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Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance

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Abstract

Artemisinin resistance in *Plasmodium falciparum* threatens global efforts to control and eliminate malaria. Polymorphisms in the kelch domain-carrying protein K13 are associated with artemisinin resistance, but the underlying molecular mechanisms are unknown. We analyzed the in vivo transcriptomes of 1043 *P. falciparum* isolates from patients with acute malaria and found that artemisinin resistance is associated with increased expression of unfolded protein response (UPR)

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pathways involving the major PROSC and TRiC chaperone complexes. Artemisinin-resistant parasites also exhibit decelerated progression through the first part of the asexual intraerythrocytic development cycle. These findings suggest that artemisinin-resistant parasites remain in a state of decelerated development at the young ring stage, whereas their up-regulated UPR pathways mitigate protein damage caused by artemisinin. The expression profiles of UPR-related genes also associate with the geographical origin of parasite isolates, further suggesting their role in emerging artemisinin resistance in the Greater Mekong Subregion.

Artemisinin resistance in *Plasmodium falciparum* is spreading rapidly throughout Southeast Asia. Since it was first detected 7 years ago in Pailin, western Cambodia (1), artemisinin resistance has become prevalent in other Cambodian provinces (2, 3), Thailand-Myanmar border areas (4, 5), and southern Vietnam (4, 6) and is emerging in southern Laos and central Myanmar (4). Artemisinin resistance threatens the efficacy of all artemisinin-based combination therapies (ACTs) (7), as its spread from Southeast Asia to Africa would derail malaria control and elimination efforts worldwide. The parasite clearance half-life, derived from the log-linear decline in parasitemia over time, is now established as the best pharmacodynamics measure of *P. falciparum* sensitivity to artemisinins (8, 9). Resistance is associated with a parasite clearance half-life of >5 hours (4) and is prevalent where standard 3-day courses of ACTs are now failing (10, 11).

Artemisinin resistance is a heritable genetic trait (12) linked with three loci on chromosomes 10, 13, and 14 (13, 14) and nonsynonymous single-nucleotide polymorphisms (SNPs) in the propeller domain of a *kelch* gene on chromosome 13 (*PF3D7_1343700*) (3). These “K13-propeller” polymorphisms are currently the best predictors of artemisinin resistance in mainland Southeast Asia, with the most common mutation, Cys⁵⁸⁰ → Tyr, approaching genetic fixation in western Cambodia (3). A large multicenter clinical investigation by the Tracking Resistance to Artemisinins Collaboration (TRAC) has shown that the artemisinin resistance phenotype that is currently spreading through the Greater Mekong Subregion (GMS) is associated with K13 polymorphisms (4). Although K13-propeller mutations are highly predictive of resistance, very little is known about the molecular mechanisms that render *P. falciparum* insensitive to artemisinins. These drugs eliminate parasitemias more rapidly than other antimalarials because they accelerate the clearance of young ring-stage parasites by the spleen (9). Several lines of evidence indicate that resistance affects the early ring stage of the parasite’s intraerythrocytic developmental cycle (8, 15, 16). Previously, we suggested that artemisinin resistance may be underlined by broad changes of the parasite’s transcriptional program that alter its physiology (17).

Here, we carried out transcriptome analyses of 1043 clinical *P. falciparum* isolates to identify the transcriptionally underlined mechanisms that mediate artemisinin resistance. The parasite samples were obtained directly from the peripheral blood of patients with acute falciparum malaria enrolled into the TRAC study in 2011–2012 (4). This study was approved by the relevant local ethics committees and the Oxford Tropical Research Ethics Committee, and all adult patients or the parents of children gave written informed consent. The samples originated from 13 malaria-endemic regions of Southeast Asia and Africa. These include regions where artemisinin resistance is well established (Pursat and Pailin,

