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Interferon-alpha receptor-1 (IFNAR1) variants are associated with protection against cerebral malaria in The Gambia

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The chromosome 21q22.11 cytokine receptor cluster contains four genes that encode subunits of the receptors for the cytokines interleukin-10 and interferon-alpha, -beta and -gamma that may have a role in malaria pathogenesis. A total of 15 polymorphic markers located within these genes were initially genotyped in 190 controls and 190 severe malaria cases from The Gambia. Two interferon-alpha receptor-1 (IFNAR1) gene SNPs (17470 and L168 V) showed evidence for an association with severe malaria phenotypes and were typed in a larger series of samples comprising 538 severe malaria cases, 338 mild malaria cases and 562 controls. Both the 17470-G/G and L168V-G/G genotypes were associated with protection against severe malaria, in general, and cerebral malaria, in particular ($P = 0.004$ and 0.003, respectively). IFNAR1 diplotypes were then constructed for these two markers using the PHASE software package. The (17470-G L168V-G/17470-G L168V-G) diplotype was found to be associated with a reduced risk of cerebral malaria and the (17470-C L168V-C/17470-G L168V-G) diplotype with an increased risk of cerebral malaria (overall $3 \times 2\ \chi^2 = 12.8$, d.f. $= 2$, $P = 0.002$ and $3 \times 2\ \chi^2 = 15.2$, d.f. $= 2$, $P = 0.0005$, respectively). These data suggest a role for the type I interferon pathway in resistance to cerebral malaria.
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Introduction

Malaria is a global health problem affecting approximately 40% of the world's population, with a prevalence of 300–500 million clinical cases per year. Around 90% of these cases are in sub-Saharan Africa, and result in over 1 million deaths per year, predominantly of children.[1] Host genetic factors have been shown to play a central role in resistance mechanisms against malaria infection and clinical malaria. Indeed, genetic linkage has been demonstrated by family-based studies between *Plasmodium falciparum* blood infection levels and the 5q31–q33 locus,[2] and between mild clinical malaria and the HLA locus.[3] Furthermore, case–control studies have detected associations between several gene variants and resistance or susceptibility to cerebral malaria and/or severe anaemia.[4] Most of the genes associated with severe malaria in these studies are coding for molecules involved in red blood cell physiology (α- and β-globin) or in the host immune response (HLA, INOS, ICAM-1, TNF-α).

Immunoepidemiological studies in endemic areas have supported the importance of these molecules in clinical malaria, especially TNF-α, high levels of which were found in children with cerebral malaria.[5,6] Studies have also shown that the TNF receptor-2 (TNFR2) was upregulated during cerebral malaria in both humans and mice,[7] and more recently interferon-gamma receptor-1 gene (IFNGR1) promoter polymorphisms have been reported to be associated with cerebral malaria.[8] These results suggest that cytokines, cytokine receptors and their downstream signalling pathways are important candidates in host susceptibility to severe malaria.

Several clusters of immune response genes have been identified within the human genome. One of these, the cytokine receptor-II family, is located on chromosome 21q22.11, and contains genes coding for interferon-alpha receptor-1 (IFNAR1), interferon-alpha receptor-2 (IFNAR2), interleukin-10 receptor-B (IL10RB) and interferon-gamma receptor-2 (IFNGR2).[9–11] IL-10 and IFN-γ responses have been extensively studied in endemic areas and are shown to be involved in malaria pathogenesis.[12–18] Type-I interferon (IFN-α/β), mainly produced by monocytes and dendritic cells, plays a central role in antiviral and antitumoural immunity. IFN-α/β stimulate both antigen presentation and cellular cytotoxicity, and play a central role in the regulation of the immune response by stimulating both pro- and anti-inflammatory cytokines (IL-2, IL-10, IL-12, IL-18, IL-10).[19] As opposed to IL-10 and IFN-γ, the role of IFN-α/β in malaria has not been extensively investigated. However,

