CORRESPONDENCE

Emergence of Indigenous Artemisinin-Resistant *Plasmodium falciparum* in Africa

TO THE EDITOR: *Plasmodium falciparum* has developed resistance to artemisinin in many countries in Southeast Asia.[1,2] Artemisinin combination therapy is the first-line treatment for malaria in the majority of countries in which the disease is endemic, and its efficacy is particularly important in Africa, where malaria is the most widespread.[3] We report here an artemisinin-resistant strain of *P. falciparum* that was contracted in Africa.

On January 28, 2013, falciparum malaria was diagnosed in a 43-year-old man (identified here as CWX) at a hospital in Jiangsu Province, China. The patient had returned to China on December 3, 2012, after working for 20 months in Equatorial Guinea, where he had been treated for malaria six times. The date and therapeutic regimen associated with each episode are unknown, with the exception of the last episode, when the patient received parenteral artesunate monotherapy starting on November 20, 2012. Before his arrival in Equatorial Guinea, the patient had no history of malaria.

On the patient's presentation in Jiangsu Province, microscopy revealed *P. falciparum* parasites in peripheral blood, with an initial parasite density of 4221 per microliter. A monospecies infection was identified on polymerase-chain-reaction assay. The patient received a course of eight tablets of a combination of dihydroartemisinin (40 mg) and piperaquine (320 mg) under direct observation. The parasitemia declined over the next 3 days, but parasites were still detected on day 3 after treatment (Fig. 1A). By day 7, no parasites were detected. In contrast, three other isolates that originated from the same country were negative for asexual parasites as of day 3.

In vitro ring-stage survival assay[3] revealed a 2.29% survival rate for the CWX isolate, which was substantially higher than the rate in control *P. falciparum* strains (including wild-type strain 3D7) and in another isolate from a Chinese worker (SBC) who had returned to China from Equatorial Guinea in 2013 but lower than the rate in an artemisinin-resistant parasite line with a C580Y kelch13 mutation (Fig. 1B, and the Methods section in the Supplementary Appendix, available with the full text of this letter at NEJM.org). Polymorphisms in the *P. falciparum* gene encoding kelch13 (*K13*) have been linked to artemisinin resistance in Southeast Asia. Sequencing of *K13* in the CWX isolate revealed a previously unreported nonsynonymous single-nucleotide polymorphism (SNP) that resulted in a switch from a methionine to an isoleucine at amino acid position 579 (M579I).

In order to determine whether the CWX strain was indigenous to Equatorial Guinea, we performed whole-genome sequencing (European Nucleotide Archive accession number, PRJEB18721), and compared the SNPs with those of 245 *P. falciparum* isolates collected worldwide.[4] Principal component analysis showed that the CWX strain was of African origin and had not been recently imported from elsewhere in the world (Fig. 1C, and Fig. S2 in the Supplementary Appendix).

There is a perennially high rate of malaria transmission throughout Equatorial Guinea, and artemisinin combination therapies are commonly used for treatment.[5] Awareness of artemisinin resistance is prudent in Equatorial Guinea and countries with similar malaria transmission dy-

