Global distribution of polymorphisms associated with delayed *Plasmodium falciparum* parasite clearance following artemisinin treatment: Genotyping of archive blood samples

Kenji Murai \(^a,b\), Richard Culleton \(^c\), Teruhiko Hisaoka \(^b\), Hiroyoshi Endo \(^d\), Toshihiro Mita \(^a,d,⁎\)

\(^a\) Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
\(^b\) Department of General Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
\(^c\) Malaria Unit, Department of Pathology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki, 852-8523, Japan
\(^d\) Department of International Affairs and Tropical Medicine, Tokyo Women's Medical University School of Medicine, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

**Abstract**

The recent emergence and spread of artemisinin-resistant *Plasmodium falciparum* isolates is a growing concern for global malaria-control efforts. A recent genome-wide analysis study identified two SNPs at genomic positions MAL10-688956 and MAL13-1718319, which are linked to delayed clearance of parasites following artemisinin combination therapy (ACT). It is expected that continuous artemisinin pressure will affect the distribution of these SNPs. Here, we investigate the worldwide distribution of these SNPs using a large number of archived samples in order to generate baseline data from the period before the emergence of ACT resistance. The presence of SNPs in MAL10-688956 and MAL13-1718319 was assessed by nested PCR RFLP and direct DNA sequencing using 653 global *P. falciparum* samples obtained before the reported emergence of ACT resistance. SNPs at MAL10-688956 and MAL13-1718319 associated with delayed parasite clearance following ACT administration were observed in 8% and 3% of parasites, respectively, mostly in Cambodia and Thailand. Parasites harbouring both SNPs were found in only eight (1%) isolates, all of which were from Cambodia and Thailand. Linkage disequilibrium was detected between MAL10-688956 and MAL13-1718319, suggesting that this SNP combination may be selected by ACT drug pressure. Neither of the SNPs associated with delayed parasite clearance were observed in samples from Africa or South America. Baseline information of the geographical difference of MAL10-688956 and MAL13-1718319 SNPs provides a solid basis for assessing whether these SNPs are selected by artemisinin-based combination therapies.

© 2014 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-SA license (http://creativecommons.org/licenses/by-nc-sa/3.0/).

1. Introduction

There were 207 million cases of malaria and 627 000 deaths due to the disease world-wide in 2012 [1]. One of the most serious threats to the successful control of malaria is the emergence of parasites that are resistant to antimalarial drugs. The World Health Organization currently recommends artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated malaria. However, there is great concern that artemisinin-resistant *Plasmodium falciparum* parasites have emerged in the Cambodia/Thailand border region, where parasites resistant to other antimalarial drugs also originated [2–6]. These ACT “resistant” strains are characterized by a delay in the time it takes parasitaemia to clear from the body following treatment [7].

For many anti-malarial drugs such as chloroquine and pyrimethamine/sulfadoxine, the genetic mutations that underlie resistance have been largely elucidated. These mutations can be used as molecular markers to monitor the appearance and geographical spread of resistant parasites. For artemisinin and its derivatives, a genetic region associated with a delay in parasite clearance following ACT treatment was identified in 2012 [8]. Soon after, four single nucleotide polymorphisms (SNPs) linked to the delayed clearance phenotype were identified on chromosomes 10, 13, and 14 [9]. Two of these, MAL10-688956 (A) and MAL13-1718319 (T), were proposed to be suitable molecular markers for the resistance phenotype.

MAL10-688956 is located on chromosome 10 in the 3′ untranslated region of the DNA polymerase delta catalytic subunit gene, and MAL13-1718319 is in a RAD5 homolog. These two proteins are thought to be involved in post-replication repair [9,10]. In particular, RAD5 is a DNA clamp that is involved in the DNA damage tolerance pathway that promotes the repair of discontinuities [11–13]. In yeast, mutations in this gene have been implicated in cell cycle arrest [13,14], and thus, a similar role may be expected in *P. falciparum* [15],

*Corresponding author at: Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel.: +81 3 5802 1043; fax: +81 3 5800 0476.
E-mail address: tmita@juntendo.ac.jp (T. Mita).*
which might lead to delayed clearance following artemisinin treatment.

