Parallel Evolution of Adaptive Mutations in *Plasmodium falciparum* Mitochondrial DNA During Atovaquone-Proguanil Treatment

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Here we provide direct evidence that two adaptive nucleotide changes in the cytochrome b gene (*pfcytb*) each occurred repeatedly in independent *Plasmodium falciparum* lineages exposed to the antimalarial drug atovaquone-proguanil (AP). We analyzed the history of 7 AP resistance alleles from clinical isolates by sequencing the mitochondrial (mt) genome that encodes the *pfcytb* gene and found that a distinct mt haplotype was associated with each AP resistance allele. By comparing mt sequences and microsatellite genotypes of the isolates both before treatment initiation and at the day of failure for each uncured patient, we observed that the AP resistance alleles occurred and spread within the patients. These data demonstrate that identical AP resistance alleles have multiple independent origins and provide an example of parallel evolution driven by drug treatment selection in *P. falciparum.*

**Introduction**

In the classical model of molecular adaptation, a beneficial allele enters a population following a unique mutational event and then increases in frequency under directional selection. In parallel evolution, the same adaptive change at the same site occurs repeatedly in independent lineages exposed to the same selection pressures (Wichman et al. 2000). Whereas parallel evolution can be easily detected in experimental replicates by survey of DNA sequences during the experiment (Cunningham et al. 1997; Wichman et al. 2000), its detection in nature is more difficult because it mostly relies on phylogenetic inferences (Zhang and Kumar 1997; Colosimo et al., 2005). Evolution of microbial populations under strong selection provides a useful model for analyzing parallel adaptation, because population sizes are large and generation times are short (Boucher et al. 1992; Wichman et al. 1999).

Here we provide direct evidence that two adaptive nucleotide changes in the cytochrome b gene of *Plasmodium falciparum* (*pfcytb*) each occurred repeatedly in independent natural lineages exposed to the antimalarial drug atovaquone-proguanil (AP). Resistance of *P. falciparum* to atovaquone is conferred by single nucleotide polymorphisms (SNP) in the *pfcytb* gene located on the 6-kb mitochondrial (mt) genome (Korsinczky et al., 2000; Kessl et al. 2005). Once atovaquone resistance is present, the synergy of the partner drug proguanil is markedly reduced (Fivelman, Adagu, and Warhurst 2004) and can lead to treatment failure (Musset et al. 2006).

To address the evolutionary origins of AP resistance (APR) alleles, we analyzed sequence variation of the mt genome within 7 resistant isolates (6 African and 1 Thai) collected at the day of atovaquone (n = 1) or AP (n = 6) treatment failure (Dfail isolates; Supplementary Material Online). All the Dfail isolates carried either the a4296g (n = 2) or the a4296c (n = 5) SNP associated with APR, replacing tyrosine 268 with a cysteine (Y268C) or a serine (Y268S), respectively, in the *pfcytb* gene, (Musset et al. 2006). For each Dfail isolate, a distinct mt haplotype could be defined with 11 variable sites, including the position 4296 (fig. 1). As a control, 28 pretreatment isolates from patients successfully cured by AP (D0control isolates) were selected to match the year and the African country of origin of the Dfail isolates. All carried the wild type *pfcytb* allele. Two susceptible mt haplotypes, S1 and S2, largely predominated and 6 haplotypes were found only once, a pattern of mt genetic diversity consistent with a previous study (Joy et al. 2003). The mean number of pairwise nucleotide differences at 5,819 aligned nucleotide sites was similar between the D0control and the Dfail isolate groups (2.43 ± 0.99 SE and 2.57 ± 0.88 SE). These data do not support the hypothesis of a recent single origin, where all the APR mt sequences would be expected to be nearly identical. Rather, the distribution of resistant haplotypes in the median-joining network suggests multiple independent origins of the selected APR alleles (fig. 2).

