Short communication

Absence of the human CYP2C8*3 allele in Ugandan children exposed to *Plasmodium falciparum* malaria

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**A B S T R A C T**

Study of host pharmacogenetics can improve our knowledge of mechanisms of drug resistance selection and spread. This issue has recently been addressed with respect to chloroquine and amodiaquine in malaria endemic areas of West and East Africa. Here we report, from surveys performed in two different areas of Uganda, that the human CYP2C8*3 allele, which had been reported to be strongly associated with parasite drug resistance in Zanzibar, is absent, being a marker of genetic admixture of the Zanzibari population with a Caucasoid component. Moreover, a retrospective analysis of CYP2C8*2 and the *Plasmodium falciparum* drug resistant pfmdr1 86Y allele does not show any association, which may be related to the high level of drug resistance.

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1. Introduction

*Plasmodium falciparum* malaria is one of the most important infectious diseases mainly affecting developing countries in the tropics. Sub-Saharan Africa shows the highest levels of malaria transmission and the lowest level of health care in general, thus accounting for more than 90% of the malaria deaths worldwide, especially amongst children and pregnant women (WMR, 2013). Despite being a treatable disease in its uncomplicated forms, still a high proportion of the population at risk does not have access to the therapy or to effective drugs and when treatment is available the compliance is often poor. For these reasons malaria remains one of the priorities for public health in many African countries.

Currently, the malaria treatment for uncomplicated cases usually consists of a three days regimen of combination of a short half-life artemisinin derivative (mainly artemesunate or artether) together with a longer-lasting drug (such as amodiaquine or lumefantrine) to try and reduce the risk of further resistance developing. Artemisinin combination therapy (ACT) has been introduced in almost all countries where malaria is endemic. However, the use of monotherapy with different drugs, sometimes consisting of fake compounds supplied by the black market, is still widespread. This when taken together with the lack of good diagnosis and misuse of drugs, have a great impact on the selection of drug resistance in the *P. falciparum* parasite.

Different parasite and host factors influence the selection and spread of malaria drug resistance (Djimde et al., 2003; Talisuna et al., 2004; Kwiatkowski, 2005). Recently, two reports have assessed the influence of host pharmacogenetics on parasite drug resistance selection (Paganotti et al., 2011; Cavaco et al., 2013). Pharmacogenetics could represent an important cofactor accelerating the selection of resistant strains of the parasite. The rationale is that, in the short course treatments, the slow drug metabolizer phenotypes could enhance the selection window that the malaria parasites experience in the blood phase of their life cycle. Because drug resistance selection depends on the half-life for a certain drug, any factor that contributes to its extension could potentially increase the window of selection for resistance parasites (Hastings et al., 2002; Stepniewska and White, 2008) by extending the time for the parasite's exposure to below $C_{min}$ drug plasma concentrations. This has been hypothesized for chloroquine (CQ) in Burkina Faso (Paganotti et al., 2011) and amodiaquine (AQ) in Zanzibar (Tanzania) (Cavaco et al., 2013), despite in the two studies plasma
concentrations data were not collected. The two studies were both focused on cytochrome P450 2C8 (CYP2C8) pharmacogenetics, in two very different contexts (Paganotti et al., 2011; Cavaco et al., 2013).

CYP2C8 enzyme metabolizes both drugs with different efficiency (Gil, 2007). Moreover, the effect of the presence of the defective CYP2C8*2 allele (T nucleotide) is an impaired drug metabolism at a lower level than that linked to the CYP2C8*3 allele. Thus, CYP2C8*3 is the most important poor metabolizer (PM) allele present in the Zanzibar population, but is virtually absent in West Africa (Alessandrini et al., 2013). However, while there are substantial data about the frequency of CYP2C8*2 in West and East Africa, the frequency of CYP2C8*3 in East Africa has been investigated only in two studies reporting a value of 2.1% in the isle of Zanzibar (Cavaco et al., 2005) and 0.0% in inland Tanzania (Staehli Hodel et al., 2013).

The aim of the present work was to search for the highly inactive allele CYP2C8*3 in populations exposed to P. falciparum malaria in another East African country such as Uganda, where the allele frequency of CYP2C8*2 is already known (Paganotti et al., 2012). Moreover, we wanted to assess the possible association between CYP2C8 alleles and the malaria drug resistance based on the parasite pfmdr1 N86Y polymorphism.