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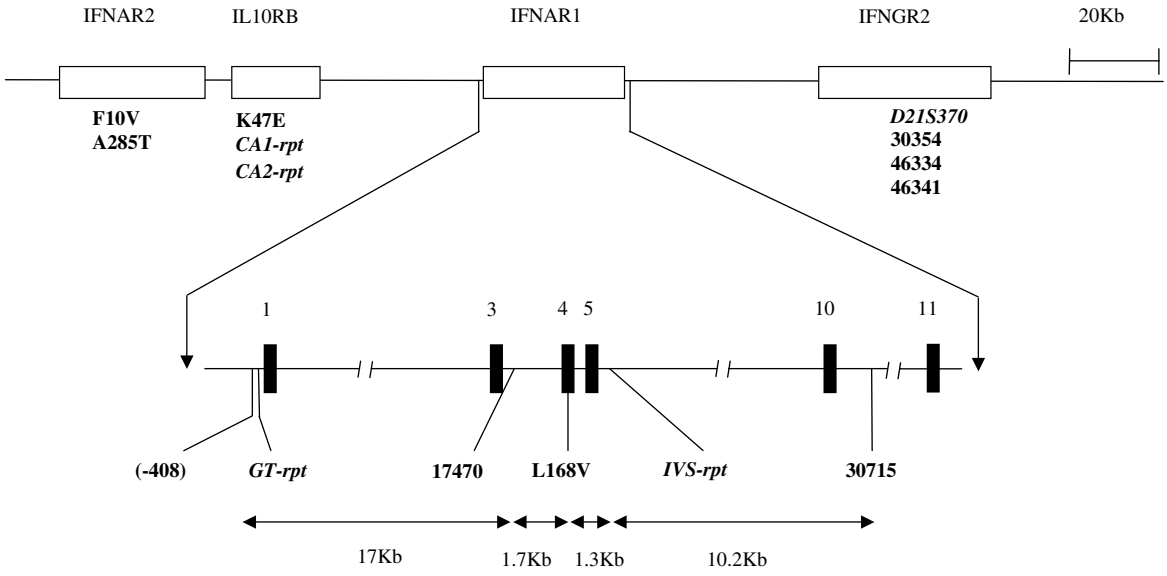

Figure 1 Chromosome 21q22.1 genes and localisation of IFNAR1 markers. Map of the chromosome 21q22.11 cytokine receptor cluster. The four genes are shown as boxes: IFNAR2 = interferon-alpha receptor-2, IL10RB = interleukin-10 receptor-B, IFNAR1 = interferon-alpha receptor-1 and IFNGR2 = interferon-gamma receptor-2. Polymorphic markers are shown in bold, with SNPs in normal typeface and microsatellite repeats in *italics*. The IFNAR1 gene is expanded to show the relative positions of the four SNPs and two microsatellite repeat markers. Exons are represented by filled boxes with the exon number above and relative distances in kilobases below.

some studies suggest that IFN-α may influence the outcome of malaria infection in mice,[20] or induce an increased immune response against malarial antigens[21] and protect against severe malaria in humans.[22]

The objective of this study was to assess, in a Gambian case–control sample of 1420 children (aged 1–10), the role of cytokine receptors IFNAR1, IFNAR2, IL10RB and IFNGR2 in the genetic control of severe malarial anaemia and cerebral malaria.

Results

Genotype distributions of 15 polymorphic markers from the 21q22. 11 locus in the Gambian malaria case–control group

A total of 15 genetic markers, five microsatellites and 10 SNPs, located in the IFNAR2, IL10RB, IFNAR1 and IFNGR2 genes in the 21q22.11 cytokine receptor cluster were analysed in this case–control study (Figure 1). No difference was observed between mild controls ($n = 248$) and severe controls ($n = 314$) for allele frequency and genotype distribution of any SNP or microsatellite (data not shown). Consequently, these control subjects were grouped as a single control group for subsequent analyses. The average heterozygosity of the five microsatellite markers was 0.75 in this stud**y.** No significant difference was observed between controls and severe malaria cases for these microsatellite markers (data not shown). Of the 10 intragenic SNPs, four result in an amino-acid change in the IFNAR2 (F10V, A285T), IL10RB (K47E) and IFNAR1 (L168V) polypeptides, and six are located in noncoding regions (Figure 1). For each SNP, allele frequencies were not significantly different between controls and mild malaria, severe malaria, cerebral malaria or severe anaemia cases (data not shown, available at http://www.well.ox.ac.uk/hill/Malaria/TABLE-IFNAR1.htm).

Genotype distributions of each SNP were compared between severe malaria cases and controls using the Pearson's 3×2 χ^2 test. No significant difference was observed for the genotype distributions of the IFNAR2, IL10RB and IFNGR2 SNPs or for IFNAR1 (−408) and 30715 markers (data not shown). However, as shown in Table 1, IFNAR1-17470 and L168V genotype distributions were significantly. different between controls and severe malaria (3×2 $\chi^2 = 8.9$, $P = 0.011$, and 3×2 $\chi^2 = 11.5$, $P = 0.003$ for IFNAR1-17470 and L168V, respectively) or cerebral malaria patients (3×2 $\chi^2 = 11.2$, $P = 0.004$, and 3×2 $\chi^2 = 11.9$, $P = 0.003$ for IFNAR1-17470 and L168V, respectively). For both IFNAR1-17470 and L168V markers, the percentage of C/G heterozygous samples was found to be higher in both severe malaria and cerebral malaria patients than in controls. Conversely, both IFNAR1-17470 G/G and IFNAR1- L168V G/G homozygous genotypes were found to be more frequent in controls than in severe and cerebral malaria cases. These results were confirmed by logistic regression analyses when cases and controls were stratified for age, sex, household location and ethnic origins ($P \leqslant 0.009$) (Table 1).