D0 parasites isolated before treatment were available for 6 of the 7 failure cases. Remarkably, the APR allele was not detected in any of the D0 corresponding isolates (Musset et al. 2006), even using a sensitive assay that detects one APR allele in the presence of up to 10^5 copies of the wild type susceptible allele (fig. S1 in supplementary data). This indicates that the APR allele was either absent or at a very low frequency in isolates before treatment onset. We then investigated the genetic diversity within each pair of D0 and Dfail isolates from the 6 patients. Remarkably, with the exception of the polymorphisms at the 4296 site associated with APR, the mt genetic pattern was identical within each pair of isolates (except for patient 2, for which we failed to amplify part of the D0 mt genome; fig. 3). Identical genetic patterns were also observed at 5 polymorphic nuclear microsatellite loci within pairs for 4 patients (fig. 3), indicating genetically identical infections before and after treatment. For the 2 remaining patients, several coinfecting alleles were detected at more than 1 locus in D0 isolates. When only predominant alleles were scored, genetic identity was observed. Because each of the 5 African D0 haplotypes was unique (defined by the mt genome and the 5 microsatellite loci) among D0control isolates sample (fig. S2 in supplementary data), it is exceedingly unlikely that, in 5 independent patients, a susceptible and a resistant allele that share an identical haplotype were transmitted by chance to the same host. Taken together, the data presented...
here show that the 2 adaptive nucleotide changes in the \textit{pfcytb} gene, a4296c and a4296g, each occurred repeatedly in different lineages from varied geographic regions. Further, we show that these APR alleles occurred and spread within at least 6 of the 7 patients under atovaquone or AP treatment selection.

Parallel evolution of APR alleles was suspected from the epidemiology of atovaquone resistance (Looareesuwan et al. 1996; Schwartz, Bujanover, and Kain 2003) and is formally demonstrated here. This situation contrasts with the very few origins and extensive spread of the \textit{pfcrt} and \textit{pfdhfr} mutant alleles that determine chloroquine and pyrimethamine resistance, respectively (Cortese et al. 2002; Wootton et al. 2002; Nair et al. 2003; Roper et al. 2003; Ariey et al. 2006). These different patterns in drug resistance evolution might be primarily explained by the different dynamics in the acquisition of the resistance mechanisms. Resistance to chloroquine and pyrimethamine arose by sequential additions of SNPs at the \textit{pfcrt} and \textit{pfdhfr} loci, respectively (Anderson and Roper 2005). It is extremely unlikely that these complex resistant haplotypes occurred during replications within a patient (Hastings 2004). In contrast, atovaquone resistance is conferred by one SNP that has a major effect on susceptibility to AP (Fivelman, Adagu, and Warhurst 2004; Musset et al. 2006). Theory predicts that the same beneficial allele might enter a population several times by recurrent mutation when the population-level mutation parameter 2Ne\(l\) > 0.01, where Ne is the effective population size and \(l\) the per generation rate of mutation to the beneficial allele (Pennings...
With an estimated \( l \) for the atovaquone resistance allele of \( 10^{-5} \) to \( 10^{-8} \) (Gassis and Rathod 1996) and an estimated \( N_e \) of \( 10^5 \) in the African \( P. falciparum \) population (Joy et al. 2003), \( 2N_e l \) is included between 0.002 and 2. This is consistent with our observation of multiple origins of the selected allele. A recent study demonstrated recurrent amplification of the \( pfmdr1 \) gene during the evolution of multidrug resistance in malaria parasites from Southeast (SE) Asia (Nair et al. 2007).

Although mutation rates in eukaryotes are generally lower for point mutations than for copy number change (Inoue and Lupski 2002), our data support that parallel evolution is also likely to occur when adaptation is conferred by simple genetic change like one SNP. However, in contrast with the evolution of \( pfmdr1 \) gene copy number in SE Asia, the evolution of APR alleles occurred in the situation where AP pressure on \( P. falciparum \) populations is not at a high level nor is it sustained, mainly because the high cost of AP precludes its widespread use. As observed by Nair et al. (2007), it is expected that the different haplotypes linked to each independent copy of the beneficial allele will be retained and will spread in the population under selection.

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