The allele CYP2C8*3 is defined by the presence of two polymorphisms, 416G>A, and 1196A>G, causing an Arg139_Lys substitution in exon 3 and a Lys399_Arg substitution in exon 8, respectively. The two polymorphisms of this allele have been found to be completely linked in previously published studies on other populations (Dai et al., 2001; Pechandova et al., 2012; Minhas et al., 2013). Overall, it seems that the allele shows its highest frequency in Caucasian populations (Pechandova et al., 2012); while its frequency in Asia varies from 4.0% in India (Minhas et al., 2013) to 0.0% in Japan (Nakajima et al., 2003). In Africa, the reported frequency of the allele is 0.4% in Burkina Faso (Parikh et al., 2007), 0.0% in Ghana (Röwer et al., 2005; Kudzi et al., 2009), 0.0% in Tanzania (inland) (Staehli Hodel et al., 2013), 2.1% in Zanzibar (Cavaco et al., 2005, 2013).

The pfmdr1 86Y allele has been shown to be associated with CQ and AQ resistance, while the 86 N allele (in combination with other pfmdr1 alleles) seems to be associated with Artemether-lumefantrine tolerance and/or resistance (Sisowath et al., 2007). Because of the spread of drug resistance against many molecules used in antimalarial therapy, the study of the pfmdr1 polymorphism at position 86 could be very important to follow the spread of P. falciparum drug resistance in Africa.

2. Material and methods

2.1. The blood samples were collected during cross-sectional surveys of children performed in 2007 in two sites in Uganda, a rural area of the Karamoja (a border region with Kenya, through Namalu and Rupa health centres, and Kakolile and Nadunget primary schools), and a suburban area of Kampala (the Makindiye children centre), defined as Rural and Urban (R and U) respectively, in the tables. We enrolled 262 children (mean age 7.8 ± 3.0) that we selected as unrelated according to family names and interviews with parents/carers. Signed informed consent for multiple genetic and epidemiologic surveys was obtained from the children's parents/carers (Romano et al., 2010, 2011). This study was conducted with the approval of the ethics committee and research committee of the “Sapienza” University of Rome.

2.2. Fingerpick blood samples were spotted on Whatman grade 1 filter papers at the time of the field survey and then air dried before being separately stored in sealed plastic containers. Human/P. falciparum parasite DNA was extracted with Chelex-100 resin (Bio-Rad).

2.3. CYP2C8*3 (defined by the presence of two SNPs: rs11572080, 416G>A and rs10509681, 1196A>G) detection was carried out using a PCR-RFLP technique for both SNPs. For the SNP 416G>A we proceeded according to the protocol of Nakajima et al. (2003). Briefly, we amplified a 467 bp fragment of the CYP2C8 gene (forward primer: 5’-AGGCAATGCCAAAATATC-3’; reverse primer: 5’-CAGGATGCAGAATAAGAC-3’); the PCR product was then digested with BseRI which cuts the wild-type allele only (G); undigested products represent the variant allele (A). For the SNP 1196A>G we proceed according to Dai et al. (2001), where we amplified a 117 bp fragment of the CYP2C8 gene (forward mismatch primer: 5’-CTCTCCGTCTCATGATGACg-3’; reverse primer: 5’-CTGCTGAGAAAGGCGATGAG-3’); the PCR product was then digested with XmnI which cuts the wild-type allele only (A); undigested products represent the variant allele (G). Controls for human genotyping were utilized for each different genotype.

2.4. DNA samples were amplified by nested PCR-RFLP to identify the N86Y point mutation in pfmdr1 (on chromosome 5), according to a protocol already available (Duraiingham et al., 2000). Appropriate P. falciparum controls (3D7 for wild-type CQ-sensitive strains and K1 for CQ-resistant strains) were used for each PCR analysis.

2.5. Genotype frequencies were compared using a χ² test. Binominal regression (BLR) odds ratios were determined when looking at the possible influences of different human factors on parasite drug-resistant 86Y allele distribution. Differences were considered to be significant for values of P = 0.05. Calculations were done using STATISTICA version 6.0 (StatSoft).

3. Results and discussion

We successfully genotyped the human CYP2C8*3 allele from 261 out of 262 collected samples. The CYP2C8*3 polymorphism showed the wild-type nucleotides both at the position 416 and 1196 (G and A, respectively) in all samples, also confirming that the two loci are in perfect linkage disequilibrium in Uganda.

