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Genetic Analysis of Primaquine Tolerance in a Patient with Relapsing Vivax Malaria

Technical Appendix

Supplemental Materials and Methods

Ethics Statement

This study was approved by the Health Research Ethics Board of the University of Alberta. The patient provided written informed consent. The consent form states in English that blood samples collected from the patient may be used to genetically characterize the parasites and *CYP* genes from the patient, and that samples may be shared with other researchers for the purpose of investigating the basis of primaquine (PQ) resistance.

Sample Collection

Whole blood samples from the first (EAC01) and second (EAC02) malaria infections were collected in EDTA tubes and stored at –20°C. For the third infection (EAC03), the red blood cell pellet was stored at –80°C. Plasma was collected on day 12 of PQ treatment.

Genotyping of Cytochrome P450 (CYP) Alleles

Human DNA extraction was performed by using the PSS GC12 instrument (Precision System Science Co. Ltd) and eluted into a 100-µL volume. Four alleles were selected for genotyping analysis based on analysis of the literature taking into account the country of origin of the patient and alleles hypothesized to play a role in drug metabolism: *CYP1A2*1C* (Dandara, Basvi, Bapiro, Sayi, & Hasler, 2004), *CYP2B6*6* (Penzak et al., 2007; Wang & Tompkins, 2008), *CYP3A4*1B* (Ferreira et al., 2008; Garsa, McLeod, & Marsh, 2005; Kedmi, Maayan, Cohen, Hauzi, & Rund, 2007) and *CYP2D6*4* (Xie, Kim, Wood, & Stein, 2001).

Regions within the *CYP1A2* and *CYP3A4* genes were amplified by PCR followed by DNA sequencing according to published methods (Nakajima et al., 1999; Paganotti et al., 2011). The *CYP2B6* allele was characterized by PCR-RFLP using the enzyme *BsrI* (Ebeshi, Bolaji, & Masimirembwa, 2011). For *CYP2D6*4*, PCR was performed with the primers: 5′-

CAAGAAGTCGCTGGAGCAGT-3′ (forward) and 5′-GAGGGTCGTCGTACTCGAAG-3′ (reverse) and the following PCR conditions: 94°C for 3 min, 30 cycles of 94°C for 30s, 60°C for 30s, 72°C for 30s, and a final extension step at 72°C for 10 min. PCR products were digested with *Eco*RII and *MvaI* analyzed by RFLP. These enzymes will digest the PCR product when there is a guanine at position 1934 but not when there is an adenine, which corresponds to the *CYP2D6*4* allele. The presence of the mutant allele was further confirmed by direct sequencing of the PCR product.

Parasite Genotyping

For the three parasite samples (EAC01-EAC03), bulk genomic DNA was isolated from frozen whole blood samples using the DNeasy Blood and Tissue kit (Qiagen) as per the manufacturer's instructions. Whole genome capture of parasite DNA for the three samples was performed as described previously (Bright et al., 2012). Captured DNA was paired-end sequenced on an Illumina HiSeq 2000 for 101 bp per read plus one 7-bp index read using Illumina v.3 chemistry. Data for each sample sequenced in this study is available in the NCBI Sequence Read Archive [SRA057904]. Fastq files obtained from sequencing were aligned to the Sal1 reference using BWA (v. 0.5.9) (Li & Durbin, 2009). Aligned reads were cleaned and analyzed by using Picard (v. 1.51) and GATK (v. 1.6+) (DePristo et al., 2011). 55,517 high confidence SNVs were genotyped in all three samples using GATK (Bright et al., submitted). Heterozygous SNV calls were excluded from downstream analysis.

Measurement of Drug Levels

Plasma concentrations of PQ and its major metabolite, carboxy-primaquine (CPQ), were measured with a newly developed stereoselective bioanalytical method (Hanpithakpong et al., manuscript in preparation). In summary, the method used solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry. Triplicates of 3 quality control samples were analyzed in the same batch to ensure that accuracy and precision were acceptable according to United States Food and Drug Administration (FDA) standards (FDA Guidance for Industry–Bioanalytical Method Validation). Measured drug concentrations were compared to simulated concentration-time profiles based on literature values for pharmacokinetic parameters in healthy male volunteers (Binh et al., 2009; Cuong et al., 2006; Elmes, Bennett, Abdalla, Carthew, & Edstein, 2006; Fletcher et al., 1981; Mihaly et al., 1985; Mihaly, Ward, Edwards,

Orme, & Breckenridge, 1984) and male patients with vivax malaria (Bangchang, Songsaeng, Thanavibul, Choroenlarp, & Karbwang, 1994.; Bhatia et al., 1986; Kim et al., 2004).

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