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SHORT REPORT: POLYMORPHISMS IN THE CHLOROQUINE RESISTANCE TRANSPORTER GENE IN *PLASMODIUM FALCIPARUM* ISOLATES FROM LOMBOK, INDONESIA

MARIA CECILIA HUAMAN, KAZUMI YOSHINAGA, AAN SURYANATHA, NYOMAN SUARSANA, AND HIROJI KANBARA

Institute of Tropical Medicine, Nagasaki University, Sakamoto, Nagasaki, Japan; Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; Meninting Health Center, West Lombok, Indonesia

Abstract. The polymorphisms in the *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) and *P. falciparum* chloroquine resistance transporter (*pfcrt*) genes, which are associated with chloroquine resistance, were examined in 48 *P. falciparum* isolates from uncomplicated malaria patients from the West Lombok District in Indonesia. The point mutation N86Y in *pfmdr1* was present in 35.4% of the isolates and mutation K76T in *pfcrt* was found in all but one of the samples studied. Identified *pfcrt* haplotypes were mainly identical to the Papua New Guinea type S_{agt}VMNT (42 of 48, 87.5%), and a few isolates had the Southeast Asia type CVIET (5 of 48, 10.4%). Moreover, one *P. falciparum* isolate harbored the K76N mutation, giving rise to the haplotype CVMNN, which was not previously reported in field isolates. Our findings suggest that chloroquine resistance in this area might have the same origin as in Papua New Guinea.

The mechanism of chloroquine (CQ) resistance in *Plasmodium falciparum* has been investigated and mutations in the *P. falciparum* CQ resistance transporter gene (*pfcrt*) located on chromosome 7, and the *P. falciparum* multidrug resistant gene 1 (*pfmdr1*), located on chromosome 5, have been implicated. The substitution of threonine for lysine in codon 76, K76T in the *pfcrt* gene, was shown *in vitro* to be associated with CQ resistance in isolates from Asia, Africa, South America, and Papua New Guinea.[1,2] Sequence polymorphisms at position 72-76 of this gene have been associated with the geographic origin of parasite samples, with the CVIET pattern in resistant isolates from Asia and Africa, and with SVMNT in resistant isolates from Papua New Guinea and South America.[1,3] The multidrug resistant gene *pfmdr1* with a mutation of asparagine to tyrosine at position 86 (N86Y) has been associated with *in vitro* resistant strains.[4] Although its participation is not clear, it has been suggested that the *pfmdr1* mutation may confer some advantage to the parasite in the presence of CQ, thus increasing the level of CQ resistance.[2,5] Furthermore, a recent study that included samples from four countries of Southeast Asia described the mutations N86Y in *pfmdr1* and K76T in *pfcrt* genes as molecular markers for predicting clinical outcome of CQ treatment.[6]

In Indonesia, the first cases of resistance were reported in the early 1970s from Kalimantan and Irian Jaya. Although resistance has been reported on several islands in Indonesia,[7] with resistance as high as 95% for *P. falciparum* and 84% for *P. vivax*,[8] CQ continues to be the first-line treatment of *P. falciparum* and *P. vivax* malaria because of its safety and availability at very low cost. Here we examined the prevalence of polymorphisms in the *pfmdr1* and *pfcrt* genes in 48 *P. falciparum* isolates from the West Lombok District of Indonesia. In addition, the possible origin of CQ resistance in Indonesia is discussed.

Blood samples were collected from 48 patients with uncomplicated *P. falciparum* malaria in sub-district Batulayar in West Lombok in the West Nusa Tenggara Province of Indonesia (Figure 1) from June to September 2002. Sub-district Batulayar has a population of approximately 35,658. The climate is tropical and malaria transmission occurs more frequently during dry season between April and October, although low-level transmission occurs throughout the year, especially in the hilly-forested ranges of Sidemen to Pusuk. Cases of *P. falciparum* and *P. vivax* malaria and a few cases of *P. malariae* malaria have been reported in the area. The recommended first-line treatment is CQ, 25 mg/kg given over a three-day period. When a treatment failure occurs, the combination of sulfadoxine/pyrimethamine is prescribed. Inclusion criteria were a fever ≥37.5°C during the last 48 hours and a positive result in the NOW ICT® (Binax, Portland, ME) rapid malaria test. Blood samples were collected on filter paper and transported to the Muninting district health center laboratory for malaria testing. Samples positive for *P. falciparum* malaria were processed thereafter in Japan at the Department of Protozoology of Nagasaki University. Informed consent was obtained from each individual. The study was reviewed and approved by the ethical committee of the Institute of Tropical Medicine of Nagasaki University and the executive committee of the Malaria Control Project in Lombok and Sumbawa under the Japanese International Cooperation Agency partnership program.

The DNA was extracted from filter paper by cutting the blood spot into pieces and soaking them in 0.5% saponin in HBS buffer (140 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.2). Thereafter, the QIAamp DNA Kit (Qiagen, Valencia, CA) was used according to the manufacturer's instructions. The parasite lines FCR3 and K1 were used as controls for the detection of polymorphism at position 86 in *pfmdr1* and direct sequencing analysis of *pfcrt* gene. For genotyping of the glutamate-rich protein (*glurp*) gene, strains K1 and 3D7 were used as controls in the amplification.