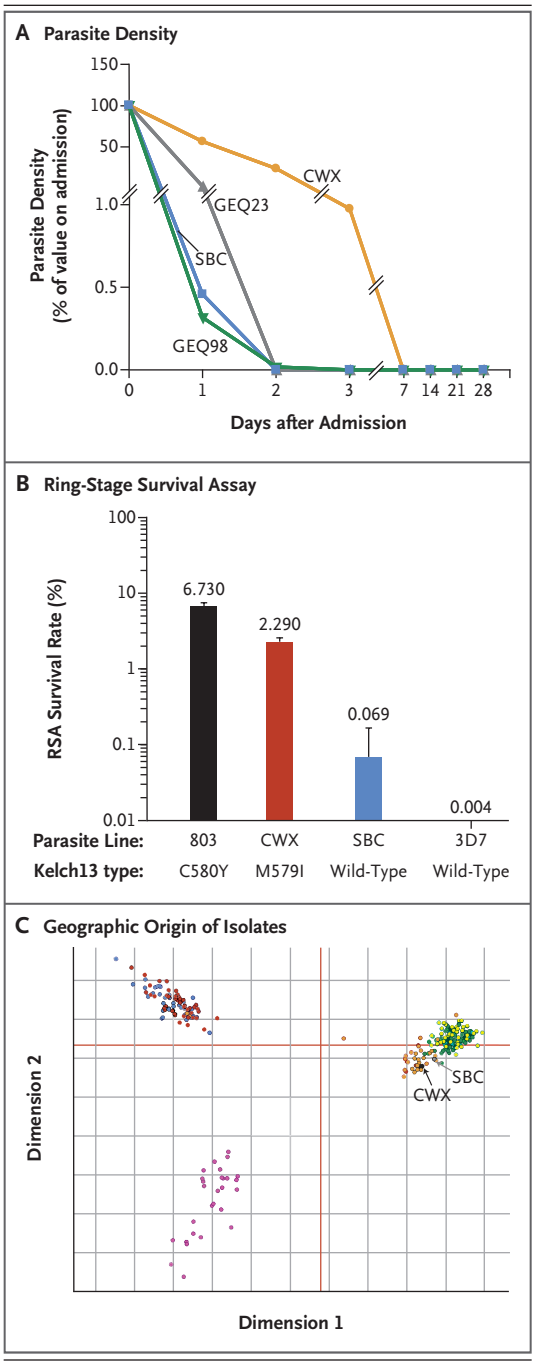

Figure 1. Artemisinin Resistance Associated with M579I Mutation in an Isolate of African Origin.

Panel A shows the rate of parasite clearance after treatment with artemisinin combination therapy in vivo in the CWX isolate and in three other isolates originating from the same country (including SBC, which was obtained from another Chinese man who had worked in Equatorial Guinea). The three other isolates were negative for asexual parasites as of day 3. Panel B shows the survival rate on ring-stage survival assay (RSA) for isolates with or without the M579I mutation. 3D7 is a wild-type strain, and 803 is an artemisinin-resistant parasite line with the C580Y kelch13 mutation. Panel C shows multidimensional scaling of CWX, SBC, and 245 other *P. falciparum* isolates with different geographic origins. Each dot represents an isolate and is spread in a three-dimensional space according to the similarities or dissimilarities among them. The distances between the isolates were inferred from 26,918 common genome-wide single-nucleotide polymorphisms and plotted with the use of a sequence variation analysis, maps, and phylogeny (SVAMP) program. The isolates are color-coded as follows: CWX, black; SBC, gray; Burkina Faso isolates, green; Mali isolates, yellow; Kenya isolates, gold; Papua New Guinea isolates, purple; Thailand isolates, red; and Cambodia isolates, blue. CWX and SBC cluster with other parasites from Africa.

namics in order to monitor for the potential emergence of artemisinin resistance in Africa.

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Disclosure forms provided by the authors are available with the full text of this letter at NEJM.org.

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Lu F, Culleton R, Zhang M, et al. Emergence of indigenous artemisinin-resistant *Plasmodium falciparum* in Africa. N Engl J Med. DOI: 10.1056/NEJMc1612765

SUPPLEMENTARY APPENDIX

Title

Emergence of Indigenous Artemisinin Resistant *Plasmodium falciparum* in Africa

Table of Contents

1. SUPPLEMENTARY METHODS

Study sites and participants

This study was carried out in Jiangsu Province, China, where only imported malaria cases have been reported in recent years. Blood samples were collected in 2013 from malaria patients with acute, uncomplicated *P. falciparum* infections at local hospitals in Jiangsu Province. Provision of informed consent from the participants was approved by the Institutional Review Board (IRB00004221) of Jiangsu Institute of Parasitic Diseases, Wuxi, China. All other patients provided written informed consent. Blood samples were collected from all participants before they received antimalarial drug treatment. For therapeutic efficacy studies and *in vitro* adaptation of parasites, the main exclusion criteria were receipt of an artemisinin antimalarial drug in the previous week or those patients with danger signs.