We have previously analysed 53 travellers’ malaria samples collected from patients that had returned to Scotland from 11 African and nine South-eastern Asia/Oceania countries. We found that two samples harboured the delayed-clearance associated SNPs in both MAL10-688956 and MAL13-1718319 [16]. These two isolates were obtained from Thailand and Cambodia, the epicentre of the apparent emergence of resistance to artemisinin as well as to other antimalarial drugs [4,17,18]. This finding supports the notion that SNPs MAL10-688956 and MAL13-1718319 could be applicable as molecular markers for the surveillance of artemisinin resistance.

However, the delayed-clearance associated SNPs are also found in several P. falciparum laboratory maintained clones, e.g., V1/S, IT, 106/1, and FCR5 (MAL10-688956-A) and V1/S and IT (MAL13-1718319-T), which were isolated from patients from diverse geographic regions and well before the first reports of artemisinin resistance. This suggests that the two proposed SNPs might be widely distributed, and their selection unrelated to ACT pressure [9]. Thus, we consider that robust information on the global prevalence of these SNPs before the widespread implementation of ACTs would provide the baseline data necessary to infer whether they are, indeed, reliable markers for the spread of ACT resistance.

Here, we determine the distribution of delayed-clearance associated SNPs MAL10-688956 and MAL13-1718319 using a large number of P. falciparum isolates from East/West Africa, Asia, Pacific Oceania and South America. All samples were obtained before the first report of the emergence of artemisinin resistance [2]. We report the complete absence of delayed-clearance associated SNPs in parasites collected from South America and Africa. Delayed-clearance associated SNPs were found singularly in a number of samples from areas outside Africa and South America, but only parasites from Cambodia and Thailand harboured both SNPs simultaneously.

2. Materials and methods

2.1. Study sites

Blood samples were obtained from P. falciparum-infected patients in all age groups, unless otherwise stated, living in 13 malaria-endemic countries as follows (Table 1):

1. Bangladesh: Samples were collected from patients infected with P. falciparum in the Bandarban district hospital in 2007. This study was approved by the Bangladesh Medical Research Council and the local health regulatory body in Bandarban, Bangladesh [19].

2. Cambodia: Samples were collected from P. falciparum-infected individuals during a cross-sectional survey of rural villages in Chumkiri, Kampong province, in 2004, 2005, and 2006. The study was approved by the National Center for Parasitology, Entomology, and Malaria Control of Cambodia [20].

3. Thailand: Samples were collected from patients infected with P. falciparum at town clinics located in the western border of Tak, Kanchanaburi, and Ratchaburi provinces from 2001 to 2002. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University.

4. Lao People’s Democratic Republic (Lao PDR): Samples were collected from P. falciparum-infected individuals during cross-sectional surveys of rural villages in Khammouane province in 1999. The study was approved by the Laos Ministry of Health [21].

5. Philippines: Samples were collected from patients infected with P. falciparum in hospitals on Palawan Island in 1997. This study was approved by the Palawan Provincial Health Office [22].

6. Papua New Guinea: Samples were collected from P. falciparum-infected individuals at villages in Dagua district, East Sepik in 2002 and 2003. The study was approved by the National Department of Health Medical Research Advisory Committee of Papua New Guinea.

7. Solomon Islands: Samples were collected from P. falciparum-infected individuals during cross-sectional surveys in northeastern Guadalcanal Island from 1995 to 1996. The study was approved by the Ethics Committee of the Solomon Islands for Medical Research.

8. Vanuatu: Samples were collected from P. falciparum-infected individuals during cross-sectional surveys in rural villages located on 4 islands; Gaua, Santo, Pentecost and Malakula, in 1996 and 1998. The study was approved by the Vanuatu Department of Health [23].