To determine the presence of tyrosine at position 86 in *pfmdr1*, a nested polymerase chain reaction (PCR)–restriction fragment length polymorphism protocol was used as previously described.[9] Digestion with the restriction endonuclease *Apo* I (New England Biolabs, Inc., Beverly, MA) detects tyrosine at position 86 after resolution of the products by electrophoresis on 1–3% agarose gels (Nusieve 3:1; BioWhittaker Molecular Applications, Rockand, ME). For *pfcrt* gene analysis, a first amplification was carried out with previously designed primers,[9] and the products obtained were used as a template in a nested PCR encompassing the polymorphic codons 72-76 and 97 in exon 2 as previously reported.[3] The PCR amplification products were purified using the QIAquick

FIGURE 1. Map of Indonesia showing the location of Lombok.

PCR purification kit (Qiagen) and directly sequenced on an ABI310 automated sequencer using ABI PRISM Big Dye Terminator Cycle kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

The *glurp* gene, located on chromosome 11, which has a high degree of polymorphism, was assessed for evaluation of diversity of the *P. falciparum* isolates population in the region.[10] The amplification products were resolved by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The *glurp* amplification product sizes were estimated using DNAfrag version 3.03 Software (John Nash, Institute for Biologic Sciences, National Research Council of Canada, Ottawa, Ontario, Canada). For comparisons, Fisher's exact test was used.

Seventeen isolates (35.4%) had the 86Y mutation in the *pfmdr1* gene, 26 had wild type N86, and 5 carried both alleles (Table 1). In previous studies in Irian Jaya and West Papua, N86Y was found to show a correlation with CQ-resistant *P. falciparum* parasites.[11,12] The mutation K76T in the *pfcrt* gene was found in all but one (47 of 48, 97.9%) of the isolates studied. A previous report showed that the K76T mutation showed a correlation with clinical resistance to CQ and as a molecular marker had a sensitivity of 93% and a specificity of 82%.[13] Both point mutations in the *pfmdr1* and *pfcrt* genes have been proposed to indicate a tendency toward reduced susceptibility to CQ. Thus, our results suggest potential CQ resistance in the region, although other factors may influence the final treatment outcome. The combination of geographic remoteness to health facilities and lack of interest in seeking medical attention driven by both financial reasons and lack of knowledge resulted in an overall follow-up rate of 23% (11 of 48). After 14 days of CQ treatment, 5 of 11 patients were not able to clear the parasites, and had tendency to harbor 86Y in the *pfmdr1* gene (Table 1). However, a larger sample size is required to obtain conclusive results.

A new mutation, K76N, which substitutes asparagine for lysine, was found in one isolate from the sub-village Pusuk, generating the haplotype CVMNN. For confirmation of this finding, independent PCR amplifications and at least three repetitions of sequencing were carried out. In all cases, unambiguous electropherograms were obtained, showing AAT that codes for asparagine at position 76. The K76N mutation could be misidentified as a K76T substitution by a PCR-restriction enzyme protocol.[14] To our knowledge, this is the first time that K76N has been reported in a field study. However, it has been reported in laboratory experiments after exposure of parasites to lethal concentrations of CQ. In those experiments, Cooper and others demonstrated that the K76N mutation confers the verapamil-reversible CQ-resistance phenotype associated with greatly reduced accumulation of the drug.[15] Contrary to those *in vitro* experiments, the patient possessing this rare *pfcrt* haplotype cleared parasites after treatment with CQ. Since other factors participate in the clinical outcome, it would be interesting to look for more isolates with CVMNN and carry out the *in vitro* susceptibility test.

The sequence analysis of codons 72-76 in the *pfcrt* gene (Table 1) allowed identification of previously reported haplotypes S_{agt}VMNT (42 of 48, 87.5%) and CVIET (5 of 48, 10.4%). The *pfcrt* SVMNT haplotype with serine coded by AGT has been found in Bougainville, Papua New Guinea,[16]

TABLE 1

Genotypes for *Pfcrt, Pfmdr1,* and *Glurp* genes in 48 *Plasmodium falciparum* isolates from Lombok, Indonesia*

