Am. J. Trop. Med. Hyg., 98(1), 2018, pp. 83–87
doi:10.4269/ajtmh.17-0557

Glucose-6-Phosphate Dehydrogenase Deficiency Genetic Variants in Malaria Patients in Southwestern Ethiopia

Tamar E. Carter,[1]* Seleshi Kebede Mekonnen,[2,3] Karen Lopez,[1] Victoria Bonnell,[1] Lambodhar Damodaran,[1] Abraham Aseffa,[3] and Daniel A. Janies[1]

[1]*Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, North Carolina;* [2]*Jimma University, Jimma, Ethiopia;* [3]*Armauer Hansen Research Institute, Addis Ababa, Ethiopia*

Abstract. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked erythrocyte enzyme disorder with relevance to malaria treatment policy. Treatment with the antimalarial primaquine can result in hemolytic anemia in G6PD-deficient patients. With increased interest in primaquine use, it is important to identify G6PD variants in Ethiopia to inform malaria treatment policy. In the present study, mutations in the *G6PD* gene are identified in a sample of patients with malaria in Jimma town in southwest Ethiopia. *Plasmodium* species of infection were confirmed using polymerase chain reaction (PCR) and gel electrophoresis. PCR and Sanger sequencing were performed to observe a portion of the *G6PD* gene where the common *G6PD* mutations (A376G, G202A, and C563T) are found. Molecular analysis revealed that most of the samples were single *Plasmodium vivax* infections (83.7%). For *G6PD* genotyping, A376G was detected in 23.26% of individuals, whereas G202A and C563T were absent. Three other uncommon mutations were identified: rs782669677 (535G→A), rs370658483, (485 + 37 G→T), and a new mutation at chrX:154535443(C→T). Bioinformatic analysis of these mutations' potential functional impact suggests minimal effect on protein function. The discovery of both common and uncommon *G6PD* mutations contributes to the discussion on G6PD deficiency and appropriate primaquine treatment in Ethiopia.

INTRODUCTION

Each year 2.8 million cases of malaria resulting in 5,000 deaths are reported in Ethiopia, showing a major shift in the burden of malaria in the country.[1] Malaria in this country is mostly seasonal and unstable. Ethiopia is one of the few countries in Africa where *Plasmodium vivax* and *Plasmodium falciparum* coexist. *Plasmodium vivax* contributes to 44% of malaria cases.[1] As the burden of malaria declines, the possibility of malaria elimination is discussed more often.[2] Antimalarial treatments are one of the main population level interventions in malaria elimination programs. Primaquine, an 8-aminoquinoline-based drug, attacks the gametocyte stage of *Plasmodium* during infection, and therefore plays a crucial role in minimizing human–mosquito transmission. Because of this advantage, primaquine use is becoming more popular in malaria control and elimination programs.[3] In addition, primaquine is the only antimalarial drug for radical cure of *P. vivax*. Although primaquine is useful in malaria control and elimination programs, it also has the potential to harm individuals who are glucose-6-phosphate dehydrogenase (G6PD) deficient. G6PD deficiency is an inherited X-linked disorder caused by mutations in the *G6PD* gene that can induce hemolytic anemia in patients when exposed to exogenous agents, like primaquine.[4] The risk for hemolytic anemia is greater in patients being treated for *P. vivax* infections because they face a higher exposure to the drug during a 14-day 0.25 to 0.5 mg dosing regimen as opposed to a single 0.25 mg dose for *P. falciparum* infections.

As a result of the balancing selective pressure from malaria infection,[5,6] G6PD deficiency is prevalent and the most common enzyme deficiency worldwide.[7] One hundred and eighty-six mutations that can cause G6PD deficiency have been characterized to date.[8] The G6PD A– variant causes the majority of the G6PD deficiency cases in Africa. The G6PD A– is caused by the two mutations, A376G (rs1050829, exon 5) and G202A (rs1050828, exon 4). An individual carrying both of these mutations has on average 12% G6PD activity compared with normal values.[9] A milder variant, G6PD A+, is caused by the single A376G mutation. An individual carrying only the A376G mutation has 80% enzyme activity compared with normal values.[9] G6PD Mediterranean (G6PD Med, rs5030868, exon 6) variant, caused by the C563T mutation, is found in Italy, Cyprus, and countries in the Middle East, and is associated with less than 10% enzyme activity. G6PD deficiency, because it is X-linked, is more frequent and more severe in males (for review, see ref. 10).