Drug therapy and follow-up

The patients were given a full course of oral Duo-Cotecxin (containing 40 mg dihydroartemisinin and 320 mg piperaquine per tablet; 8 tablets totally; Holleypharma, China). With the first day of treatment being day 0, body temperature and thick/thin blood smears for parasite counts were obtained on days 0, 1, 2, 3, 7, 14, 21, and 28 for Duo-Cotecxin treated patients. Parasites were counted against white blood cells (WBCs).

Ring stage survival assay and *kelch13* gene assessment

After culture-adapted parasites, the ring-stage survival assay ($RSA_{0\text{-}3h}$) was performed as described previously (1). The $RSA_{0\text{-}3h}$ was performed independently in two laboratories in two separate research institutes. The first RSA was performed in Tongi University, the results of which are given in **Figure 1B** in the main manuscript, the second experiment was performed at the JIPD, and the data is given in **Figure S1**. Genomic DNA of *P. falciparum* isolates was used for PCR amplification with gene-specific primers to amplify the C-terminal nucleotide of the *P. falciparum kelch13* gene. Pfk13-F3 5'-AGTGGAAGACATCATGTAACCAG-3' and PfK13-R1 5'-CCAAGCTGCCATTCATTTGT-3' were used as primers for amplification of the target fragment. Double-strand capillary sequencing of PCR products was performed on an Applied Biosystems 3730 sequence analyser with both the sense and antisense primers, respectively. The deduced amino acid sequences were aligned and analyzed with the Lasergene® software (DNASTAR, Madison, WI).

Whole genome sequencing and analysis

Genomic DNA was extracted from culture-adapted parasites, and then sequenced with an Illumina MiSeq. Paired sequence reads of length 300 base pairs were aligned to the Pf3D7 v3 reference sequence (from GeneDB) using *bwa*. Non-uniquely mapped reads and read duplicates were removed. SNPs were inferred using *samtools mpileup* and only SNPs with quality, QUAL >70 and Depth, d >100 were retained. From these datasets, SNP/REF calls were extracted from only the 26,918 positions that differentiate 245 *Plasmodium* samples into their respective geographical origins (2, 3). Within the 26,918 positions, CWX had a total of 559 SNPs predicted by samtools mpileup, pipeline. The SBC had a total of 548 SNPs predicted by samtools mpileup. All

the samples were merged and their distances inferred and visualized using SVAMP (2) in order to determine the geographic origin of CWX and SBC isolates. Geographic origin was also independently confirmed based on a 23-SNP barcode within the apicoplast and mitochondrial genomes (**Figure S2**) (4).

2. ACCESSION NUMBERS

a) Sanger capillary sequences of the K13 mutation in CWX and SBC isolates have been submitted to EMBL-Bank and can be accessed through the Study accession: PRJEB18721.

b) Whole genome sequence files for CWX and SBC isolates.

Study accession: PRJEB18721

Sample accession	**Secondary accession**	**Sample unique name**	
ERS1485661	SAMEA35659918	Pfalc_CWX	
ERS1485662	SAMEA35660668	Pfalc_SBC	
Study accession	**Sample accession**	**Experiment accession**	**Run accession**
PRJEB18721	ERS1485661	ERX1833378	ERR1767828
PRJEB18721	ERS1485662	ERX1833379	ERR1767829