Subvillage	*Pfcrt* haplotype†	Codon 86 in *Pfmdr1*	*Glurp* genotype‡	Parasite clearance at 14 days
Bunean	SVMNT	Tyr and Asn	650	Failure
Bunean	SVMNT	Tyr	750	Failure
Batu Penyu	SVMNT	Tyr	750	Failure
Batu Penyu Atas	SVMNT	Asn	750	Failure
Pelolat	SVMNT	Tyr	1000	Failure
Kedondong Atas	SVMNT	Asn	700	Success
Batu Penyu	SVMNT	Tyr	750	Success
Batulayar	SVMNT	Asn	700	Success
Pusuk	CVMNN	Asn	950	Success
Senggigi	SVMNT	Asn	700	Success
Senggigi	SVMNT	Asn	700 and 850	Success
Penanggak	SVMNT	Asn	600	ND
Penanggak	SVMNT	Asn	800	ND
Penanggak	SVMNT	Tyr	900	ND
Penanggak	SVMNT	Asn	1100	ND
Penanggak	SVMNT	Asn	1000	ND
Penanggak	SVMNT	Tyr	900	ND
Kekeran	SVMNT	Tyr	450	ND
Kekeran	SVMNT	Tyr	750	ND
Kekeran	SVMNT	Asn	900	ND
Kekeran	SVMNT	Asn	700	ND
Sidemen Daye	CVIET	Tyr	450	ND
Sidemen Daye	SVMNT	Asn	900	ND
Sidemen Lauk	SVMNT	Asn	750	ND
Sidemen Lauk	SVMNT	Asn	550	ND
Sidemen Lauk	SVMNT	Tyr	550	ND
Seraye	SVMNT	Asn	700	ND
Seraye	SVMNT	Asn	600	ND
Seraye	SVMNT	Asn	600	ND
Kodondong Atas	SVMNT	Asn	700	ND
Kedondong Atas	CVIET	Tyr	450	ND
Kedondong Atas	CVIET	Tyr	450	ND
Kedondong Atas	CVIET	Tyr	450	ND
Kedondong Atas	SVMNT	Tyr	900	ND
Kedondong Atas	CVIET	Tyr and Asn	450	ND
Apit Aik	SVMNT	Tyr and Asn	750	ND
Apit Aik	SVMNT	Asn	750	ND
Apit Aik	SVMNT	Asn	800	ND
Batu Bolong	SVMNT	Asn	750	ND
Batu Bolong	SVMNT	Asn	650	ND
Batu Penyu	SVMNT	Tyr	750	ND
Batu Penyu	SVMNT	Asn	750	ND
Batu Penyu Atas	SVMNT	Tyr	750	ND
Batu Penyu Atas	SVMNT	Asn	750	ND
Duduk Atas	SVMNT	Tyr and Asn	600	ND
Duduk Atas	SVMNT	Asn	950	ND
Duduk Atas	SVMNT	Tyr	1000	ND
Sd. Kedondong	SVMNT	Tyr and Asn	700	ND

* *Pfcrt* = *P. falciparum* chloroquine resistance transporter; *Pfmdr1* = *P. falciparum* multidrug resistance 1; *Glurp* = glutamine-rich protein; ND = no data.
† Codons 72–76 in *Pfcrt* gene.
‡ Polymerase chain reaction product sizes in basepairs.

the main island of Papua New Guinea,[3] and East Timor.[17] The haplotype CVIET has been reported in countries of Southeast Asia.[1,18] Since Lombok, Indonesia is located near Papua New Guinea (Figure 1), it is not unexpected that both the SVMNT and CVIET haplotypes were detected.

Furthermore, in our attempt to evaluate the diversity among the isolates studied, we assessed the *glurp* gene and 11 *glurp* genotypes were found in West Lombok, ranging from 450 to 1,100 basepairs. The West Lombok District, despite its small area, shows a high degree of diversity in the *P. falciparum* population that might be a product of high rate of transmission of malaria or human transmigration.

Upon examination for any linkage among the alleles studied in the *pfmdr1*, *pfcrt*, and *glurp* genes, significant associations were found between the *pfcrt* CVIET haplotype and *pfmdr1* 86Y ($P = 0.0193$), the CVIET haplotype and *glurp* 450 ($P < 0.001$), and *pfmdr1* 86Y and *glurp* 450 ($P = 0.0057$). Our findings showed that the majority of isolates have *pfcrt* haplotype SVMNT, *pfmdr1* 86N, and *glurp* with molecular masses greater than 450 homogeneously distributed in all the villages from West Lombok, indicating that these might be indigenous in the area. A few isolates harboring *pfcrt* haplotype CVIET, *pfmdr1* 86Y, and *glurp* 450, found mainly in Kedondong Atas village, were most likely introduced recently. Therefore, CQ resistance in Lombok might have the same origin as the Papua New Guinea strains, and the Southeast Asian *pfcrt* haplotype CVIET might have been introduced only recently in a particular region. Further studies are being carried out in isolates from Lombok and other Indonesian islands to determine the prevalence of the novel K76N mutation and its association with clinical outcome/*in vitro* susceptibility to CQ.

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Authors' addresses: Maria Cecilia Huaman, Institute of Tropical Medicine, Nagasaki University, Sakamoto 1-12-4, Nagasaki 852-8523, Japan and Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru, E-mail: ceci_huaman@yahoo.com. Kazumi Yoshinaga and Hiroji Kanbara, Institute of Tropical Medicine, Nagasaki University, Sakamoto 1-12-4, Nagasaki 852-8523, Japan, Telephone: 81-95-849-7838, Fax: 81-95-849-7805. E-mail: f0512@cc.nagasaki-u.ac.jp. Aan Suryanatha and Nyoman Suarsana, Meninting Health Center, West Lombok, Indonesia.

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