The values of the Hosmer–Lemeshow goodness-of-fit statistic were not significant for the models with IFNAR1-17470 data, indicating that the phenotypes predicted by our models fitted with the observed phenotypes ($P = 0.26$ and 0.29 for comparisons between controls, and severe malaria and cerebral malaria cases, respectively) (data not shown). This test was significant ($P = 0.022$) for the IFNAR1-L168V data, when controls and severe malaria cases were studied, indicating that this model poorly fits the data (data not shown). However, the Hosmer–Lemeshow statistic was not significant ($P = 0.69$) for the IFNAR1-L168V marker when controls and cerebral malaria phenotypes were studied, suggesting that the regression model including IFNAR1-L168V data was improved when the cerebral

Table 1 IFNAR1 SNP genotypes in The Gambia

Subjects[a]	*Severe malaria*	*Cerebral malaria*[b]	*Severe malarial anemia*[b]	*Mild malaria*	*Controls*
Total	528	319	136	338	554
(a) IFNAR1 17470					
C/C	57 (10.8)	36 (11.3)	16 (11.8)	45 (13.3)	72 (13.0)
C/G	268 (50.8)	170 (53.3)	62 (45.6)	148 (43.8)	231 (41.7)
G/G	203 (38.4)	113 (35.4)	58 (42.6)	145 (42.9)	251 (45.3)
Wald[c]	9.32	10.95			—
P[d]	0.009	0.004	NS	NS	—
Total	538	327	137	335	562
(b) IFNAR1 L168V					
C/C	29 (5.4)	19 (5.8)	7 (5.1)	27 (8.1)	44 (7.8)
C/G	226 (42.0)	144 (44.0)	57 (41.6)	133 (39.7)	183 (32.6)
G/G	283 (52.6)	164 (50.2)	73 (53.3)	175 (52.2)	335 (59.6)
Wald[c]	12.5	11.9			—
P[d]	0.002	0.003	NS	NS	—

[a]Numbers given. Percentages within each disease or control group are given within parentheses.
[b]Children with both cerebral malaria and severe malarial anaemia were excluded from this analysis (n=73 for 17470 and n=74 for L168V SNP).
[c]Binary logistic regression Wald statistic (d.f.=2). For this analysis, confounding factors age, ethnic origin, household location and sex were included. For both 17470 and L168V markers, individuals sharing the G/G genotypes were used as the reference group.
[d]NS=non-significant; *P*-values>0.05.

Table 2 Associations between IFNAR1 SNP genotypes and severe or cerebral malaria

Models[a]	*Severe malaria*			*Cerebral malaria*		
	Wald	*P*	*OR (95% CI)*	*Wald*	*P*	*OR (95% CI)*
IFNAR1-17470 17470-G/G *vs* 17470-(C/G and C/C)	5.6	0.018	0.74 (0.57–0.95)	8.7	0.003	0.64 (0.47–0.86)
IFNAR1-L168V L168V- G/G *vs* L168V-(C/G and C/C)	4.7	0.031	0.76 (0.59–0.97)	6.5	0.011	0.69 (0.51–0.92)

[a]Binary logistic regression of the G/G homozygotes to the other genotypes for the IFNAR1- 17470 and IFNAR1-L168V SNPs was performed to assess the risk of severe malaria or cerebral malaria of these genotypes. Age, sex, household location and ethnic origin were included in the analysis. Only significant *P*-values (P>0.05) are given.

malaria subphenotype was analysed separately (data not shown). No statistically significant difference was observed between controls and severe anaemia or mild malaria cases.

