RED BLOOD CELL DUFFY ANTIGEN RECEPTOR FOR CHEMOKINES AND SUSCEPTIBILITY TO *PLASMODIUM VIVAX* INFECTION IN THAIS

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Abstract. *Plasmodium vivax* is the most prevalent malaria infection in Thailand. *P. vivax* uses Duffy Antigen Receptor for Chemokines (DARC) or Duffy antigen (Fy) as a receptor for entry into reticulocytes. Polymorphism of DARC exon 2 gene (FYA/FYB) in 40 *P. vivax*-infected subjects were investigated using nested PCR of blood samples spotted on filter paper collected during August 2013 to November 2013 from various malaria clinics in Thailand. Distribution of DARC genotypes was FYA 62.5%, FYB 20% and FYAB 17.5%, consistent with that of Hardy-Weinberg equation. Mutation G17A was found in both FYA and FYB alleles, resulting in Gyl48 and Asp48 of Fy^a and Fy^b antigen, respectively. Mean parasitemia among the three groups is not statistically different. To the best of our knowledge, this is the first such study in Thailand.

Keywords: *Plasmodium vivax*, Duffy Antigen Receptor for Chemokines (DARC), Fy^a, Fy^b

INTRODUCTION

Malaria remains one of the most important parasitic infections in tropical and sub-tropical regions of the world and Thailand is no exception. It has been estimated that in 2015 there were 214 million new clinical cases of malaria and 438,000 deaths (WHO, 2015). Among the five species of human malaria parasites, *Plasmodium vivax* remains the most prevalent because of re-emergence of the parasites, lack of vaccine and new drug treatment, and appearance of parasite resistant to antimalarials and insect vectors to insecticides (Zhou *et al*, 2005). The incidence of malaria in 2014 in Thailand was 23,749, with *P. vivax* accounting for 46.71%, *P. falciparum* 41.05% and *P. malariae* 0.13% (Bureau of Epidemiology, 2014).

In the human host blood circulation, *P. vivax* merozoites invade reticulocytes, enucleated precursors of erythrocytes, a process which depends primarily on the presence of red cell surface Duffy antigen receptor for chemokines (DARC or FY) (Gelpi and King, 1976; Miller *et al*, 1976). DARC is a glycosylated membrane protein encoded by the Duffy gene located on the long arm of chromosome 1 at q22-q23

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(Donahue *et al*, 1968; Collins *et al*, 1992). Although Duffy antigen-negative red cells are refractory to *P. vivax* infection, differences in red cell surface Fy^a and Fy^b antigens influence host susceptibility to *P. vivax* malaria (King *et al*, 2011). The basis of this polymorphism of FYA and FYB alleles is due to a single nucleotide variation at position 125: guanine (G) in FYA and adenine (A) is in FYB resulting in Gly42 in Fy(a+b-) or Fy^a antigen and Asp42 in Fy(a-b+) or Fy^b antigen (Chaudhuri *et al*, 1993).

This study investigated DARC gene polymorphisms of individuals infected with *P. vivax* and their relationship with susceptibility to vivax malaria. The data should provide insight into reticulocyte surface Fy antigens required for *P. vivax* binding and subsequent invasion, which may lead to development of invasion-blocking vaccines and/or discovery of interfering small molecules.

MATERIALS AND METHODS

Blood samples collection

Blood samples (20 μl) taken by finger prick from 40 Thai individuals, age >18 years, with symptomatic malaria were spotted individually onto filter paper (1.5 x 1.5 cm) (Whatman; Thomas Scientific, Swedesboro, NJ) and allowed to air dry. Subjects were diagnosed with vivax malaria by thin film examination and parasitemia determined [per 200 white blood cells (WBC)] at malaria clinics in Mae Sot, Tak, Mae Hong Son and Chantaburi Provinces, Thailand during August to November 2013. Subjects with positive thick-smear microscopy results were treated according to the type and stage of the malaria by Public Health official at malaria clinics as routine according to the current malaria therapy guidelines in Thailand.

The research protocol was approved by the Human Research Ethics Committee of the Education Committee for Human Research, Ministry of Public Health, Thailand (approval no. No 22/2556). Written consents were obtained prior to enrolment into the study.

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of DARC gene

Genomic DNA was extracted from the dried blood sample using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). The presence of *P. vivax* in the blood samples was verified by nested PCR (120 bp amplicon) as described previously (Snounou *et al*, 1993). Primers specific for exon 2 of DARC gene were FYAB1 forward (5´ TCCCCCTCAACTGAGAACTC 3´) and FYAB2 reverse (5´ AAGGCTGAGCCATACCAGAC 3´) (Castilho *et al*, 2004; Reid and Lomas-Francis, 2004). PCR was performed in a 60-μl reaction mixture containing 3 μl of DNA and 57 μl of a solution consisting of 200 μM each dNTP, 2.0 mM $MgCl_2$, 50 pM each primer, and 1 U *Taq* DNA polymerase (Biolabs, Herts, UK). Thermocycling was conducted in a BioRad iCycler (Cary, NC) as follows: 95ºC for 5 minutes; followed by 35 cycles of 95ºC for 20 seconds, 62ºC for 20 seconds and 72ºC for 20 seconds; and a final step of 72ºC for 10 minutes. Amplicons (392 bp) were analyzed by 1.2 % agarose gel-electrophoresis mixed with RedSafe™ Nucleic Acid Staining Solution in 1X Tris-borate-EDTA buffer (TBE) to verify amplification efficiency before treatment with restriction enzymes. PCR-RFLP was performed as previously described (Geneaid, 2012). In brief, amplicons were digested overnight with 5.0 U *Ban*I (New England Biolabs, Ipswich, MA) (restriction site 5´ G˘GYRCC 3´) generating fragments of 86, 96 and 210 bp for FYA and 86 and 306 bp for FYB.

