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Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 in the China–Myanmar border area

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ABSTRACT

Deletion of the *Plasmodium falciparum histidine-rich protein 2* (*pfhrp2*) gene may affect the performance of *PfHRP2*-based rapid diagnostic tests (RDTs). Here we investigated the genetic diversity of the *pfhrp2* gene in clinical parasite isolates collected in recent years from the China–Myanmar border area. Deletion of *pfhrp2* has been identified in 4 out of 97 parasite isolates. Sequencing of the *pfhrp2* exon2 from 67 isolates revealed a high level of genetic diversity in *pfhrp2*, which is reflected in the presence of many repeat types and their variants, as well as variable copy numbers and different arrangements of these repeats in parasite isolates. In addition, we observed *pfhrp3* deletion in three of the four parasites harboring *pfhrp2* deletion, suggesting of double deletions of both genes in these three isolates. Analysis of two cases, which were *P. falciparum*-positive by microscopy and PCR but failed by two *PfHRP2*-based RDTs, did not find *pfhrp2* deletion. Further correlational studies of *pfhrp2* polymorphisms with detection sensitivity are needed to identify factors influencing the performance of RDTs in malaria-endemic areas.

1. Introduction

Malaria remains endemic in 104 countries and estimated 3.4 billion people are at risk of malaria globally (WHO, 2013). Case management and treatment of malaria is highly dependent on accurate early diagnosis. Rapid diagnostic tests (RDTs) for malaria have the potential to improve case management and thereby reduce morbidity and mortality (Maltha et al., 2013; Mouatcho and Goldring, 2013). At least four target antigens are captured by various available RDTs: *Plasmodium falciparum* histidine-rich protein 2 (*PfHRP2*) and lactate dehydrogenase (LDH); the pan-plasmodial aldolase and LDH (Abba et al., 2011). *PfHRP2* is an abundant antigen produced during the blood stages of *P. falciparum*. It consists of a number of Ala- and His-rich amino acid repeats (Wellems and Howard, 1986) and shares epitopes with another histidine-rich protein, *PfHRP3*. Thus, antibodies specific for *PfHRP2* have been found to also across react with *PfHRP3* (Lee et al., 2006).

The performance of malaria RDTs is generally associated with the product quality, storage conditions, parasite or operator factors and parasite/antigen concentrations. One important factor is the variability within the parasite antigens detected by the RDTs, which includes presence or absence of the target epitopes and variations in the number of epitopes in a particular parasite isolate (Lee et al., 2006, 2012; Maltha et al., 2014; Talman et al., 2007). Genetic diversity may be particularly important for *PfHRP2*-based RDTs, since most of the RDTs are based on this antigen (Mouatcho and Goldring, 2013). Studies to date have documented extensive size variations between parasite strains and a high degree of genetic diversity within this antigen (Baker et al., 2010, 2005; Deme et al., 2014; Kumar et al., 2012; Lee et al., 2006; Rock et al., 1987). An extreme situation is that certain parasite isolates even harbor a deletion of the *pfhrp2* gene, leading to false negative results in diagnosis with *PfHRP2*-based RDTs. This phenomenon was first discovered in the Peruvian Amazon in 2010 (Gamboa et al., 2010). Since then, *pfhrp2*

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deletion-associated poor performance of RDTs has been reported in many malaria-endemic regions (Cheng et al., 2014; Koita et al., 2012; Maltha et al., 2012; Pava et al., 2010; Wurtz et al., 2013). To date, unequivocal evidence for *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum* isolates has been obtained in Peru (Gamboa et al., 2010; Maltha et al., 2012), Brazil (Houze et al., 2011), Senegal (Wurtz et al., 2013), and India (Kumar et al., 2012). Apart from gene deletion, *PfHRP2* sequences in different regions showed differences in the number of amino acid repeats and even some rare amino acid variants (Baker et al., 2010, 2005; Deme et al., 2014; Kumar et al., 2012; Lee et al., 2006). Given the significance of *PfHRP2*-based RDTs in malaria diagnosis, systematic mapping of *PfHRP2* diversity in global malaria endemic regions is highly demanded, especially in areas where poor performance or failure of *PfHRP2*-based RDTs have been reported (Cheng et al., 2014).

In the remote border regions of Southeast Asian countries, malaria remains prevalent (Cui et al., 2012) and RDTs have been widely used for malaria diagnosis as a case management practice. Recently, we compared two different RDTs in malaria diagnosis at the China–Myanmar border (Yan et al., 2013). From a limited analysis, we identified two *P. falciparum* cases, which were positive by both microscopy and PCR but consistently failed two *PfHRP2*-based RDTs. This has prompted us to examine *pfhrp2* genetic diversity in greater detail and determine whether the two false-negative cases were due to *pfhrp2* gene deletions.

