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Associations of multi-locus polymorphisms in an immune network with susceptibility to uncomplicated *Plasmodium falciparum* malaria in Daraweesh village, Eastern Sudan

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ABSTRACT

Susceptibility to uncomplicated malaria (UM), as to other forms of the disease, is genetically determined. Over 9-years of clinical and parasitological follow up of inhabitants of Daraweesh, in Eastern Sudan, the relative susceptibility to UM was estimated in terms of number of episodes experienced by each individual. Previously, we reported that the levels of IgG2 and IgG3 to Pf332-C231 malaria antigen are negatively correlated with number of malaria episodes. In addition, four molecular markers for malaria susceptibility (CRP −286, GM/KM haplotypes, FcγRIIa131 and HbAS) were tested. In this study, the above data were combined and reanalysed. The CRP −286A allele and GM 1,17 5,13,14,6 phenotype were previously found to be associated with increased susceptibility to malaria; however, individuals have both polymorphism together were not more susceptible to UM than the non-carriers of the same double polymorphism. The FcγRIIa-RR131 and HbAA genotypes taken individually or as double polymorphism were not associated with malaria susceptibility; however, their combination with any or both of the former polymorphisms was mostly associated with increased susceptibility to malaria. None of the four markers were associated with the levels of IgG2 and IgG3 against Pf332-C231. In conclusion, while our data support the polygenic nature of susceptibility to UM and highlighted the role of immune markers polymorphisms, the combinations of these markers were not predictable, i.e. the combination of the susceptibility markers will not necessarily render the carriers more susceptible to UM.

1. Introduction

The influence of combined polymorphisms of genes encoding immune effector molecules on malaria susceptibility and severity is largely unknown and rarely investigated (Patel et al., 2004; Basu et al., 2010). The defence mechanisms, including the immune system, are generally functioning as networks, thus, alteration (polymorphism) of one or more members (molecular markers) in the network would influence the other markers. As well as, the combined effects of two or more markers would be expected to have an overall positive, negative or balanced effect. However, combination of polymorphisms that are already in association with each other, due to linkage disequilibrium (Machado et al., 2010), is unlikely to have an additional or different effect. Thus, the study of combined effect of both associated and un-associated polymorphisms in a functional network is of interest. In this study the examined network included (1) gamma immunoglobulin (IgG) subclasses, (2) C-reactive protein – CRP, (3) GM/KM allotypes, (4) Fcγ receptor (FcγRIIa), and (5) sickle cell trait (HbAS). The IgG, recognises parasite antigens via fragment antigen binding (Fab) in one hand and activate phagocytic cells (opsonisation) and complement cascade via IgG constant fragment (Fc) on the other hand. Variations of the IgG subclasses are to some extent due to polymorphism of GM allotypes, part of constant fragment (Fc) of IgG heavy chain, and KM allotypes in kappa light chain in Fab region

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of the IgG (De Lange, 1989). The IgG can be typed for 18 different GM allotypes, IgG3 subclass is the most polymorphic, with 13 GM3 allotypes (Van Loghem, 1986). There are four IgG1 allotypes and one IgG2 allotype, whereas no allotypes have been detected on the heavy chains of IgG4 (De Lange, 1989). Recently, we reported an association between GM 1,17 5,13,14,6 phenotype and susceptibility to malaria (Giha et al., 2009), while another study showed the opposite (Migot-Nabias et al., 2008).

The IgG antibodies activate immune effector cells, e.g. T-cells and macrophages, through Fcγ receptors, e.g. FcγRIIa. The FcγRIIa shows genetic polymorphism that result in two allotypes, FcγRIIa-H131 and FcγRIIa-R131 (Ernst et al., 1991; Bredius et al., 1995). This functional polymorphism is critical for IgG binding, although the FcγRIIa does not bind IgG4. The FcγRIIa-H131 has a much higher affinity for complexed IgG2 than the FcγRIIa-R131 allotype (Bredius et al., 1995). The HH genotype was found to be protective against malaria manifestations (Sinha et al., 2008), however, in one study the RR genotype was found to be protective against high-density parasitaemia (Ouma et al., 2006). Another study demonstrated that the HH genotype was associated with susceptibility to severe malaria while the presence of the R allele was important in protection from malaria (Cooke et al., 2003; Braga et al., 2005).

C-reactive protein (CRP), the fourth member in this network, joins the network through the FcγRs as it has a sequence homology to the FcγR binding regions of IgG (Bang et al., 2005). The CRP has a high affinity to the R variant of FcγRIIa-H/R131, thus, the CRP is speculated to compete with anti-malarial IgG antibodies for binding to FcγR-containing phagocytic cells (Stein et al., 2000). A triallelic upstream mutation (−286 C > T > A) of the CRP has been shown to be associated with blood levels of CRP (Israelsson et al., 2009). Recently, we reported strong association between the CRP −286A allele and increased malaria susceptibility (Giha et al., 2010a).

