shipped frozen to Oxford University, UK. After arrival, the sample manifest was confirmed and all samples were relabelled and recoded with new sample codes to a standardized format bearing no relationship to the original coding. Sample volumes were recorded and the DNA was quantified using the PicoGreen reagent (Invitrogen, Paisley, UK) as per manufacturer's instruction.

Primer-extension amplification (PEP)

gDNA was diluted to 1 ng μl^{-1} in 96-well plates. A PCR reagent mixture (45 μl comprising 2.2 μl of 1:10 diluted N15 primers (Molecular Devices Ltd, Wokingham, UK), 1.25 μl 8 mM pooled dNTPs, 2.5 μl 50 mM MgCl$_2$, 5 μl of 10 × buffer, 0.5 μl 5U μl^{-1} Biotaq polymerase (Bioline, London, UK), 33.55 μl MilliQ water) was added to each well. gDNA (5 μl of 1 ng μl^{-1}) was added to the PEP PCR mixture, the plates were sealed and thermocycled with the following programme: 94 °C for 3 min; 50 cycles of 94 °C for 1 min, 37 °C for 2 min with a 0.1 °C increase per cycle up to 55 °C; 55 °C for 4 min; and a final extension of 72 °C for 5 min. PEP DNA was stored at −20 °C until used. Quality and performance of the PEP reaction and products was assessed by PCR using a primer pair selected from the genotyping assays described below and run out on 2% agarose gels to check band intensity and fidelity.

Selection of SNPs

The selection of SNPs for genotyping was undertaken by the MalariaGEN consortium. This provided a common set of SNPs typed on all samples

within the MalariaGEN consortium as part of a process for quality control of samples included in the Consortium's projects. These SNPs were selected by interrogation of the literature and ongoing Consortium experiments for evidence of association with severe malaria. The two Sequenom iPLEX reactions designed also included gender-typing SNPs. Full details can be found at www.malariagen.net and the manuscript is in preparation.

Genotyping

Genotyping of PEP DNA samples diluted 1:10 was performed using the SEQUENOM iPLEX Gold platform and were performed according to the manufacturers specifications (http://www.sequenom.com). All primers were purchased and lyophilized from Metabion International AG (Martinsried, Germany).

Data analysis

Genotypic deviations from Hardy–Weinberg equilibrium were assessed using a χ^2 statistical test. SNPs were excluded from analysis if they had at least 15% of genotype calls missing or there was significant deviation from Hardy–Weinberg equilibrium ($P < 0.01$). Subjects were removed from the data set if they had >10% of their total genotype data missing, or their self-reported gender deviated from the gender-specific genetic markers (amelogenin gene, three SNPs). Univariate analysis was performed for categorical variables with Pearson's χ^2 test to assess associations between disease phenotypes and allele or genotype frequencies. All association analysis of the cases and controls was adjusted by ethnic group, where a categorical variable encoding the ethnicities is included as a covariate in the logistic regression. The logistic regression also allows the odds ratios for the genotypes to be estimated. In this approach we modelled the SNP of interest assuming several related genotypic mechanisms (additive, dominant, recessive, heterozygous advantage and general models) and report the minimum P-value from these correlated tests. More specifically, the general model was used to compare genotype frequencies. Briefly, the hypothesis of this model is that one genotype group (for example, AA group) is the baseline, and there is no pattern at all between the odds ratios for the genotype groups AB and BB. Therefore, there are separate estimates for the odds ratio for genotype AB, and another odds ratio for genotype BB. This model that uses the three genotype classes makes no assumptions about the risk or mean for AB heterozygotes compared with AA and BB homozygotes. We calculated the F_{st} between the cord blood controls and the community controls using allele frequencies and the following formula (p1–p2)^2/[(p1 + p2)(2−p1−p2)]. Continuous outcomes (for example, parasite count μl^{-1}) were tested for association using a linear model. All analyses were performed using the R statistical package (http://www.r-project.org). Performing multiple statistical tests leads to inflation in the occurrence of false positives, therefore this study should be regarded as exploratory. To be able to use these associated variants for any translational purposes it is essential to replicate these SNP associations in independent cohorts. Therefore, the data presented here are unadjusted, but given the number of SNPs tested ($N = 65$), many SNPs would not remain significant after conservative adjustment (for example, Bonferroni). However, given that all tests are not independent (related phenotypes, SNPs not independent due to linkage disequilibrium and so on) Bonferroni adjustment may not be appropriate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)