3. SUPPLEMENTARY FIGURES

S1

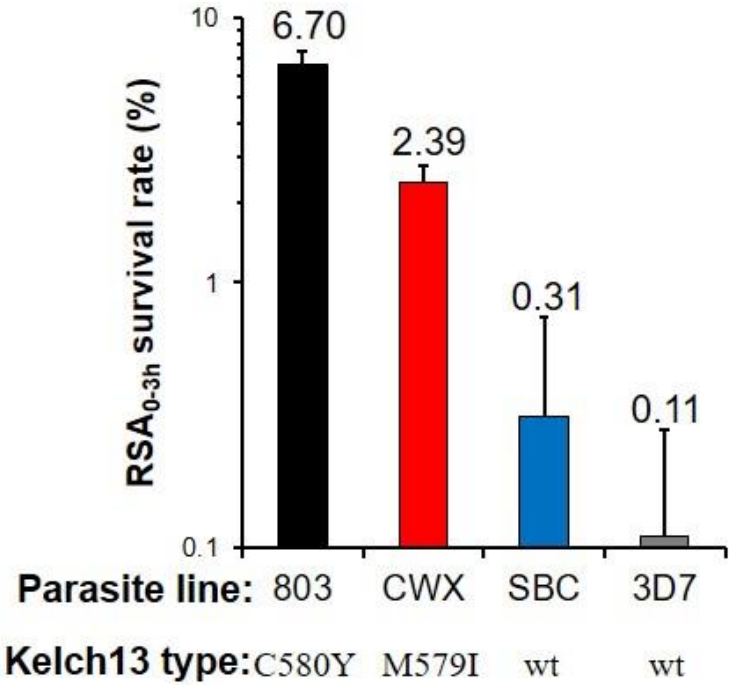

Figure S1. Survival rate in the Ring-stage Survival Assay ($RSA_{0\text{-}3h}$) for isolates with or without the M579I mutation. 3D7, wild-type (wt) strain; 803, artemisinin-resistant parasite line with C580Y kelch 13 mutation.