9. Kenya: Samples were collected from P. falciparum-infected individuals during cross-sectional surveys at 4 villages in Kisii District in 1998. The study was approved by the Kenyan Ministry of Health and Education [24].

10. Tanzania: Samples were collected from P. falciparum-infected individuals during cross-sectional surveys in the Rufiji River Delta in eastern coastal Tanzania in 1998 and 2003. The study was approved by the Ethics Committee of the National Institute for Medical Research of Tanzania [25].

11. Republic of the Congo: Samples were collected from patients with P. falciparum in Pointe-Noire, Brazzaville, and Gamboma in 2006. The study was approved by the Ministry of Research and Ministry of Health of the Republic of the Congo [26].

12. Ghana: Samples were collected from P. falciparum-infected children during cross-sectional surveys in 3 villages near Winneba, a western coastal region, in 2004. This study was approved by the Ministry of Health/Ghana Health Service.

13. Brazil: Samples were collected from P. falciparum-infected individuals in the eastern part of Acre state in 1985–1986, 1999, and 2004–2005. The study protocol was approved by the ethics review board of the Institute of Biomedical Sciences, University of São Paulo.

All studies were conducted before the official implementation of ACT except in Cambodia and Thailand and before the first official report of artemisinin resistance [2]. Finger-prick blood samples were collected and transferred on filter paper (ET31CHR, Whatman) in all studied regions except Thailand in which venous blood samples were used. Parasite DNA was purified using a QIAamp DNA blood mini kit (QIAGEN) or the EZ1 BioRobot™ (QIAGEN, Hilden, Germany) according
to the manufacturer’s instructions. In all study sites, informed consent was obtained from individual patients or their guardians and antimalarial treatment was provided if necessary.

2.2. Determination of polymorphisms in MAL10-688956 and MAL13-1718319

Nestled polymerase chain reaction (PCR) for Plasmodium species typing was conducted to confirm the presence of *P. falciparum* parasites [27,28]. All *P. falciparum* positive samples were assayed for the presence of SNPs in MAL10-688956 and MAL13-1718319 [9]. A PCR and restriction fragment length polymorphism (RFLP) protocol was used as described on the worldwide antimalarial resistance network (WWARN) website: http://www.wwarn.org/toolkit/procedures with one modification (1.5 mM MgCl₂ in the nested PCR for MAL13-1718319). Briefly, genotyping was conducted by nested PCR followed by RFLP analysis. *NsiI* (New England Biolabs), which digests the amplified product when the allele (T, non-delayed clearance associated SNP) is present at the polymorphic site, was used for MAL10-688956. *Msfl* (New England Biolabs), which produces two digested products when the allele (A, non-delayed clearance associated SNP) is present, was used for MAL13-1718319. In all undigested samples, in order to confirm the presence of delayed-clearance associated SNP, nested PCR amplicons were purified with ExoSAP–IT Kit (Amersham Biosciences, Buckinghamshire, UK) and were directly sequenced (50 cycles of 95 °C for 20 s, 50 °C for 30 s, and 60 °C for 1 min) in one direction using the reverse primer of the nested PCR (TTATATGTAATGGGTGAAAAGATA) with a BigDye Terminator 1.1 cycle sequencing kit in the Applied Biosystems 3500xl genetic analyzer (Life Technologies, Carlsbad, California, U.S.).

2.3. Microsatellite analysis

Ten neutral microsatellite markers with no evidence of genetic hitchhiking were genotyped to examine the possibility that linkage disequilibrium is found between evolutionary neutral markers in the Cambodian parasite population. The loci used were as follows: TA42 and TA81 (chromosome 5); TA1, TA87, and TA109 (chromosome 6); TA60 and 2490 (chromosome 10); ARA2 (chromosome 11); and PfG377 and PfPK2 (chromosome 12). These markers were amplified by semi-nested PCR using fluorescent end-labelled primers as previously described [29]. Size variations of the amplified products were determined by electrophoresis on a DNA sequencer and analysed with GeneScan software (Applied Biosystems). Samples with minor peaks at least 50% in peak height compared to the major peak were considered mixed genotype, and were excluded from the analysis.