2. Materials and methods

2.1. Parasite isolates and DNA extraction

A total of 97 *P. falciparum* parasite isolates from the China–Myanmar border and western Thailand were analyzed in this study. Among them, 87 were obtained from malaria patients in the China–Myanmar border area between May 2011 and December 2012, of which 36 were also evaluated by two *PfHRP2*-based RDTs (Wondfo, China and Tycolpharm Co. Limited, United Kingdom) (Yan et al., 2013). Based on microscopy of Giemsa stained thick smears, parasite densities ranged from 40 to 105,920 parasites/μL of blood, assuming 8000 leukocytes/μL. The remaining ten parasite isolates were collected in Tak Province, western Thailand during mass blood surveys conducted between August 2011 and May 2012 (Li et al., 2014). Parasite DNA was extracted from dried blood spots on Whatman 3 M filter paper using the Qiagen DNA Mini Kit (Qiagen, Valencia, CA, USA). DNA was eluted in 50 μL of distilled water and stored under −20 °C.

2.2. Amplification of the pfhrp2 and pfhrp3 genes

P. falciparum infections in 97 samples were verified by a nested PCR method (Yan et al., 2013). Previously established protocols were used for amplifications of *pfhrp2* and *pfhrp3* (Table S1). Specifically, the A262/A264 primers were used in the primary PCR reaction for *pfhrp2* (Trouvay et al., 2013). For nest PCR, two sets of primers were used to amplify the exon1/intron 1 part (+29nt to +155nt) and the exon2 (+135nt to 7nt after the stop codon) of the *pfhrp2* gene, respectively (Baker et al., 2005; Trouvay et al., 2013). All amplification conditions used were the same as the reference articles. The PCR products of the exon2 were cloned in the T-vector (Takara Biotechnology, Dalian, China) for sequencing. For amplification of *pfhrp3*, primers targeting exon2 (+77nt to +180nt) were used (Baker et al., 2005; Mariette et al., 2008; Trouvay et al., 2013). Samples were considered *pfhrp2*-negtive or *pfhrp3*-negtive if amplifications were not successful after two attempts.

2.3. Flanking genes and genotyping of pfhrp2-negative samples

To determine whether *pfhrp2*-negative samples by PCR were due to lack of parasite DNA in the samples, three polymorphic genes *pfmsp1*, *pfmsp2* and *pfglurp* were amplified using established protocols (Meng et al., 2010). DNA from the reference strains 3D7, 7G8 and HB3 were used as positive controls. Two genes flanking *pfhrp2*, *PF3D7_0831900* and *PF3D7_0831700*, were further detected by the nested-PCR using modified primers and conditions as described earlier (Akinyi et al., 2013).

2.4. DNA sequencing and analysis

The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia) and cloned into the pMD18-T vector. Cloned PCR products were sequenced on an ABI 3730XL DNA Analyzer with primers M13-47 (CGCCAGGGTTTTCCCAGTCA CGAC) and RV-M (GAGCGGATAACAATTTCACACAGG) (Takara, Dalian, China). The nucleotide sequences of *pfhrp2* exon2 were assembled using DNASTAR (Madison, WI, USA). Different types of amino acid repeats within exon 2 were identified and given a numeric code(1–18) as described earlier (Baker et al., 2005). These repeat types were aligned to visualize the similarity between parasite isolates and sequence from 3D7 was used as the reference. *Pfhrp2* sequences were deposited in GenBank under accession numbers KP712709–KP712775.

3. Results

3.1. Pfhrp2 gene size variation and deletion

Of the 97 *P. falciparum* isolates, amplification of *pfhrp2* exon1/intron 1 and exon2 were successful in 93 isolates; 4 samples (M0100441, M0N00199, M0N00529, and M0N00302) failed to amplify any *pfhrp2* fragments. Sixty seven *pfhrp2* PCR fragments were cloned and sequenced. The results showed that the amplified exon 2 fragments ranged from 319 to 902 bp, with most of them (~79%) being 500–900 bp (Fig. 1). Sequencing analysis revealed that 65.7% (44/67) isolates had unique sequences, whereas 23 (34.3%) sequences were present in >1 parasite isolate. It is noteworthy that nine identical sequences were shared among nine parasite isolates collected in a single village, an indication of the same clone in circulation.

For the four parasite isolates that failed to amplify any *pfhrp2* fragments, three (M0100441, M0N00199, and M0N00302) also failed to yield a PCR product for *pfhrp3* exon2, suggesting possible deletions of both *pfhrp2* and *pfhrp3* genes in these samples. The presence of parasite DNA in these four samples was confirmed by successful amplifications of three polymorphic genes *pfmsp1*, *pfmsp2* and *pfglurp* (data not shown). In addition, two genes (*PF3D7_0831700* and *PF3D7_0831900*) flanking *pfhrp2* were also successfully amplified from these four samples (Table S2).

3.2. Correlation between pfhrp2 gene deletion and RDT results

Thirty-six of the samples from the China–Myanmar border area have been evaluated by two *PfHRP2*-based RDTs (Yan et al., 2013). Two samples (M0100304 and M0500035) were slide-positive for *P. falciparum* infections but were negative by both RDTs. Based on microscopy, parasite densities of these two samples were estimated at 480 (M0100304) and 120 parasites/μL (M0500035), respectively. *P. falciparum* infections in these two samples were further confirmed by PCR. PCR and sequencing of *pfhrp2* exon2 from these two samples yielded *pfhrp2* products of 633 and 690 bp, respectively. This result indicated that these two RDT-negative