Cambodia; Mae Sot, Thailand; and Binh Phuoc, Vietnam), emerging (Preah Vihear, Cambodia, and Shwe Kyin, Myanmar), or currently not detected [Ramu, Bangladesh, and Kinshasa, Democratic Republic of Congo (DR Congo)] (Fig. 1A and table S1). By measuring the overall abundance of mRNA transcripts for 4978 of the ~5591 genes in the *P. falciparum* genome, we identified three types of transcription profiles among the parasite isolates (GrpA, GrpB, and GrpC) (Fig. 1B). Projecting the field isolate transcription profiles onto the in vitro reference (17) revealed that the three transcriptional groups are mainly defined by parasite developmental stage ($P < 2 \times 10^{-16}$) (fig. S1). This analysis showed that GrpA consisted mostly of early ring-stage parasites [8 to 10 hours post-invasion (hpi)], and GrpB consisted predominantly of middle and late ring-stage parasites (10 to 20 hpi). GrpC also consisted predominantly of early ring-stage parasites (8 to 10 hpi); however, in these isolates the density of gametocytemia was significantly higher than in the two other groups (fig. S1).

The three transcriptional patterns were significantly associated with several clinical and parasitological parameters (fig. S1). Artemisinin-resistant parasites were unevenly distributed among the three groups. GrpA was associated with a longer parasite clearance half-life (mean 4.16 hours) relative to GrpB (3.43 hours). However, GrpB contained a higher proportion of parasites with a half-life of <5 hours (fig. S1), largely due to its overrepresentation of parasites from areas of Bangladesh and DR Congo that are apparently free of artemisinin resistance (Fig. 1B). The transcriptional profiles of GrpA and GrpC parasites were found to be associated with parasite clearance times elapsed time until parasitemia is reduced by 50% (PC50) and 90% (PC90) of its initial admission value; $P < 2 \times 10^{-16}$] and the duration of the lag phase of the parasite clearance curve (9) ($P = 4 \times 10^{-15}$) (fig. S1). The lag phase represents the period immediately after administration of the first artemisinin dose when the initial parasite density remains constant before commencing a first-order decline. The extended lag phases in GrpA and GrpC patients may be caused by higher densities of early ring-stage parasites in the peripheral blood, resulting from recent schizont rupture. This is suggested by the fact that these patients presented to clinic with higher temperatures, likely caused by pyrogenic factors released from rupturing schizonts into the bloodstream (18).

GrpB patients were significantly less likely to present to clinic in the evening hours than GrpA and GrpC patients ($P = 8 \times 10^{-11}$) (fig. S1). This suggests that in areas where primary care is readily available, patients tend to seek medical care for fever shortly after synchronous schizont rupture anytime throughout the day, including the evening hours. Patients with high fever at clinical presentation may thus have a higher chance of developing a longer lag phase that prolongs the overall clearance time, regardless of their artemisinin resistance status.

Next, we focused on the uniformly young ring-stage parasites in GrpA ($n = 549$) and carried out linear regression between mRNA levels and parasite clearance half-life (Fig. 2A and fig. S2A). We found that 487 (9.6%) and 511 (10.1%) genes were significantly up-regulated and down-regulated, respectively, in association with the clearance half-life and thus artemisinin resistance [$P < 0.01$, false discovery rate (FDR) < 0.05] (fig. S2A and table S2). In particular, artemisinin resistance appears to be associated with up-regulation of genes

involved in protein metabolism, such as endoplasmic reticulum retention sequences, unfolded protein binding, protein folding, protein export, posttranslational translocation, signal recognition particle (SRP), proteasome, and phagosome ($P < 0.05$, FDR < 0.25) (fig. S2A). A nearly identical set of functional pathways was found to be up-regulated in GrpA parasites with K13-propeller mutations (fig. S2B). There was no correlation between K13 mRNA levels and artemisinin resistance (Fig. 2A). Most of the up-regulated pathways are known to participate in the overall unfolded protein response (UPR) in other eukaryotic species. Hence, these results indicate that an up-regulated UPR may be a major mediator of artemisinin resistance in *P. falciparum* and is caused by single K13-propeller mutations. Similar functional representation of artemisinin resistance- and K13-linked up-regulated genes was found in GrpC, although the statistical significance was reduced because of greater gene expression variability caused by varying levels of gametocytes (fig. S2C).