For P. falciparum parasite genotyping of the pfmdr1 N86Y polymorphism, we showed a success rate of 36.8% (96 positive PCRs out of 261 DNA samples) and the genotypes are distributed as shown in Table 1. The parasite rate was higher in rural than in the urban areas (54% and 25%, respectively), OR: 3.47 (1.98–6.10), P < 0.001.

We retrospectively analyzed the CYP2C8*2 data from the same survey (Paganotti et al., 2012) with the new P. falciparum drug resistance analysis. The results are shown in Table 2. No association was revealed from the analysis of human genetic variation and pfmdr1 allele distribution after stratification for age and site. The results of our study showed that CYP2C8*3 is apparently absent in the Ugandan population, and that there is no association between CYP2C8*2 and pfmdr1 86Y allele and that could be related to the very high frequency of malaria parasite drug resistance. However, these results may be biased by the limited number of samples analyzed and shall be received with caution. In addition, there is an inverse association between parasite rate and drug resistance frequency between urban and rural areas that could be due to different malaria transmission/immunity and drug usage in the two different settings.

From the point of view of population genetics our results contribute to a better understanding of the spread of described alleles in African populations. While CYP2C8*2 is found to be present in Africans and in African-Americans at relatively high frequency (9.9–24.2% allele frequency, Alessandrini et al., 2013) CYP2C8*3 is more prevalent in Caucasian populations (5.9–20% allele frequency, Bahadur et al., 2002; Henningsson et al., 2005; Cavaco...
Comparison of drug resistant rate among genotypes AA (normal metabolizers) and AT (poor metabolizers) by binary logistic regression, OR: 0.72 (0.14–3.78); explanation for these results could be due to the less defective similar to the findings of Cavaco et al. (2013) in Zanzibar. The included higher pharmacological pressure.

mission in the urban zone as result of a better control of malaria less intensive drug pressure, compared with the less intense tran-

section (Cavaco et al., 2005).

Our results confirm the previous findings about the CYP2C8*3 allele. The fact that CYP2C8*3 was found in Zanzibar, where a long history of colonization from Arabs and Persian has taken place (Chittick, 1965; http://www.afrika.upenn.edu/NEH/tethnic.htm), could be due to genetic admixture of the Zanzibar population with a Caucasoid component, a hypothesis that was already confirmed by the finding of another Caucasoid allele, CYP2C8*4, in the same context (Cavaco et al., 2005).

The lack of association between CYP2C8*2 and pfmdr1 86Y was similar to the findings of Cavaco et al. (2013) in Zanzibar. The explanation for these results could be due to the less defective effect of the CYP2C8*2 allele in a context of very high P. falciparum drug resistance spread. We can speculate that at a lower level of drug resistance in the P. falciparum population, host factors could partition the parasite drug resistance population according to the presence of the CYP2C8*2 allele. For example, in Burkina Faso the drug resistance frequency was between 29.6% and 46.0% with CYP2C8*2 allele frequency between 9.9% and 24.2%, according to ethnicity (Paganotti et al., 2011). In Uganda, the overall frequency of CYP2C8*2 was 10.5%, but the overall frequency of pfmdr1 86Y was much higher (81.2%), similarly to that of Zanzibar (Cavaco et al., 2013) and in line with other studies from Uganda (Kamugisha et al., 2012).

Lastly, the rate of P. falciparum drug resistance was higher in the urban context than in the rural zone. This is largely expected as the result of more intense transmission in the rural zone together with less intensive drug pressure, compared with the less intense transmission in the urban zone as result of a better control of malaria included higher pharmacological pressure.

4. Conclusions

We described a meso-endemic area for P. falciparum malaria transmission (Smith et al., 2007), with significant differences in the dynamic of parasite rate and drug resistance spread between the urban and rural context. Moreover, we found a lack of presence of the human defective allele CYP2C8*3 that support its absence from East-African countries. This requires further investigation in other parts of the continent to assess the presence of the allele. Moreover, the link between CYP2C8*3 and pfmdr1 should be tested in countries where the Caucasoid component is strong and malaria numbers are still enough to perform this association, as in all countries where Fulani ethnic group is present since there is a Caucasoid contribution in their gene pool (Modiano et al., 2001; Lulli et al., 2009).

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