2.4. Statistical analysis

$D'$ and $r^2$ were measured to assess potential linkage disequilibrium between delayed-clearance associated SNPs [30,31]. Linkage disequilibrium for all pairs of ten microsatellite loci was also examined using Genepop version 4.1 under the following Markov chain parameters: dememorization number = 20000, number of batches = 500, and number of iterations per batch = 10000. The significance of linkage disequilibrium was assessed using the two-tailed Chi-squared test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MAL10-688956

Among a total of 653 *P. falciparum* isolates, we successfully determined allele types for 637 isolates (98%) at MAL10-688956 (Fig. 1A). The overall prevalence of the delayed-clearance associated SNP was 8% (52/637). Ten isolates (2%) were found to be dimorphic with both T and A nucleotides present at this position, indicating a mixed infection. The delayed-clearance associated SNP was not detected in any isolates from Africa or South America but was prevalent in Cambodia (38%) and Thailand (26%). In both countries, ACTs were already in use as first-line therapy for uncomplicated malaria when the samples were obtained. However, there were very few cases of the presence of this delayed-clearance associated SNP in neighbouring countries, Laos PDR (0%) and Bangladesh (2%). Notably, the unexpectedly high prevalence of the delayed-clearance associated SNP was observed in Pacific countries, 14% in Papua New Guinea, 18% in Vanuatu, and 2% in the Solomon Islands. In these regions, artesinin or its derivatives were not implemented at the time of sampling.

3.2. MAL13-1718319

We successfully genotyped MAL13-1718319 in 637 isolates (98%) (Fig. 1B). The delayed-clearance associated SNP (T) was observed in only 18 isolates (3%), none of which were from Africa or South America. This SNP was almost exclusively confined to samples from Cambodia and Thailand, with the exception of one sample from Papua New Guinea, which harboured both alleles.

3.3. Combinations of delayed-clearance associated SNPs at MAL10-688956 and MAL13-1718319

Those samples harbouring a delayed-clearance associated SNP at both MAL10-688956 and MAL13-1718319 are shown in Fig. 2. Isolates with mixed alleles at either locus (n = 11) were excluded. Among 615 isolates, eight isolates (1%) harboured delayed-clearance associated SNPs at both loci. Nearly all of these isolates (7/8) were localised in Cambodia, and one was from Thailand.

3.4. Analysis of linkage disequilibrium

Linkage disequilibrium (non-random association) between MAL10-688956 and MAL13-1718319 was observed only in Cambodia ($D' = 0.5174$ and $r^2 = 0.1629$) (Table 2) with statistical significance ($p = 0.0146$, chi-square test). We then assessed the existence of linkage disequilibrium between ten putatively neutral microsatellite loci to clarify whether the observed linkage disequilibrium was an inherent feature of the Cambodian parasite population. Among a total of 36 Cambodian isolates, 25 showed multiple alleles at least one microsatellite locus and were excluded from this analysis (Table S1). Analysis of linkage disequilibrium between each microsatellite locus produced 45 comparisons, but two results were not obtained because only one allele combination was observed (Fig. S1). No linkage disequilibrium was observed in the remaining 43 comparisons with the smallest $p$ value 0.18 (comparison between TA60 and TA42), which indicates that linkage disequilibrium is not an inherent feature of the Cambodian isolate population. Rather, the observed linkage disequilibrium may be produced by the selection of parasites that harbour both delayed-clearance associated SNPs.