IFNAR1-17470 G/G and IFNAR1-L168V G/G genotypes are associated with reduced risk of severe malaria and cerebral malaria

Further logistic regression analyses stratified as previously described for age, sex, household location and ethnic origins were performed to assess the differential associations between single genotypes and severe disease phenotypes. As shown in Table 2 for IFNAR1-17470 marker, G/G genotype was associated with a reduced risk of severe malaria (OR = 0.74, $P = 0.018$) and cerebral malaria (OR = 0.64, $P = 0.003$). Similarly, individuals with the G/G homozygous genotype for the IFNAR1-L168V marker were found to be less likely to have severe malaria in general (OR = 0.76, $P = 0.031$) or cerebral malaria in particular (OR = 0.69, $P = 0.011$), as compared with individuals sharing at least one L168V-C allele. Overall, Hosmer–Lemeshow statistics indicate that our models fitted the observed phenotypes ($P > 0.17$). An exception was observed with the model including IFNAR1-17470 data in which cerebral malaria phenotype was included ($P = 0.022$) (data not shown).

IFNAR1-17470 and L168V haplotype and diplotype analysis

The SNP marker data within the IFNAR1 gene was used to construct a linkage disequilibrium map using the PHASE and GOLD software packages. Highly polymorphic markers GT-rpt (17 alleles) and IVS-rpt (10 alleles) were not included in this analysis to minimise the number of haplotypes. Subdivision of cases and controls into too many genotype groups would have resulted in a loss of power to detect an association between particular haplotypes and resistance or susceptibility to severe malaria. Using the four IFNAR1 SNP markers, strong linkage disequilibrium was observed between IFNAR1-17470 and IFNAR1-L168V. No linkage disequilibrium was detected between these markers and either the

Table 3 Haplotypes constructed using PHASE for IFNAR1-17470 and L168V markers.

Subjects[a] *(Haplotype)*	*17470*	*L168V*	*Severe malaria*	*Cerebral malaria*	*Controls*
(a) Haplotype frequencies					
1	C	C	275 (26.1)	177 (27.8)	254 (23.1)
2	G	G	666 (63.3)	394 (61.9)	723 (65.7)
3	C	G	107 (10.2)	65 (10.2)	116 (10.5)
4	G	C	4 (0.4)	0 (0.0)	7 (0.6)
Total			1052	636	1100

Subjects[b]	*Severe malaria*	*Cerebral malaria*	*Controls*
(b) Diplotype frequencies			
C-C/C-C	27 (5.1)	18 (5.7)	39 (7.1)
C-C/G-G	197 (37.5)	127 (39.9)	150 (27.3)
C-C/C-G	24 (4.6)	14 (4.4)	24 (4.4)
C-C/G-C	— —	— —	2 (0.4)
G-G/G-G	198 (37.6)	112 (35.2)	245 (44.5)
G-G/C-G	71 (13.5)	43 (13.5)	78 (14.2)
G-G/G-C	2 (0.4)	— —	5 (0.9)
C-G/C-G	6 (1.1)	4 (1.3)	7 (1.3)
C-G/G-C	— —	— —	— —
G-C/G-C	1 (0.2)	— —	— —
Total	526	318	550

[a]Numbers of haplotypes are given. Percentages within each disease or control group are given within parentheses. Sixteen diplotypes are possible with the four different haplotypes observed, however, six of these are duplicates. The 10 unique diplotypes are listed in Table 3b.
[b]Haplotype on chromosome 21a/haplotype on chromosome 21b. Markers are shown in chromosomal order (ie 17470 and L168V). Numbers are given. Percentages within each disease or control group are given within parentheses.

IFNAR1-(-408) or the IFNAR1-(30715) SNP (data not shown). Haplotypes were analysed using the IFNAR1-17470 and IFNAR1-L168V data in order to identify those associated with resistance or susceptibility to severe or cerebral malaria. As shown in Table 3a, the 17470-G L168V-G haplotype was found to be the most frequent haplotype in both severe malaria (63.3%) and controls (65.7%). However, haplotype frequencies were not significantly different between controls and severe malaria or cerebral malaria cases (Table 3a).

Data were then analysed according to the two-chromosome haplotype combinations of individuals, or diplotypes, for cases and controls. As shown in Table 3b, the diplotype (17470-C L168V-C/17470-G L168V-G) was found to be more common in children with severe malaria (37.5%) and cerebral malaria (39.9%) than in controls (27.3%). Conversely, the diplotype (17470-G L168V-G/17470-G L168V-G) was found to be more common in controls (44.5%) than in severe malaria (37.6%) and cerebral malaria cases (35.2%) (3×2 $\chi^2 = 12.8$, d.f. $= 2$, $P = 0.002$ and 3×2 $\chi^2 = 15.2$, d.f. $= 2$, $P = 0.0005$, for comparison between controls and severe malaria or cerebral malaria cases, respectively).