Presently, primaquine and G6PD deficiency screenings are not a part of the national policy for the treatment of *P. vivax* infections; however, there are reports of its use in Ethiopia.[11] With the increased interest in using primaquine for malaria control and elimination programs, there is a concomitant need to determine the *G6PD* mutations that exist in Ethiopia. Ethiopia houses very genetically and linguistically diverse populations, with a sizable portion of genetic ancestry shared with populations in neighboring regions.[12] Therefore, it is likely that other G6PD variants, like the G6PD Med, could be observed in Ethiopia.

To date, little is known about the mutations that cause G6PD deficiency in Ethiopia. A study of suspected malaria patients in southwest Ethiopia revealed that G6PD deficiency was as high as 17% based on CareStart™ fluorescence spot test[13] and a restriction enzyme–based study demonstrated the absence of the G6PD A– variant Malo in southwest Ethiopia.[14] Here, direct sequencing methods were applied to identify G6PD mutations in a sample of malaria patients in southwest Ethiopia. This study focused on the portion of the *G6PD* gene that contains the mutations that cause the G6PD A– and the G6PD Med variants and could potentially contain

* Address correspondence to Tamar E. Carter, Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, 9201 University City Blvd, Charlotte, NC 28233. E-mail: tcarte47@uncc.edu

other mutations that would have the major impact of G6PD enzyme function.

METHODS

Sample collection. Samples included in the study came from an epidemiological investigation on malaria and G6PD deficiency in Jimma town. Jimma is located 350 km southwest of Addis Ababa with an altitude of 1,720–2,010 m above sea level. Jimma zone is a malaria-endemic region in Ethiopia. The population of Jimma is ethnically diverse including primarily Oromo, but also Dawro, Amhara, Yem, Guraage, Tigire, and Kefa peoples. Prevalence of malaria infection in Jimma has reached as high as 5.2%.[15] Blood samples were taken during the rainy season between August and October 2014 for genetic testing from patients who were positive for *Plasmodium* infection based on microscopy. Two to four drops of blood were collected on 3-mm Whatman filter paper and air-dried.

Ethics approval and consent to participate. The study was approved by the Institutional Review Board of Aklilu Lemma Institute of Pathobiology, Addis Ababa University (IRB Red. No/2/2011-2012), the Ethical Review Board of Jimma University (Ref. No RPGC/185/2011), and the University of North Carolina at Charlotte Institution Review Board (protocol #15-11-27). Informed consent was obtained from all participants.

Molecular analysis. Samples were transported to the University of North Carolina at Charlotte for genetic analysis. Punches were taken from blood spot samples, and DNA was extracted using QIAamp DNA Investigator Kit (Qiagen, Valencia, CA). Extractions were conducted according to the manufacturer's recommendation. Three polymerase chain reaction (PCR) assays were conducted: 1) identification of *Plasmodium* species of infection, 2) identification of sex of the patients, and 3) amplification of *G6PD* for genetic mutation analysis (detailed below). The reagent components and concentrations for the three PCR assays were 1× Promega Hot-Start Master Mix (Promega, Madison, WI) and 0.4 mM for each primer, plus 1 μL of isolated DNA template.

Plasmodium *species identification.* Molecular test was used to confirm the species of infection for each sample collected. Species-specific PCR assays were used that target the plasmodial *ssrRNA* genes in *P. falciparum* and *P. vivax* as described in Snounou.[16] PCR temperature protocol was carried out as detailed except the annealing temperature for the *P. falciparum* assay was increased to 62°C. Each species-specific PCR assay was run separately. PCR products were separated using gel electrophoresis with a 3% agarose gel run at 120 V for 1.5 hours. Gels were visualized using ultraviolet radiation (UV) illumination and *Plasmodium* species was determined by the presence/absence of a band.

Sex identification of patients. Because the *G6PD* gene is located on the X-chromosome, it is important to have accurate sex information to determine genotypes. There were a few instances in this study where recorded gender was not consistent with the *G6PD* calls (i.e., heterozygous genotypes for participants recorded as male), likely because of recording error. To ensure *G6PD* genotypes could be correctly called, a PCR-based protocol to assess sex was implemented that utilizes the amelogenin genes found on both the X and Y chromosome.[17,18] These genes differ in length by 6 bp in males and females. PCR reaction components and concentrations differed from the original protocol (see description above) but temperature protocol remained as described in Mannucci et al.[17] PCR products were separated by gel electrophoresis with 4% agarose gel at 130 V for 2.5 hours. Gels were visualized using UV illumination and sex was determined by the number of bands (one for females and two for males).