4. Discussion

We assessed the distribution of SNPs at MAL10-688956 and MAL13-1718319 using a large number of archived worldwide *P. falciparum* isolates prior to the reported emergence of ACT resistance [2]. Parasites harbouring a combination of two delayed-clearance associated SNPs (A allele in MAL10-688956 and T allele in MAL13-1718319) were found exclusively in Thailand and Cambodia. In both countries, ACT was already implemented when our sampling was carried out. This may be due to the fact that there were no clear criteria for assessing ACT ‘resistance’ until the mid-2000s [32], and thus, the potential emergence of ACT-resistant parasites might have been missed at the time our sampling was carried out. Additionally/alternatively, delayed-clearance
associated SNPs at MAL10-688956 and MAL13-1718319 alone may not be sufficient for the acquisition of the ‘resistance’ phenotype; rather, these polymorphisms might be necessary prior to the attainment of ‘resistance’ through mutation elsewhere in the genome. A similar mechanism is thought to be involved in the attainment of resistance to pyrimethamine/sulfadoxine. Amino acid substitutions at position 108 in the dihydrofolate reductase gene and at position 437 in dihydropteroate synthase gene are not, in themselves, sufficient to confer a high degree of in vivo resistance, but they are required as an initial step for the further acquisition of other mutations in these genes that eventually result in pyrimethamine and sulfadoxine resistance, respectively [33,34]. It is possible that resistance to artemisinin would require
Linkage disequilibrium in the \textit{Plasmodium falciparum} between MAL10-688956 and MAL13-1718319 in 13 countries is shown (\(n = 615\)). MAL10-688956 delayed-clearance associated SNP + MAL13-1718319 delayed-clearance associated SNP (red), MAL10-688956 delayed-clearance associated SNP + MAL13-1718319 non-delayed clearance associated SNP (orange), MAL10-688956 non-delayed clearance associated SNP + MAL13-1718319 delayed-clearance associated SNP (yellow) and MAL10-688956 non-delayed clearance associated SNP + MAL13-1718319 non-delayed clearance associated SNP (white). Isolates harbouring mixed genotypes at either MAL10-688956 or MAL13-1718319 were excluded.

The delayed-clearance associated SNP combination was mainly distributed in Cambodia, and these SNPs were significantly linked. Since the two SNPs are located on different chromosomes (10 and 13), physical linkage does not explain the observed linkage disequilibrium. Thus, the following two mechanisms are proposed for the observed linkage disequilibrium: (1) low diversity within the Cambodian population, leading to the signature of linkage disequilibrium between markers separated by large physical distances on the genome, and (2) natural selection of a particular allele combination that has a sufficient selective advantage over others [36]. To test the former possibility, we examined linkage disequilibrium using 10 microsatellite markers that are thought to be selectively neutral. We did not find linkage disequilibrium between any of the possible pairs of microsatellite loci, suggesting that the observed linkage disequilibrium between two delayed-clearance associated SNPs is not the result of low diversity within the Cambodian parasite population. Hence, it is probable that the observed linkage disequilibrium is a result of selective pressure favouring parasites with both delayed-clearance associated SNPs.

The continuous use of artemisinin in this area is one candidate for possible selecting factors.

We found no evidence for the existence of delayed-clearance associated SNPs at either MAL10-688956 or MAL13-1718319 in Africa or South America. All the samples from African regions were collected between 1998 and 2006, at the time period before the wide-scale implementation of artemisinin derivatives. Practically, the development of a credible molecular marker of ACT resistance is urgently required, particularly in sub-Saharan Africa. Since many individuals living in this region develop protective immunity to malaria after repeated infections, this immunity would enhance the effectiveness of antimalarial drugs [37]. As a result, these patients may respond to antimalarial drugs even if they are infected with drug-resistant parasites [38–40] and, in such cases, it would be impossible to monitor the emergence of artemisinin-resistant parasites based on clinical information alone [16]. In this regard, the absence of either delayed-clearance associated SNPs before wide-scale implementation of artemisinin derivatives in Africa suggests that assessment of MAL10-688956 or MAL13-1718319 may allow the detection of the emergence of artemisinin resistance before the appearance of clinical failure cases.

