IJP: Drugs and Drug Resistance 8 (2018) 320–328

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Longitudinal surveillance of drug resistance in *Plasmodium falciparum* isolates from the China-Myanmar border reveals persistent circulation of multidrug resistant parasites

Yao Bai[a,b,1], Jiaqi Zhang[a,1], Jinting Geng[a], Shiling Xu[a], Shuang Deng[c], Weilin Zeng[a], Zenglei Wang[d], Huguette Gaelle Ngassa Mbenda[d], Jie Zhang[a], Na Li[a,b], Yanrui Wu[e], Cuiying Li[a], Huae Liu[a], Yonghua Ruan[c], Yaming Cao[f], Zhaoqing Yang[a,*], Liwang Cui[a,d,**]

[a] Department of Pathogen Biology and Immunology, Kunming Medical University, Kunming, China
[b] Department of Pharmacology, Kunming Medical University, Kunming, China
[c] Department of Pathology, Kunming Medical University, Kunming, China
[d] Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA
[e] Department of Cell Biology and Medical Genetics, Kunming Medical University, Kunming, Yunnan Province 650500, China
[f] Department of Immunology, China Medical University, Shenyang, Liaoning 110122 China

ARTICLE INFO

Keywords:
Drug resistance
Plasmodium falciparum
Mutation
In vitro assay
China-Myanmar border

ABSTRACT

Multidrug-resistant *Plasmodium falciparum* in the Greater Mekong Subregion of Southeast Asia is a major threat to malaria elimination and requires close surveillance. In this study, we collected 107 longitudinal clinical samples of *P. falciparum* in 2007–2012 from the malaria hypoendemic region of the China-Myanmar border and measured their in vitro susceptibilities to 10 antimalarial drugs. Overall, parasites had significantly different IC_{50} values to all the drugs tested as compared to the reference 3D7 strain. Parasites were also genotyped in seven genes that were associated with drug resistance including *pfcrt, pfmdr1, pfmrp1, pfdhfr, pfdhps, pfnhe1*, and *PfK13* genes. Despite withdrawal of chloroquine and antifolates from treating *P. falciparum*, parasites remained highly resistant to these drugs and mutations in *pfcrt, pfdhfr*, and *pfdhps* genes were highly prevalent and almost reached fixation in the study parasite population. Except for pyronaridine, quinine and lumefantrine, all other tested drugs exhibited significant temporal variations at least between some years, but only chloroquine and piperaquine had a clear temporal trend of continuous increase of IC_{50}s. For the *pfmrp1* gene, several mutations were associated with altered sensitivity to a number of drugs tested including chloroquine, piperaquine, lumefantrine and dihydroartemisinin. The association of *PfK13* mutations with resistance to multiple drugs suggests potential evolution of *PfK13* mutations amid multidrug resistance genetic background. Furthermore, network analysis of drug resistance genes indicated that certain haplotypes associated multidrug resistance persisted in these years, albeit there were year-to-year fluctuations of the predominant haplotypes.

1. Introduction

Malaria, a life-threatening disease caused by the *Plasmodium* parasites, has claimed over 400 000 human lives globally in 2016 (WHO, 2017). In the tropical and subtropical areas of the Greater Mekong Subregion (GMS), recent achievements in malaria control have encouraged countries within this region to pursue malaria elimination, aiming to reach this goal by 2030. Chemotherapy is an essential tool for malaria management, but its effectiveness is compromised by the emergence and spread of drug-resistant *Plasmodium falciparum* strains. Chloroquine (CQ) was one of the most widely used antimalarial drugs. Only several years after its introduction, CQ-resistant cases emerged firstly in Southeast Asia, then appeared in Latin America, and spread to all other endemic areas (Wellems and Plowe, 2001). This also happened to the antifolates drug pyrimethamine (PY). The GMS is a breeding ground of antimalarial drug resistance, and *P. falciparum* has developed resistance to essentially all commonly used antimalarial drugs (Fairhurst and Dondorp, 2016). Multidrug-resistant (MDR) parasites

