# Emergence of Indigenous Artemisinin-Resistant Plasmodium falciparum in Africa

# 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

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# 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**

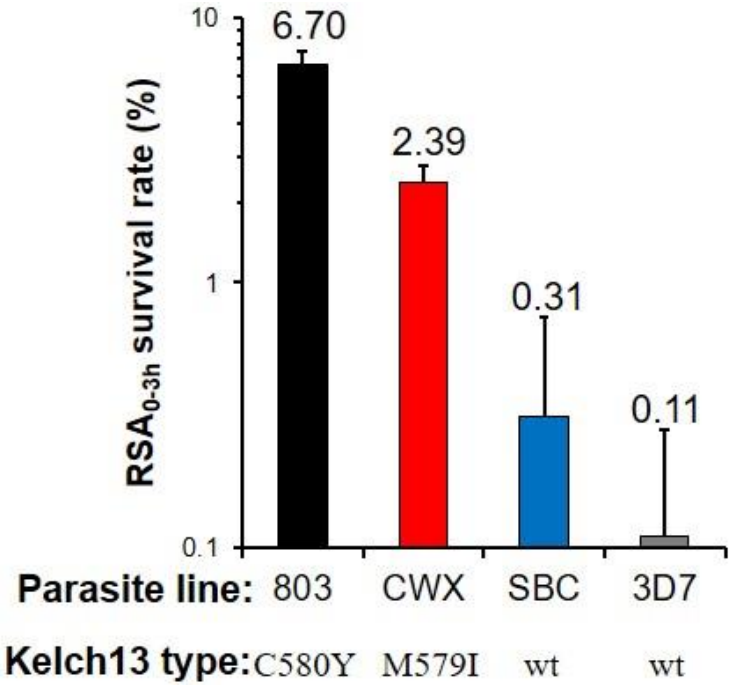


**Figure S1. Survival rate in the Ring-stage Survival Assay ($RSA_{0\text{-}3h}$) for isolates with or without the M579I mutation.** 3D7, wild-type (wt) strain; 803, artemisinin-resistant parasite line with C580Y kelch 13 mutation.