Discussion

Previous studies have shown the importance of cytokines and their receptors in malaria pathogenesis (see Ref. 23 for a review). The chromosome 21q22.11 locus contains several genes coding for such molecules including IFNAR1, IFNAR2, IFNGR2 and IL10RB. A total of 15 polymorphic markers located in those genes were analysed in order to evaluate their role in the genetic control of malaria pathogenesis in a Gambian case–control study. Five of these markers were microsatellite repeats, six were noncoding SNPs located in intronic regions, and four were coding SNPs.

For all markers studied, the allele frequencies were not different between samples from the groups of controls, mild malaria, severe malaria or the severe malaria subphenotypes of cerebral malaria and severe anaemia. However, when the genotype distributions were analysed for each marker, a significant difference was observed between the control group and the severe malaria group for two adjacent markers located within the IFNAR1 gene. For those markers, IFNAR1-17470 G/G and IFNAR1-L168V G/G genotypes were found to be less common in severe malaria cases than in controls. In addition, when the severe malaria data were subdivided into cerebral malaria and severe anaemia, it was observed that IFNAR1-17470 G/G and IFNAR1-L168V G/G genotypes were correlated with a reduced risk of cerebral malaria. Genotype frequencies were not found to be significantly different between severe anaemia cases and controls. However, as observed for the cerebral malaria phenotype, both IFNAR1-17470 G/G and IFNAR1-L168V G/G genotypes were found to be more common in controls (45.3 and 59.6%, respectively) than in severe anaemia cases (42.6 and 53.3%, respectively). Conversely, heterozygous genotypes were found to be less frequent in controls than in severe anaemia cases (Table 1). However, associations between IFNAR1 markers and the severe anaemia phenotype may have gone

undetected due to the relatively low number of severe anaemia cases ($n = 137$) compared to cerebral malaria cases ($n = 327$).

These associations were still significant when other factors, age, ethnic origin, sex and household location were included in logistic regression analyses. Therefore, as opposed to other reports (8, 24) but in accordance with previous analysis of this Gambian case–control sample (25–27), neither ethnicity, age, household location nor sex were found to influence the associations that were observed between IFNAR1 polymorphisms and susceptibility to severe malaria.

Haplotypes were constructed using SNP markers using the PHASE and GOLD programs in order to evaluate the associations between haplotypes and the risk of severe malaria. Strong linkage disequilibrium was observed between IFNAR1-17470 and IFNAR1-L168V but not between any of the other markers. No significant differences were observed between the IFNAR1-17470 and IFNAR1-L168V haplotype frequencies of the control and severe malaria groups. Analysis of the diplotypes demonstrated that the distribution of the two most common diplotypes (17470-C L168V-C/17470-G L168V-G) and (17470-G L168V-G/17470-G L168V-G) was significantly different between controls and severe malaria or cerebral malaria cases. Indeed, in accordance with the individual marker data, the diplotype with homozygous G/G genotypes for both markers was associated with a reduced risk of severe malaria and cerebral malaria. Conversely, the diplotype (17470-C L168V-C/17470-G L168V-G) was associated with an increased risk of severe malaria and cerebral malaria.

These results suggest that the IFNAR1-17470 G/G and IFNAR1-L168V-G/G genotypes are associated with protection against severe malaria or cerebral malaria, and that the heterozygous genotype for both markers may be associated with increased susceptibility to severe malaria or cerebral malaria. These results showing heterozygous disadvantages in our study contrast with previous findings showing associations between heterozygous genotypes and protection against severe malaria.[8,25,28] Taken together our results suggest that both IFNAR1-17470 C/C and IFNAR1-L168V C/C genotypes are dominant negative alleles and are associated with an increased risk of severe malaria. However, given the low frequency of these particular alleles, it is possible that such discrepancies between case and control genotype frequencies were not detected (Table 1).

The functional significance of these associations is unclear. Indeed, it is not known if the intronic (intron 3) IFNAR1-17470 SNP has a functional effect on IFNAR1 gene expression, mRNA stability or mRNA splicing. Similarly, in spite of the fact that several functional domains have been identified within the intracellular part of IFNAR1,[29,30] the functional role of the L168V amino-acid substitution in the extracellular domain of the IFNAR1 is uncertain.

The IFNAR1-17470 and IFNAR1-L168V polymorphisms may have no effect on the IFNAR1 function but may be proximal to a causative polymorphism. Further IFNAR1 sequencing experiments and functional studies are required to rule out this possibility. However, the lack of association with flanking gene variants suggests that such a variant affecting IFNAR1 function must be genetically close to the associated SNPs.