S2

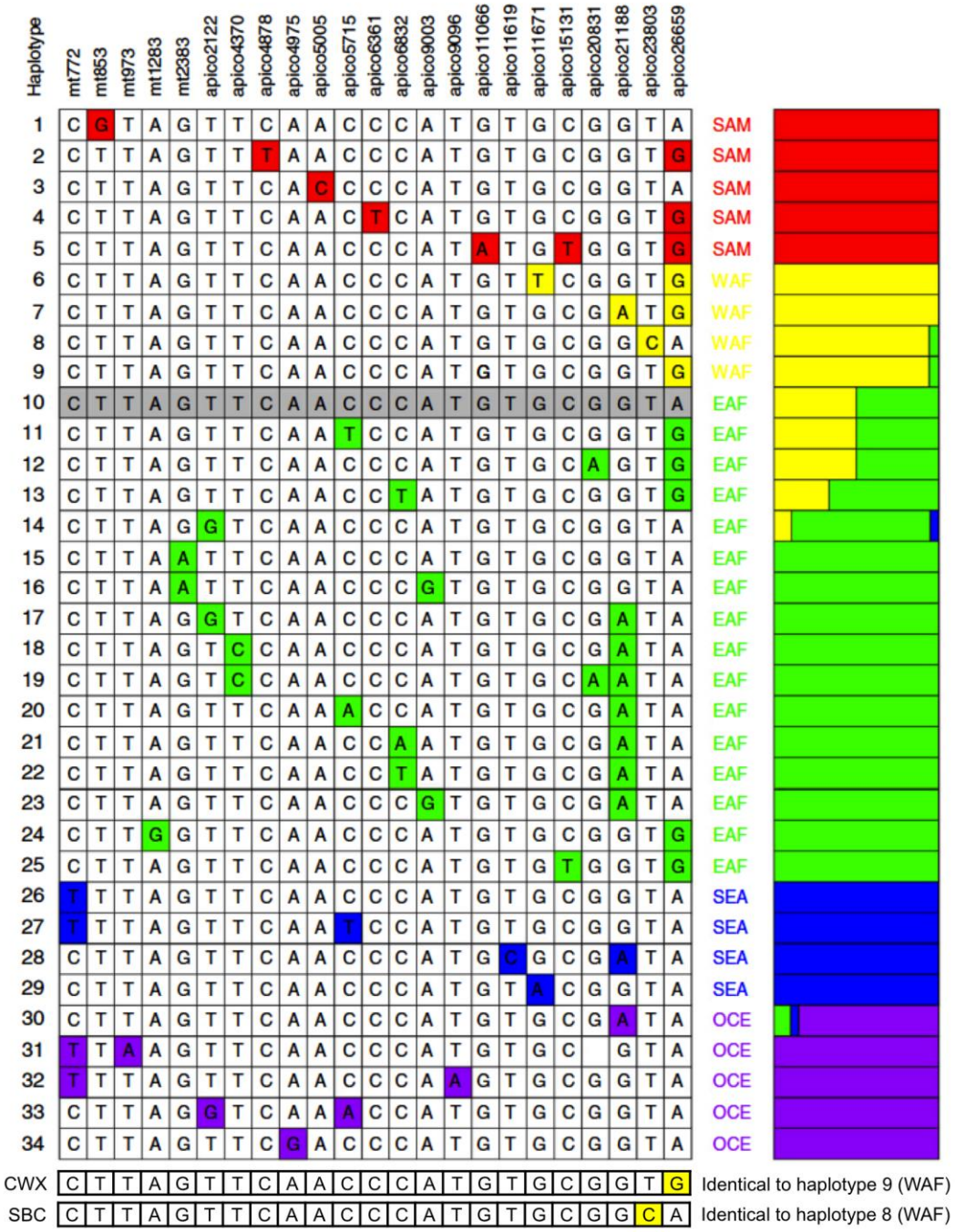

Figure S2. 23-SNP Barcode Adapted from (4) and based on SNPs present in the mitochondrial and apicoplast genomes. SAM, South America; WAF, West Africa; EAF, East Africa; SEA, Southeast Asia; OCE, Oceania. Bottom two panels show the CWX and SBC mapped reads at their SNP positions in the mitochondrial and apicoplast genomes. CWX and SBC belong to haplotype 9 and 8 respectively, both of West African origin.

S3

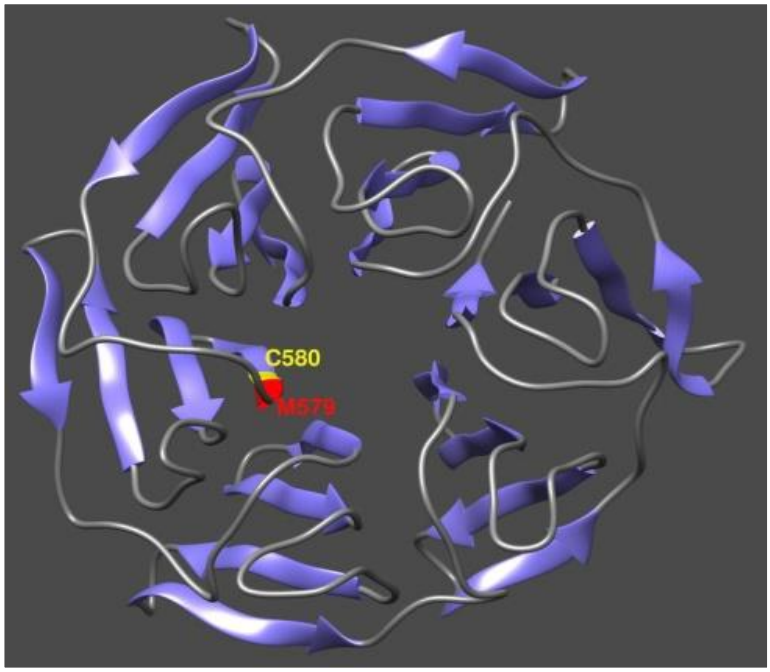

Figure S3. Distribution of the M579 and C580 in the predicted 3D model of the K13 propeller domain.

4. SUPPLEMENTARY REFERENCES

(1) Witkowski B, Amaratunga C, Khim N, *et al*. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: *in-vitro* and *ex-vivo* drug-response studies. Lancet Infect Dis, 2013;13:1043-9.
(2) Naeem R, Hidayah L, Preston MD, *et al*. SVAMP: sequence variation analysis, maps and phylogeny. Bioinformatics, 2014; 30(15): 2227-9.
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(4) Preston MD, Campino S, Assefa SA, *et al.* A barcode of organellar genome polymorphisms identifies the geographic origin of *Plasmodium falciparum* strains. Nat Commun 2014; 5: 4052.