**S2**

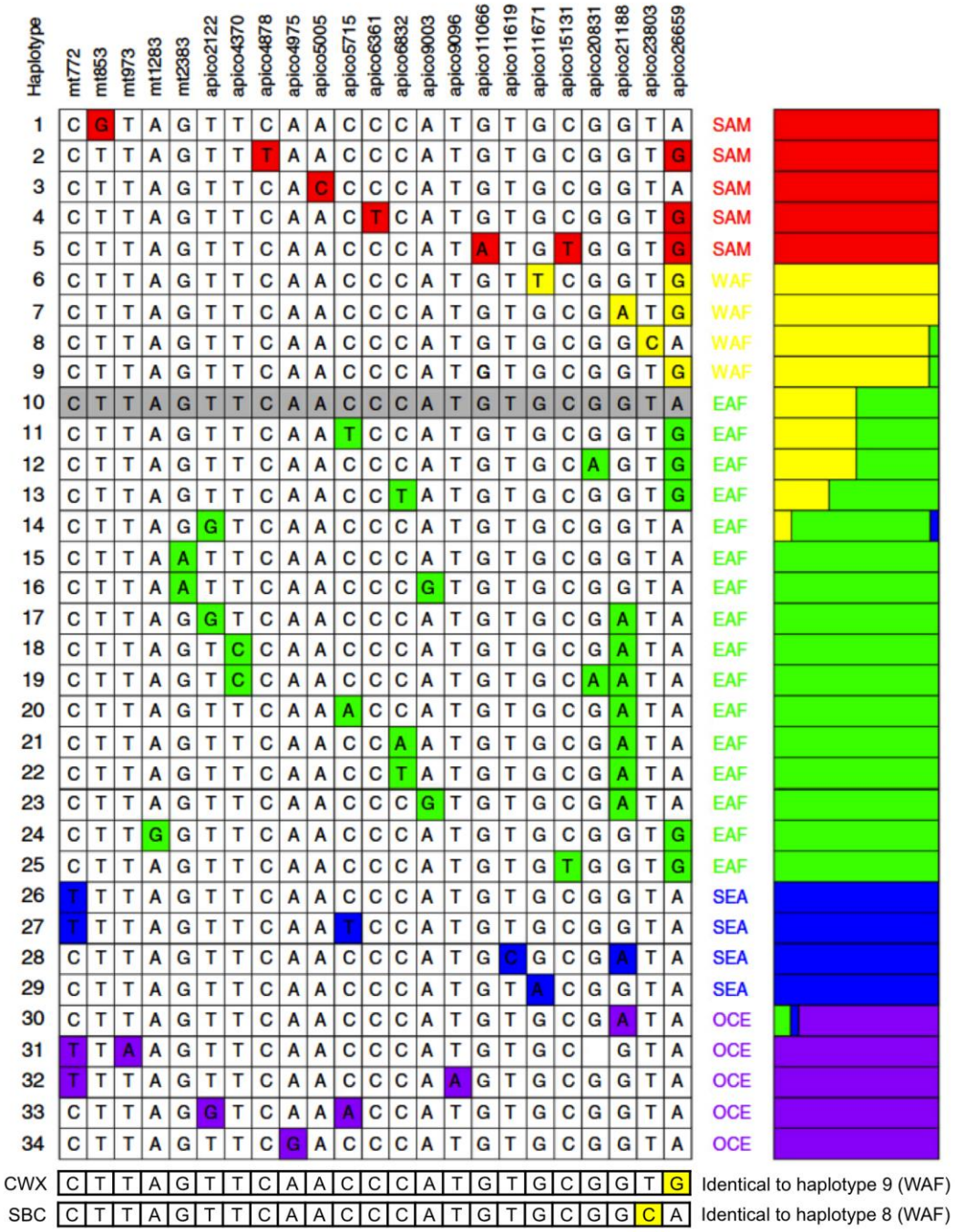


**Figure S2. 23-SNP Barcode Adapted from (4) and based on SNPs present in the mitochondrial and apicoplast genomes.** SAM, South America; WAF, West Africa; EAF, East Africa; SEA, Southeast Asia; OCE, Oceania. Bottom two panels show the CWX and SBC mapped reads at their SNP positions in the mitochondrial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

**S3**

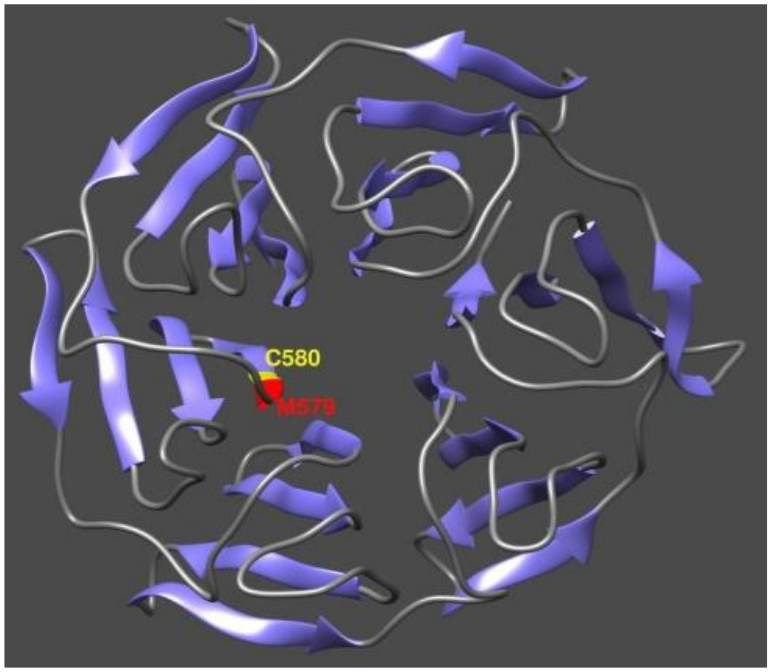


**Figure S3. Distribution of the M579 and C580 in the predicted 3D model of the K13 propeller domain.**

## 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.
(3) Manske M, et al. Analysis of *Plasmodium falciparum* diversity in natural infections by deep sequencing. Nature. 2012;487:375–379
(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.