G6PD genotyping and sequence analysis. Portions of the *G6PD* gene, including exons 3, 4, 5, and 6 were PCR amplified using primers previously detailed in Kim et al.[19] PCR temperature protocol was conducted as described in Kim et al.,[19] with the exception of the initial extension time increased to 60 seconds. PCR products were cleaned with 10 units of Exonuclease I (Thermo Scientific, Pittsburgh, PA) and 0.5 units of Shrimp Alkaline Phosphatase (Affymetrix, Cleveland, OH) at 37°C for 1 hour followed by a 15-minutes incubation at 65°C to deactivate the enzymes and a 4°C hold. PCR products were sequenced with ABI BigDye™ Terminator v3.1 (Thermo Fisher Scientific) in a 20 μL reaction containing 0.4 μL 10 μM primer, 1.3 μL BigDye Ready Reaction Mix, 2.7 μL BigDye 5X Sequencing Buffer, 11.1 μL molecular-grade water, and 4.5 μL PCR product. Sequencing reactions were run at 1) 95°C for 10 seconds, 2) 95°C for 10 seconds, 3) 51°C for 5 seconds, 4) 60°C for 4 minutes, 5) repeat steps 2–4 for 39 more cycles, and a 4°C hold. Sequencing reaction was purified using Sephadex® G-50 (Sigma-Aldrich) rehydrated in a Multiscreen Durapore® Filter Plate (EMD Millipore) before sequencing on a 3130 Genetic Analyzer (Applied Biosystems).

Sequences were analyzed using Codon Code Aligner Program V. 6.0.2 (CodonCode Corporation, Centerville, MA). All sequences were aligned to the GenBank reference sequence (GRCh38.p7 Primary Assembly, NC_000023.11) and single nucleotide polymorphisms (SNPs) were identified. Samples with poor sequence quality and those exhibiting rare SNPs were sequenced again. Identified mutations were further analyzed for their functional properties using a bioinformatic approach. Mutations found in the exon regions were analyzed for their potential impact of protein stability using the tool "Impact of Non-synonymous mutations on Protein Stability - Multi Dimension" (INPS; http://inpsmd.biocomp.unibo.it/inpsSuite).[20] Locations of mutations were analyzed to determine whether they were found in transcription factor binding sites (TFBS) or locations with histone modification peaks using the tool RegulomeDB (http://www.regulomedb.org).[21] All analyses involving SNPs were stratified by sex. Hardy–Weinberg equilibrium tests were performed in the female samples for SNPs using a χ^2 statistical test.

RESULTS

In total, blood spot samples were collected from 86 patients. The microscopic examination revealed 73 *P. vivax* infections, 10 *P. falciparum infections*, and three undetermined conditions (Table 1). The PCR-based test showed that most of the samples were *P. vivax* infections, whereas some contained *P. falciparum* and/or both. Of the 86 samples, 73 were positive for *P. vivax* alone, four were positive for *P. falciparum* alone, three were positive for both, and six were negative for either *P. falciparum* or *P. vivax* (Table 1). Samples that were neither *P. vivax* nor *P. falciparum* were tested for *Plasmodium ovale* or *Plasmodium malariae* sample and neither of the latter species were detected. The sex-typing assays determined that of 86 patients, 34 were male and 52 were female.

TABLE 1
Malaria parasite species detected by microscopy and polymerase chain reaction (PCR) genotyping

	Microscopy		PCR	
	n	Percent	*n*	Percent
Plasmodium vivax positive only	73	84.9	72	83.7
Plasmodium falciparum positive only	10	11.7	4	4.7
P. vivax and *P. falciparum* positive	0	0	4	4.7
Negative	0	0	6	7.0
Unknown	3	3.5	0	0
Total	86	–	86	–

Exons 3, 4, and 5 of *G6PD* were sequenced for all 86 samples and exon 6 for 44 samples; all SNPs observed were recorded in these regions. Four SNPs were detected in Exon 5 (Table 2). The common A376G mutation (rs1050829, 376 A→G, chrX:154535277; 156 asn → asp) was detected in 23.26% (20/86) of patients. Of these 20 patients, 19 were females heterozygous for A376G, and one male carried the A376G mutation (i.e., was hemizygous).

A missense mutation was detected (rs782669677, 535 G→A, chrX:154535208; 179: ala → thr) in two heterozygous females. This mutation was located within the exonic region upstream of rs1050829. The change in protein stability caused by the 179: ala → thr amino acid change was predicted using INPS. The resulting ΔΔG was 0.455361 kcal/mol, indicating that the change would result in a neutral to slight increase in protein stability.

Two additional mutations in the intronic region were observed. One mutation (485 + 37 G→T, rs370658483, chrX: 154535131) was observed in one male in this study. This SNP has been identified in another individual, a participant from Gambia in the 1000 Genomes Project.[22] This SNP is located in the intronic region upstream of Exon 5. Another mutation observed was located at chrX:154535443 (C→T) in an intronic region. This mutation was observed in two individuals, both females and in heterozygous state. This mutation has not been recorded in GenBank, and therefore has no rs-ID. No individual carried more than one mutation described. No mutations were observed in Exons 3, 4, or 6, including G202A and C563T. Representative sequences for each genotype observed in this study have been deposited into the National Center for Biotechnology Information GenBank (Accession No. MF796522–MF796526).