* Corresponding author. Department of Pathogen Biology and Immunology, Kunming Medical University, Kunming, China
** Corresponding author. Department of Entomology, Pennsylvania State University, University Park, PA, USA
E-mail addresses: zhaoqingy92@hotmail.com (Z. Yang), luc2@psu.edu (L. Cui).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.ijpddr.2018.05.003
Received 1 April 2018; Received in revised form 4 May 2018; Accepted 13 May 2018

have led to the deployment of artemisinin combination therapies (ACTs). However, artemisinin resistance in *P. falciparum* has also emerged in the same place of the GMS (Noedl et al., 2008; Dondorp et al., 2009), where CQ and PY resistance first emerged. Furthermore, resistance to the partner drug mefloquine (MQ) and recently piperaquine (PQ) have resulted in increased clinical failures of the artesunate (AS)-MQ and dihydroartemisinin (DHA)-PQ, respectively (Wongsrichanalai and Meshnick, 2008; Saunders et al., 2014; Spring et al., 2015). With the unfolding of malaria elimination campaign in the GMS, heightened surveillance of drug resistance in *P. falciparum* is required in order to monitor the situation, prevent the spread of resistant parasites, and make timely changes of the national drug treatment policies.

The identification of drug resistance mechanisms facilitates molecular surveillance of antimalarial drug resistance (Ekland and Fidock, 2007). The K76T mutation in the *P. falciparum* chloroquine resistance transporter, pfcrt, is a major determinant of CQ resistance (Fidock et al., 2000). Point mutations in dihydropteroate synthase (dhps) and dihydrofolate reductase (dhfr), two key enzymes in the folate biosynthesis pathway, mediate resistance to the antifolates sulfadoxine and PY, respectively (Gregson and Plowe, 2005). As its name indicates, point mutations in the multidrug resistance 1 (*mdr*1) gene confer resistance to a number of drugs, while *mdr1* gene amplification is responsible for clinical resistance to MQ (Price et al., 2004), and in vitro resistance to other amino alcohol drugs (Sidhu et al., 2006). In recent years, the advancement of genomic tools allowed accelerated identification of resistance mechanisms to artemisinin and PQ. Through a combination of in vitro selection, genomics and population biology, artemisinin resistance was found to be associated with point mutations in the propeller domain of the *PfK13* gene (Ariey et al., 2014), which were subsequently confirmed by genetic manipulations (Ghorbal et al., 2014; Straimer et al., 2015). Similarly, genome-wide association studies revealed that amplification of two protease genes *plasmepsin 2/3* was associated with clinical resistance to PQ in Cambodia (Amato et al., 2017; Witkowski et al., 2017). In areas of low transmission where host immunity against the malaria parasites is low, molecular markers serve as proxies for the prediction of efficacies of antimalarial drugs and provide convenient assessment of the epidemiology of drug resistance in malaria parasites.

“Border malaria” – concentrated malaria transmission along international borders – brings extreme difficulties for surveillance, and malaria re-introduction by cross-border migratory human populations could plunge people of malaria eliminating countries into malaria resurgence (Delacollette et al., 2009; Cui et al., 2012). Since drug policies in neighboring countries may differ considerably, parasite populations at the border may experience divergent drug selection pressures, favoring the emergence of MDR parasites (Zeng et al., 2017). The China-Myanmar border used to be a malaria hyperendemic region with a distinct antimalarial drug use history. Since 1979, PQ has been used extensively as a replacement drug of CQ in China, which has led to clinical resistance to PQ (Gao et al., 1993; Yang et al., 1999). Also, artemisinin drugs had been deployed mostly as monotherapies prior to 2005. After 2005, the national antimalarial drug policy has changed to ACT, mostly DHA-PQ, as the frontline treatment for uncomplicated *P. falciparum* cases in this region. Clinical follow-ups in recent years showed that DHA–PQ remained highly efficacious for treating uncomplicated falciparum malaria (Liu et al., 2015; Wang et al., 2015a). Further studies of parasites from this region also showed that day-3 parasite positivity as well as delayed parasite clearance were associated with *PfK13* mutations (Huang et al., 2015; Wang et al., 2015c). Moreover, consistent with extensive deployment of ACT, the proportions of parasites carrying the *PfK13* mutations have been increasing (Wang et al., 2015b). Possibly reflecting the divergent antimalarial drug histories, parasites from this region showed a *PfK13* mutation pattern that is distinct from that in Cambodia (Ariey et al., 2014; Tun et al., 2015; Wang et al., 2015b). Thus, continuous monitoring of antimalarial drug resistance in this region is warranted.