The results presented here were not corrected for multiple genes or phenotypes. Such corrections are necessary in case–control studies but it is likely that methods based on Bonferroni's test are too conservative because they do not take into account the fact that each gene was not randomly chosen but carefully selected according to various criteria. In addition, these corrections do not take into account the type of test statistic performed with the data. As an alternative to these statistical procedures, further studies confirming our results in other populations would be more convincing.

This study has demonstrated an association between two IFNAR1 polymorphisms and cerebral malaria. There has been little investigation of the relevance of the type-1 interferons in malaria and our data suggest that the role of these cytokines and their signalling pathway in malaria pathogenesis merit further study.

Materials and methods

Subjects

The details of this case–control study have been described previously.[25] Briefly, children aged 1-10 years were enrolled, between August 1989 and September 1990 at the Royal Victoria Hospital of Banjul, and the Medical Research Council Hospital of Fajara, in The Gambia. Malaria was diagnosed if a patient with an appropriate clinical picture had parasitaemia $>2500/\mu l$ and relevant laboratory investigations did not suggest other diagnoses. Cerebral malaria was defined by a Blantyre coma score of $\leqslant 2$ (persisting for >30 min after effective treatment of hypoglycaemia or convulsions) or repeated prolonged seizure (>30 min) in a child with *P. falciparum* parasitaemia and no other apparent cause of fits or coma. Severe malaria anaemia was defined as a haemoglobin level <50 g/l on admission in a child with parasitaemia. Children with mild malaria had an uncomplicated febrile illness with *P. falciparum* parasitaemia and no other apparent cause of fever. The group of severe malaria patients was matched to two control groups of children for age and area of residence. The mild controls ($n = 248$) were recruited at both hospitals and health centres in the study area. These children had mild, mostly infectious, illnesses that did not require hospital admission, and did not have malarial parasites in their blood on microscopy. Severe controls ($n = 314$) were inpatients at the two hospitals with a large range of other acute, mainly infectious, illnesses but without evidence of current or recent malaria infection. The ethnic composition of the population in this area is mixed: Mandinka (42%), Jola (14%), Wolof (14%), Fula (12%) and several less common ethnic groups. The children from the different groups were not matched for ethnic group; instead, analyses stratified with this variable were done.

Screening strategy

A two-stage screening strategy was adopted. A set of DNA samples was screened initially, containing 190 severe malaria cases and 190 mild controls. If statistically significant association could be demonstrated for a marker using these extremes of phenotype, then the whole set of DNA samples was screened and the data

reanalysed as a whole (538 children with severe malaria, 338 children with mild malaria, 562 controls).

Sample typing

A total of 15 polymorphic markers were studied in the four genes: IFNAR2 ($n=2$), IL10RB ($n=3$), IFNAR1 ($n=6$), and IFNGR2 ($n=4$). Five were microsatellites and 10 were single-nucleotide polymorphisms (SNPs) (Figure 1). Polymerase chain reaction (PCR) was performed in a 1 μl reaction mixture containing 50 ng of genomic DNA, 10 mM Tris-HCl, 50 mM KCl, pH 8.3 (GeneAmp 10X Buffer II, Perkin-Elmer), 1.0–3.0 mM $MgCl_2$, 50–200 μM of each dNTP, 0.5–0.75 U *AmpliTaq Gold DNA polymerase* (Perkin-Elmer), and 0.2–0.3 μM each of forward and reverse primers (Sigma-Genosys Ltd). Amplification reactions were performed using an MJ Research tetrad thermal cycler as follows: 94°C for 14 min, 35–39 cycles at (94°C for 15–30 s, 48–65°C for 30–40 s, 72°C for 30–60 s), and finally 72°C for 2–7 min. For microsatellite markers, one of each primer pair was fluorescently labelled using FAM or HEX (Sigma-Genosys Ltd), and PCR products were analysed on an ABI 3700 sequencer (Applied Biosystems). The SNP genotyping was performed using the Ligase Detection Reaction (LDR)[31] (http://www.well.ox.ac.uk/hill/Protocols.shtml). For each SNP, the allele-specific oligonucleotide probe pairs were distinguished by different fluorescent labels (FAM/HEX or FAM/TET) and by their lengths for some SNPs. LDR reactions were performed in a final volume of 15 μl containing 20 mM Tris-HCl, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100, pH 7.6 (*Taq* DNA Ligase Buffer, New England Biolabs), 10 nM of 5′-phosphorylated common probe, 10 nM of each allelic probe, 2 μl of Proteinase K-treated PCR product and four units of *Taq* DNA Ligase (New England Biolabs). The LDR reactions were cycled as follows: 95°C for 1 min followed by 15 cycles at 95°C for 15 s, 54–72°C for 4 min. LDR reactions were stopped by the addition of 3 μl of 100 mM EDTA pH 8.0, and LDR products were analysed using either an ABI 3700 or 373 sequencer (Applied Biosystems) (Further genotyping assay details are available on request).