The association between the above polymorphisms and their combined effects were investigated in this study using a unique clinical and parasitological material collected over 9 years from Daraweesh village in Eastern Sudan. Several polymorphisms were analysed but only those found to be significantly associated with or showed a trend towards susceptibility to malaria were included in this study.

2. Materials and methods

2.1. Study area and population

This cohort based study was conducted between 1991 and 2004 in Daraweesh, a small village in Eastern Sudan of an estimated total population of >550 individuals in 2005. Malaria transmission in the region is moderate and seasonal, it starts in September, peaks in October/November and declines by January. However, there is marked variation in malaria incidence between years. Villagers are Fulani originally from Burkina Faso; they maintained ethnicity through intermarriage, and culture and habits by living in extended families in the same compounds (Creasey et al., 2004). More than 98% of malaria infections are due to *Plasmodium falciparum*, which are transmitted by *Anopheles arabiensis*. Individuals in Daraweesh rarely develop more than one malaria episode in one season. Chloroquine was the first line and sulphadoxine/pyrimethamine (SP) was the second line for malaria treatment until 2004 when artesunate/SP combination was introduced. More details were reported elsewhere (A-Elbasit et al., 2006; Creasey et al., 2004).

2.2. Study design, clinical examination and malaria diagnosis

The malaria infections were detected by active and passive surveillances during the malaria season. Generally, individual complained of fever or symptoms suggestive for malaria was clinically examined, had oral temperature measured and blood smears taken. Thin and thick blood films were stained with Giemsa and examined microscopically for detection of both sexual and asexual stages of *P. falciparum* parasite. Thereafter, the slides were read and revised by expert microscopist using light microscopy as described by the WHO (1991). Malaria was defined as measurable fever ($\geqslant$37.5 °C) or recent history of fever coupled with microscopically detectable asexual *P. falciparum* parasitaemia. The clinical and parasitological follow up, the passive and active surveillances and the overall morbidity and mortality in Daraweesh during the study period was presented in details before (Giha et al., 2000; Creasey et al., 2004). In May 2005, blood samples were obtained from 250 individuals; all donors were malaria-free at the time of sampling that was confirmed by screening of the samples for detection of malaria parasites by PCR, as reported before (Nasr et al., 2009).

2.3. Measurement of plasma antibody levels

Plasma (250 samples) levels of total IgG and IgG subclasses against four malaria antigens; MSP2-3D7, MSP2-FC27, AMA-1 and Pf332-C231, were measured by ELISA using original protocol (Perlmann et al., 1989) with some modifications (Nasr et al., 2007). The response to Pf332-C231 antigen was the only one found to be associated with protection from malaria (Giha et al., 2010b), thus, it was used in this study.

2.4. Typing of polymorphisms

- *GM/KM allotypes:* Haemagglutination–inhibition test was used for GM/KM allotyping using original protocol (Field and Dugoujon, 1989), as reported before (Pandey et al., 2007).
- *Single nucleotide polymorphisms (SNP) analysis:* A restriction fragment length polymorphism (RFLP) analysis was used for determination of SNPs of FcγRIIA gene (131 H/R), using a published protocol (Jiang et al., 1996) with some modification (Nasr et al., 2007).
- Pyrosequencing was used for genotyping the CRP −286 (C > T > A) triallelic gene, as reported before (Israelsson et al., 2009). The sickle haemoglobin trait (HbAS) was determined by PCR genotyping using established protocol (Ayatollahi et al., 2005; Nasr et al., 2008).

2.5. Data analysis

Associations of malaria morbidity with individual polymorphisms, CRP −286A and GM 1,17 5,13,14,6 and with the IgG2 and IgG3 to Pf332-C231 antigen, were published separately (Giha et al., 2009, 2010a,b). In this study, data from the above studies was combined with unpublished data and reanalysed, using Sigma stat software. Not all samples were analysed for all parameters. The associations between individual polymorphisms were analysed by Chi-square test. Comparisons of IgG levels and of number of malaria episodes between study groups (carriers vs. non-carriers of double, triple and quadruple polymorphisms) were done by Mann–Whitney Rank Sum Test or Kruskal–Wallis One Way Analysis of Variance on Ranks.

3. Results

3.1. Review of previous data: associations of gene polymorphisms and of IgG subclass response with number of malaria episodes

As seen in Table 1, the CRP −286A allele was found to be strongly associated with the number of malaria episodes,