Among the genes with the highest correlation with artemisinin resistance are those involved in protein folding and repair, including cyclophilin19B ($P < 2 \times 10^{-16}$), dolichyl-phosphate-mannose protein mannosyltransferase ($P = 3 \times 10^{-13}$), endoplasmic reticulum-resident calcium binding protein ($P = 0.004$), BiP/grp78 ($P = 0.004$), and a protein disulfide isomerase ($P = 10^{-6}$) (Fig. 2A and table S2). Consequently, we used previous experimental and bioinformatics data (19, 20) to assemble a network of molecular chaperones consisting of two major protein complexes and other chaperones in the *P. falciparum* cell (fig. S3). These include subunits of two putative chaperonin complexes, *Plasmodium* reactive oxidative stress complex (PROSC) (table S3) and TCP-1 ring complex (TRiC), that participate in the UPR of other species (21, 22). The mRNA levels for the PROSC and TRiC subunits correlated not only with each other but also with artemisinin resistance (Fig. 2B and fig. S3). Moreover, these mRNA levels inversely correlated with those of other chaperones such as Pf14-3-3, cyclophilin19A, and PF3D7_1024800 (protein of unknown function, UF), all of which are components of the chaperone network in *Plasmodium* (Fig. 2B). We also observed strong correlation between parasite clearance half-life and the relative expression ratio of PROSC and TRiC (average mRNA level) versus Pf14-3-3 ($r = 0.39$, $P < 2.2 \times 10^{-16}$) (Fig. 2B). This correlation is significantly stronger ($P = 0.01$) than that between half-life and expression of any individual gene within or outside the chaperone network (e.g., maximum $r = 0.35$ for *cyclophilin19B*), indicating that artemisinin resistance is associated with the coordinated transcription of multiple chaperone partners.

In GrpA, artemisinin resistance and K13-propeller polymorphisms also associated with down-regulation of genes involved in DNA replication (Fig. 2A and fig. S2, A and B). Although DNA replication occurs later than 8 to 10 hpi (23), this down-regulation may represent a developmental stalling of resistant parasites in which transcription of the DNA replication genes is “halted.” Accordingly, the age (hpi) of GrpB parasites correlated inversely with clearance half-life ($r = -0.39$, $P < 0.001$) (Fig. 2C). To analyze this relationship further, we investigated the ex vivo transcriptomes of 9 sensitive and 10 resistant parasites (median half-lives, 4.99 and 7.35 hours) from Pailin, sampled from patients at 0 hours and during ex vivo cultivation at 8, 16, 24, 32, and 40 hours (fig. S4). Estimating the parasite age at each time point, we observed that the resistant isolates progressed from rings to trophozoites to early schizonts at a slower rate than sensitive isolates ($P < 0.05$) (Fig. 2D). This deceleration in blood-stage development occurred during the first 32 hours. At 40

hours, presumably at the end of DNA replication, the resistant isolates reached the same stage of development as their sensitive counterparts, thus completing their intraerythrocytic life cycle at the same time.

Up-regulation of the UPR may enable resistant parasites to better withstand the deleterious effect of Fe²⁺-activated artemisinins, which are believed to damage intracellular structures via direct alkylation and oxidation (24). An up-regulated UPR is likely to increase the capacity of parasites to quickly repair or degrade proteins (or other cellular components) that are damaged by brief artemisinin exposures in patients. The two chaperonin complexes, PROSC and TRiC, are likely to play a pivotal role in this process, as they do in the UPR of other eukaryotes (21, 22). Upregulation of the UPR is also consistent with the putative function of kelch proteins as negative regulators of signal transduction, triggering the UPR upon protein damage (25). In resistant parasites, mutant K13 proteins may lose their ability to negatively regulate this pathway, leading to its constitutive activation at baseline, increased activation upon artemisinin exposure, or both. Finally, inverse relationships between members of the *Plasmodium* chaperone network—especially that between cyclophilin19B and cyclophilin19A—may reflect specific adjustments in protein-folding activities in the *Plasmodium* cytoplasm. These proteins have been suggested to function as rate-limiting steps in protein folding in other eukaryotes, and their differential expression is also thought to reflect readjustments of the UPR (26).