In the present study, we performed a longitudinal follow-up of in vitro sensitivities in *P. falciparum* clinical isolates collected during 2007–2012 to commonly-used antimalarial drugs and determined dynamic changes in drug sensitivities and polymorphisms of genes associated with drug resistance. These data combined allowed us to further detect and confirm associations between drug-resistant genes and in vitro sensitivities to several antimalarial drugs. Our results revealed the persistent circulation of MDR parasites and further highlighted the necessity of close drug-resistance monitoring in the GMS in order to use updated drug policy for a specific region and period.

2. Material and methods

2.1. Parasite sample collection

To longitudinally follow *P. falciparum* in vitro sensitivities to antimalarial drugs at the China-Myanmar border, we collected 107 clinical parasite samples from acute, uncomplicated *P. falciparum* infections from malaria clinics located near the Nabang township in west Yunnan Province, China, and the Laiza township, Kachin State, Myanmar, during 2007–2012. Malaria diagnosis was based on microscopy of Giemsa-stained blood smears, and 2–5 ml venous blood was drawn from patients with falciparum malaria. Blood samples were stored in liquid nitrogen and used for culture adaptation. All patients in this study signed informed consent forms voluntarily and the research project was approved by the institutional review board of Kunming Medical University.

2.2. Parasite culture and in vitro drug assay

Culture-adapted parasite isolates were assayed for their in vitro sensitivities to 10 antimalarial drugs. CQ, MQ, quinine (QN), and PY were purchased from Sigma (St. Louis, MO, USA). PQ was from Chongqing Kangle Pharmaceutical Co. (Chongqing, China), pyronaridine (PND) was obtained from the China Institute of Pharmaceutical and Biological Products (Beijing, China), while naphthoquine (NQ), lumefantrine (LMF), AS, and DHA were from Kunming Pharmaceutical Co. (Kunming, Yunnan, China). Stock solutions of CQ, NQ, PND, and PQ were prepared in distilled water, MQ, QN, LMF, AS and DHA in ethanol, and PY in 1% acetic acid. Only monoclonal isolates were used for drug assays (Meng et al., 2010; Yuan et al., 2013). Parasite culture, synchronization and drug assay using the SYBR Green I-based method were performed as described (Smilkstein et al., 2004; Wang et al., 2016). Drugs were added to each well of a 96-well microplate at an initial concentration of 3.75 μM for CQ and PY, 256 nM for NQ and MQ, 1.5 μM for AS and DHA, 160 nM for PND, 320 nM for PQ, 10.24 μM for QN, and 800 nM for LMF, which were serially diluted. Each parasite strain was assayed with three technical repeats and two biological replications, and the 3D7 strain was included in all assays as an internal reference.

2.3. Sequencing analysis of drug resistance genes

Parasite genomic DNA was extracted from cultured parasites using a QiaAmp DNA minikit (Qiagen). Polymorphisms in drug resistance genes were determined by PCR and sequencing as previously reported (Yang et al., 2011; Gupta et al., 2014; Wang et al., 2015b). These include two *pfcrt* fragments covering codons 72–76 and 220, two *pfmdr1* fragments including codons 86, 184, 1042 and 1246, a *pfdhfr* fragments containing codons 51, 59, 108 and 164, two *pfdhps* fragments containing codons 436, 437, 540, and 581, a *pfnhe1* fragment containing the ms4760 minisatellite, and two *pfmrp1* gene fragments containing codons 191, 325, 437, 785, 876, 1007, 1390 and the complete sequence of *PfK13* gene.