PCR primers

For IFNAR2 polymorphisms: F10V-F 5′-AGA CCA GGC TCA CTT GAA TAA ATG-3′, F10V-R 5′-GGG TGG TAC TGG GTC CTC TA-3′, A285T-F 5′-GAG CAGTTG CAA GGG AAA GGT AAA-3′, A285T-R 5′-AGG AAA TGG CCA GGC TAA AAA GTT-3′. For IL10RB polymorphisms: K47E-F 5′-CAT GGG CAT CTG TTT TGA GG-3′, K47E-R 5′-GGC CAC AGA ATT TCC CAG ACA-3′, CA-1-F 5′-(FAM) AGG ATG GAG TGA GAT GTG G-3′, CA-1-R 5′-ACA AGA GAA TGG ACC AGC AC-3′, CA-2-F 5′-(HEX) CTC AAG GAA GCA TCA GC-3′, CA-2-R 5′-TAG GGA GGT TCA TCC TAC AC-3′. For IFNAR1 polymorphisms: (-408)-F 5′-ACT CCC AAC GCC ACT GTC CA-3′, (-408)-R 5′-CTC CTA GCT AGC ACC CCT TCT CCA-3′, GT-repeat-F 5′-CCA CCC GCG CCC TCC GAC TG-3′, GT-repeat- R 5′-(FAM) GCA CGC GCC GCC TCT TCT GAC AC -3′, 17470-F 5′-CTT TCC CTG TAG TAG TGG TTC T-3′, 17470-R 5′-CTG TAG TGA GCC GTG ATT GT-3′, L168V-F 5′-AGC TTT CTA TCC TAT CTG TAT G-3′, L168V-R 5′-TTC GCC TAA TTT TTC TCT-3′, IVS-repeat-F 5′-(FAM) TGC TTA CTT AAC CCA. GTG TG-3′, IVS-repeat-R 5′-CAG AGG TTG CGG TGA GC-3′, 30715-F 5′-GCA AGT CAC TAA GGG CAG GTA AGC-3′, 30715-R 5′-GGT AGG CGG GGA AGA ACA CG-3′. For IFNGR2 polymorphisms: D21S370-F 5′-(FAM) GAG GAC TTC AGG CTC CAG CA-3′, D21S370-R 5′-GTG TGA TTT GGG GCA TGC TC-3′, 30354-F 5′-GGG GAA CTG TAT GGT ACA TAT AAA TTG-3′, 30354-R 5′-TCG ATA CCG CAA GTG AAG AAA ACC-3′, 46334/41-F 5′-GGG CTG AGC AGT CAG AAG AC-3′, 46334/41-R 5′-CAT TTT AAG CCA GCA CAC CA-3′.