Intriguingly, UPR-related genes, including those encoding PROSC and TRiC, show high variability among the GMS field sites. In investigating the geographical distribution of transcription profiles, we first noted two distinct clades of parasites based on Pearson correlation of the individual transcriptome in all three transcriptional groups (fig. S5). Whereas one clade contained mainly parasites from DR Congo and Bangladesh, the other was strongly enriched for parasites from the GMS. In the GMS clade for GrpA, we found 659 differentially expressed genes that define parasite populations in distinct areas. Among these, there was a high enrichment of biological processes associated with artemisinin resistance, including protein turnover and oxidative damage response (Fig. 3). The transcriptional variability of these genes is particularly evident in Pailin, where *P. falciparum* resistance to several classes of drugs (quinolones, antifolates, and artemisinins) has originated (7, 27) and where the parasite population exhibits a distinct genetic structure (28). This suggests that the propensity of Pailin parasites to develop drug resistance may be maintained by differential expression of genes involved in response to oxidative or other types of stress. It seems reasonable to speculate that this transcriptional heterogeneity is driven by frequent exposure to oxidative stress due to the high prevalence of hemoglobinopathies in this region (2).

The decelerated progression of ring-stage development is also consistent with the artemisinin mode of action and putative mechanism of resistance. Ring-stage arrest has been associated with lower levels of endocytosis and hemoglobin digestion, which decreases free heme-mediated activation of artemisinin and enhances the parasite's tolerance to this drug (29). This phenotype may also relate to *P. falciparum* dormancy, which has been suggested to enable the parasite to withstand the drug pressure and resume growth in favorable conditions (16, 30). Up-regulation of the UPR may be directly linked with decelerated

parasite development as a mechanism to connect the repair of damaged proteins to cell cycle progression, as observed in other eukaryotes (25). Investigating these two phenomena in *P. falciparum* may improve our understanding of the molecular basis of artemisinin resistance and facilitate the development of new strategies to counter the threat it poses to global malaria control and elimination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Artemisinin response and transcription profiles of *P. falciparum* isolates from patients with acute malaria.

(A) The pie chart and boxplots show the distribution of parasite isolates and parasite clearance half-lives, respectively, according to field site. (B) The heat map shows mean-centered relative expression levels (log₂ ratios) grouped by *k*-means clustering: GrpA (*n* = 549), GrpB (*n* = 272), and GrpC (*n* = 222). Corresponding gametocyte densities per microliter (pink squares) (4), relative expression levels of gametocyte-specific genes (blue bars), and parasite age (hpi) are shown above; distributions of geographical origins according to parasite group (colored bars) are shown below. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Fig. 2. Transcriptional features associated with artemisinin resistance.

(A) Scatterplots depict examples of the linear regression analysis between mRNA levels and parasite clearance half-life for two genes with positive, negative, or no correlation. The middle left panel shows the correlation for the K13 artemisinin resistance marker. (B) The heatmap shows the Pearson correlation coefficients of pairwise comparisons of the 13 differentially expressed genes within the chaperone network. The scatterplot shows the ratio of the average expression of *Plasmodium* PROSC and TRiC to that of the three down-regulated genes (14-3-3, Cyp19A, and UF). (C) Distribution of parasite age (hpi) and clearance half-life for 272 GrpB isolates. (D) Boxplots show the age (hpi) of parasites over 40 hours in ex vivo culture, stratified by their artemisinin resistance status (* $P < 0.05$).

GrpA parasites in the Greater Mekong Subregion

Fig. 3. Population transcriptomics analysis of *P. falciparum* isolates.

Cladogram of the 348 GrpA isolates from the Greater Mekong Subregion, clustered by the Euclidean distance metric of 659 marker genes. The geographic origin of each isolate is indicated by the color key in the map. Stars indicate significant overrepresentation of isolates from a particular field site within the corresponding node ($P < 0.05$, hypergeometric test). Histograms of functional enrichment analysis show overrepresented and differentially expressed pathways in the demarcated Pailin clade ($P < 0.05$, hypergeometric test). Bars represent the proportion of Pailin clade-specific genes (light gray) relative to the total number of genes (dark gray).