The following points, however, should be further considered for the application of MAL10-688956 and MAL13-1718319 as molecular markers for the surveillance of the emergence of ACT resistance. In the Pacific region, the prevalence of the MAL10-688956 delayed-clearance associated SNP was unexpectedly high, although nearly all parasites harbored the non-delayed clearance associated SNP at MAL13-1718319. Artemisinin combination therapies were not implemented in this region.

Table 2

<table>
<thead>
<tr>
<th>Country</th>
<th>(n)</th>
<th>(D')</th>
<th>(r^2)</th>
<th>(p) value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>112</td>
<td>ND</td>
<td>0.00016</td>
<td>0.8491</td>
</tr>
<tr>
<td>Cambodia</td>
<td>37</td>
<td>0.5174</td>
<td>0.1629</td>
<td>0.0146</td>
</tr>
<tr>
<td>Thailand</td>
<td>44</td>
<td>0.3231</td>
<td>0.00561</td>
<td>0.6074</td>
</tr>
</tbody>
</table>

\(\text{ND, not determined. Bold letters show the significance (}P < 0.05\)).\nIsolates in which allele types were not determined were excluded (\(n = 4\)), and then isolates harbouring both delayed-clearance associated SNP/non-delayed-clearance associated SNP in either MAL10-688956 or MAL13-1718319 were excluded (\(n = 8\)).

\(^a\) \(\chi^2\) test.
at the time of sampling in any of the countries considered here. Hence, it seems likely that the MAL10-688956 delayed-clearance associated SNP is a parasite polymorphism that exists naturally in the parasite populations of this region and was not, initially, selected by ACT pressure. The other possibility is the migration of the MAL10-688956 delayed-clearance associated SNP from Southeast Asia. However, since the prevalence of this SNP was high, this may be unlikely, especially in the absence of ACT selection pressure. Further analysis using microsatellite alleles flanking the SNP will clarify the possible migration of the MAL10-688956 SNP to Southeast Asia.

Recently, another artemisinin-resistance related marker, PF3D7_1343700 kelch propeller domain (‘K13-propeller’) gene, has been identified using whole-genome sequencing of an artemisinin-resistant parasite line [41]. A number of SNPs (at least 17) were described in the propeller domains of K13, some of which might be predictive SNPs of resistance in different geographical settings. This gene may prove more suitable as a molecular marker for ACT-resistant parasites than the two described here.

5. Conclusions
Parasites bearing delayed-clearance associated SNPs at both MAL10-688956 and MAL13-1718319 are localised to Cambodia and Thailand. Both the delayed-clearance associated SNPs were entirely absent from Africa or South America prior to the introduction of ACTs as first-line treatments for malaria in these countries. This study provides baseline information for geographical differences in the distribution of malaria parasites carrying delayed-clearance associated SNPs at both MAL10-688956 and MAL13-1718319, and so lays the groundwork for developing molecular markers for monitoring the emergence and spread of ACT resistance. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2014.11.002.

Authors’ contributions
KM conducted data collection and prepared the manuscript. RC conducted a survey and made substantial corrections to the manuscript and helped with the interpretation of the data.TH and HE organized the study. TM was involved in the study design, sampling collection, data analysis, and manuscript preparation. All authors read and approved the final manuscript.

Transparency declarations
None to declare.

Acknowledgements
We thank Francis Hombhanje, Hitoko Osawa, Takahiro Tsukahara, Hideaki Eto, Akira Kaneko, Hiroshi Ohmoe, Masatoshi Nakamura, Lek Dysoley, Jun Kobayashi, Aung S, Marma, Willis S, Akhwaile, Anders Bjorkman, Mathieu Ndounga, Mawuli Dzdzomenyo, and Marcelo U. Ferreira for assistance with sample collection, Nobuyuki Takahashia, Makio Okochi for technical assistance, and Takatoshi Kobayakawa for the organization of the study. This study was supported by a Cooperative Research Grant(s) of NEKKEN, 2013. grants-in-aid for scientific research (23659211, 23590498) and the Foundation of Strategic Research Projects in Private Universities (S0991013) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

PF Global plan for artemisinin resistance containment 2011; 2011.