DNA sequencing and data analysis

PCR-RFLP amplicons were purified using QIAquick PCR Purification Kit (Geneaid, New Taipei City, Taiwan) and sequenced using BigDye Terminator chemistry with universal primers in ABI 3730XL DNA sequencer (Ramathibodi Hospital, Mahidol University). The nucleotide sequences were analyzed using Bioedit ver. 7.0.5.3 (Hall, 1999) in comparison with the *Homo sapiens* atypical chemokine receptor 1 (Duffy blood group) (ACKR1), RefSeqGene on chromosome 1, GenBank accession no NG_011626.1). The sequences were submitted to GenBank (Banklt1927081; 1-40 isolates; DARC gene, exon2 and partial cds) and were assigned Accession Nos. KX368986-KX369025.

Statistical analysis

Calculation of percent similarity and comparison of rate of nonsynonymous and synonymous substitutions were conducted using BLASTN (NCBI, Bethesda, MD).

RESULTS

DARC genotype detection by PCR-RFLP

FYA and FYB genotypes of 40 *P. vivax*-infected individuals were identified by PCR-RFLP analysis of *Ban*I-digested 392 bp amplicon from exon 2 of DARC gene. There were 25 (62.5%) FYA homozygotes, 8 (20%) FYB homozygotes and 7 (17.5%) FYAB heterozygotes. The observed frequencies are in close agreement with Hardy-Weinberg predicted values.

Diversity of DARC partial exon 2 sequences

The 129 individual *Ban*I-generated fragments from 40 DARC exon 2, 392 bp amplicons were sequenced and compared with DARC GenBank reference sequence. Nucleotide diversity of the 392-bp amplicon was 0.00172. Number of nonsynonymous substitutions per nonsynonymous site (dN) and number of synonymous substitutions per synonymous site (dS) were the same, 0.00172. Tajima's *D* test for hypothesis of mutation was 0.87547, which is not significantly different from Fu and Li's *F* (0.75079) and Fu and Li's *D* (0.56129) tests (Fu and Li, 1993). There was a nucleotide variation (G → A) at nt position 17 resulting in Gly and Asp in codon 6 of Fy^a and Fy^b, respectively.

Relationship between DARC genotypes and *P. vivax* infection

Mean (range) *P. vivax* parasitemia of the 40 infected subjects was 172 (73-385) parasites/200 WBC. The mean (range) parasitemia of infected Fy(a+b-), Fy(a-b+) and Fy(a+b+) individuals was 153 (73-322), 208 (90-348) and 198 (148-385) parasites/200 WBC, respectively, but the values are not statistically different among the three groups ($p > 0.05$ using one-way ANOVA followed by Scheffé post hoc test).

DISCUSSION

There are three major erythrocyte Duffy phenotypes, namely, Fy(a+b-), Fy(a-b+) and Fy(a+b+). Reid *et al* (2004) showed that the frequencies of Duffy antigens vary in different populations. Among Caucasians the ratio of red cell Fy(a+b-):Fy(a-b+), *ie*, Fy^a:Fy^b, antigen is 0.8:1, while among Asians the ratio is 5:1. In this study, the predominance of Fy^a among a small (*P. vivax*-infected) cohort of the Thai population appears to have reached equilibrium as determined using the Hardy-Weinberg equation.

A region covering nt 5540 to 6781 in DARC exon 2 gene was chosen for this study because *P. vivax* binds this exon encoded region of reticulocyte DARC and also is where Fy^a/Fy^b polymorphism

is located (Chaudhuri *et al*, 1993). In this study, mean *P. vivax* parasitemia is not statistically different among the three Fy phenotype groups [Fy(a+b-), Fy(a-b+) and Fy(a+b+)], the first such study in Thailand.

In a previous study conducted in a Brazilian Amazon region, individuals with FYA/FYB genotype have higher susceptibility to vivax malaria (Cavasini *et al*, 2007). The distribution of DARC genotypes in that study was consistent with the heterogeneous ethnic origins of the Amazon population, with a predominance of FYA over FYB (Souza-Silva *et al*, 2014). Previously, Cavasini *et al* (2007) reported that in Brazil the distribution among *P. vivax*-infected individuals with Fy(a+b-) phenotype is 20.19%, Fy(a-b+) 16.3% and Fy(a+b+) 34.93%, but no parasitemia data were recorded. The predominance Fy(a+b-) phenotype, which apparently is the preferred receptor for *P. vivax* attachment to reticulocytes, may account for the higher prevalence of *P. vivax* infection in regions where a high frequency of this red cell surface antigen exists (Shimizu *et al*, 1999).

In conclusion, results from this study have implications in the development of malaria vaccines targeting *P. vivax* merozoite surface Duffy-binding protein. In addition, investigations into the role of Fy variants due to substitutions/indels in exon 2 and other exons in populations with malaria transmission may provide important information regarding evolution of Fy antigen that might afford selective advantage against vivax malaria.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the directors of the malaria clinics involved and all participants. The study was partially supported for publication by the China Medical Board (CMB), Faculty of Public Health, Mahidol University (to UL).

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