LDR probes

For IFNAR2 polymorphisms: F10V-T 5′-(FAM) ATG CTT TTG AGC CAG AAT GCC TTC ATC T-3′, F10V-G 5′-(TET) ATG CTT TTG AGC CAG AAT GCC TTC ATC G-3′, F10V-com 5′-(P) TCA GAT CAC TTA ATT TGG TTC TCA TGG GTA AGT G-3′, A285T-G 5′-(FAM) TTC AAT CTC ATT AAG TTT ATT TTT TAT TTT TTT AGA GGC AAG GTC TCG-3′, A285T-A 5′-(TET) TTC AAT CTC ATT AAG TTT ATT TTT TAT TTT TTT AGA GGC AAG GTC TCA-3′, A285T-com 5′-(P) CTA AGG GCT GGA ATG CAG TGG CTA TTC ACA GGT C-3′. For IL10RB polymorphisms: K47E-A 5′-(FAM) AAA AAA GTG GGA GTC ACC TGC TTT TGC CA-3′, K47E-G 5′-(HEX) AAA AAA ATG GGA GTC ACC TGC TTT TGC CG-3′, K47E-com 5′-(P) AAG GGA ACC TGA CTT TCA CAG CTC AGT ACC T-3′. For IFNAR1 polymorphisms: (-408)-T 5′-(FAM) ATA ATA ATA GAG CGC CGG GCC GCG ACT-3′, (-408)-C 5′-(HEX) AAT AAT TAA GCG CCG GGC CGC GAC C-3′, (-408)-com 5′-(P) AGG AGC CCA CCC GCG CCC TCA ATA AAA T-3′, 17470-C 5′-(FAM) TAG GTT TAT CAT TGT TAT TTC TTT CTT TTT TTT TTT GAC-3′, 17470-G 5′-(HEX) ATA GGT TTA TCA TTG TTA TTT CTT TCT TTT TTT TTT TGA G-3′, 17470-com 5′-(P) ACA GAG TTT GTC TTG TGC CAG GCT G-3′, L168V-C 5′-(FAM) AGG CTT TGG ATG GTT TAA GCT TTA CAT ATA. GCT TAC-3′, L168V-G 5′-(TET) AGG CTT TGG ATG GTT TAA GCT TTA CAT ATA GCT TAG-3′, L168V-com 5′-(P) TTA TCT GGA AAA ACT CTT CAG GTG TAG AAG TAA GCA TT-3′, 30175-G 5′-(FAM) ACT AGT TAC AAT AGC TAA TAA TTT CTC AAT TGT GCT G-3′, 30175-T 5′-(HEX) AAC TAG TTA CAA TAG CTA ATA ATT TCT CAA TTG TGC TT-3′, 30175-com 5′-(P) CTT CTG GAT ATA TAT GTG TTG GAT ACA AAC ATT TT-3′. For IFNGR2 polymorphisms: 46334-G 5′-(FAM) AGG TCA CAC AAC CTG TCC CAG CGA G-3′, 46334-A 5′-(TET) AGG TCA CAC AAC CTG TCC CAG CGA A-3′, 46334-com 5′-(P) GGA CAC YGA GTG GCC CTT CAT GTA CAT CC-3′, 46341-T 5′-(FAM) AAC CTG TCC CAG CGA GGG ACA CT-3′, 46341-C 5′-(TET) AAC CTG TCC CAG CGA GGG ACA CC-3′, 46341-com 5′-(P) GAG TGG CCC TTC ATG TAC ATC CAT GGT-3′. 30354-C 5′-(HEX) AGA ACT GTA TGG TAC ATA TAA AAT TGT ATC TCA ATA AAC CTG C-3′, 30354-T 5′-(FAM) GGA ACT GTA TGG TAC ATA TAA AAT TGT ATC TCA ATA AAC CTG T-3′, 30354-com 5′-(P) GTT TTG AAC AAA AGC TCT GGG GAA ACT ATT ACA CAT-3′.

Statistical analysis

Initially, markers were analysed individually. Pearson's χ^2 test was used to compare cases and controls, the allele frequencies and the number of individuals sharing a particular genotype to the number of individuals who did not have that genotype. Data were analysed using a 3×2 χ^2 test for the overall difference in genotype frequencies between malaria cases and controls. Logistic

regression analysis was performed when the Pearson's χ^2 test demonstrated a statistically significant association between genotypes and phenotypes. For logistic regression analysis,[32] the effects of different confounding factors, such as age, sex, ethnicity and household location were taken into account. Age was used as a continuous variable. SNP genotypes were recoded '1' for individuals who possessed a particular genotype and '0' for those who did not. For severe malaria, subjects having severe malaria were recoded as '1' and compared to controls recoded as '0'. The same strategy was used to evaluate the associations between genetic markers and severe malaria subphenotypes (severe anaemia and cerebral malaria). An odds ratio greater than one indicated a positive correlation between the genotype of interest and an increased risk of severe malaria. The goodness of fit of the model was tested by the Hosmer–Lemeshow statistic; a significant result with the test indicated that the model fitted the data poorly.[32] Statistical analysis was performed using SPSS 11 for Windows (SPSS Inc., USA).

When associations were observed between one or more genetic markers and severe malaria subphenotypes, the marker data were used to construct haplotypes. The haplotype construction was performed using the PHASE program,[33] which is designed for haplotype estimation from case–control data. This haplotype data were then analysed in a similar fashion to the single marker data using Pearson χ^2 tests and logistic regression analysis if appropriate. The combination of haplotypes in each sample, ie the diplotype, was analysed in a similar fashion. The haplotype data was also analysed using the GOLD package[34] to investigate the distribution of linkage disequilibrium in the vicinity of markers that showed statistically significant association to malaria phenotypes.

GenBank accession numbers
L42238, L42243 for IFNAR2 gene, AP001716 for IL10RB gene, X60459 for IFNAR1 gene and AP001717 for IFNGR2 gene.

Web sites

http://www. incyte.com/
http://genome.ucsc.edu/
http://snp.well.ox.ac.uk/
http://www.ncbi.nlm.nih.gov/
http://www.well.ox.ac.uk/hill

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