RegulomeDB was used to examine whether any of the mutations had a predicted impact on function. With this tool, each mutation receives a score in the range of 1–6. Lower scores indicate more evidence that a particular mutation is functionally significant. Rs1050829 (A376G) is located on binding sites for transcription factors ZNF263, ZBTB33, YY1, and POLR2A. Rs370658483 is located in the suspected binding site for multiple transcription factors, most notably CTCF, FOXP2, MAX, MAZ, MXI1, MYC, NFKB1, POLR2a, YY1, and ZNF143, which were confirmed in multiple cell types. Rs782669677 is located in the binding site for POLR2A in multiple cell types. For the undescribed SNP at chrX: 154535443, only a binding site for ZNF263 was found in a single cell type. Overall, all four mutations had a RegulomeDB score of 4, indicating minimal evidence for disruption at the site of transcription factor binding.

DISCUSSION

The data presented here demonstrate that both common mutations and uncommon *G6PD* mutations are present in southwest Ethiopian population. Whereas the A376G mutation that serves as the background mutation to the G6PD A– variant is present in 23.26% of individuals in the study, the companion mutation G202A was not observed. In addition, the C563T mutation was not observed. In addition to the G6PD A376G mutation, three mutations in and near Exon 5 were observed and explored for their impact on protein function and structure. Rs782669677 and rs370658483 have been reported at low frequencies in the Gambia sample of the 1000 Genomes project. Rs782669677 was found in the coding regions of the gene and results in an amino acid change. The mutation at chrX:154535443 has yet to be reported in any other population.

The computational-based protein function analysis indicates that the mutations observed in this study have minimal functional impact. Although all were located near several TFBS, the analysis indicates that the mutations do not affect transcription binding. Whether these mutations in concert with other mutations not observed in this study could lead to functional changes is yet to be determined. In the case of the well-studied rs1050829 mutation, which had a low RegulomeDB score, studies have shown that this mutation does reduce G6PD enzyme activity. Additional tests that determine G6PD enzyme activity levels in individuals with these

TABLE 2
Frequency of the *G6PD* mutations observed in Exon 5

		rs1050829 (A376G)		rs370658483		rs782669677		chrX: 154535443	
Sex	Genotype	*n*	Percent	*n*	Percent	*n*	Percent	*n*	Percent
Male									
	Hemizygote for mutation	1	2.94	1	2.94	0	0.00	0	0.00
	Normal	33	97.06	33	97.06	34	100.00	34	100.00
	Total	34	–	34	–	34	–	34	–
Female									
	Heterozygote	19	36.54	0	0.00	2	3.85	1	1.92
	Normal	33	63.46	52	100.00	50	96.15	51	98.08
	Total	52	–	52	–	52	–	52	–
All	Heterozygote/Hemizygote	20	23.26	1	1.16	2	2.33	1	1.16
	Normal	66	76.74	85	98.84	84	97.67	85	98.84
		86	–	86	–	86	–	86	–

G6PD = glucose-6-phosphate dehydrogenase.

mutations are necessary to further evaluate the functional impact of these mutations on the G6PD enzyme and their risk for inducing hemolytic anemia in response to primaquine treatment. In addition, more studies are needed on the remaining portion of the *G6PD* gene to determine what other variants may exist. Given the four mutations in the *G6PD* gene observed in this study, there are likely other mutations present in other parts of the gene.

In this study, the majority of the infections detected were *P. vivax*. This result is unusual given that the majority of malaria cases in Ethiopia are due to *P. falciparum*. A study conducted in Jimma between 2000 and 2009 revealed that whereas *P. falciparum* was the primary infectious agent, *P. vivax* infections were increasing and topped *P. falciparum* in 2009.[15] This present result corroborates the shift in the proportion of *P. vivax* infections compared with *P. falciparum* in southwest region of Ethiopia. With the high number of *P. vivax* cases, knowledge of the prevalence and genetic characteristics of G6PD deficiency in this region is needed for safe malaria treatment practices.

Several limitations exist in this study. The sample size is small, and focuses on malaria patients, which limit how widely the estimates observed here, can be applied to the Ethiopian population. Although there is value in evaluating these mutations in the population that is most likely at risk for primaquine exposure (i.e., malaria patients) as done in this study, more data are needed from the general population to accurately estimate the prevalence of G6PD deficiency in the Ethiopia. Whereas the focus of this study was on G6PD genetic variation, phenotypic data related to G6PD enzyme activity, combined with genotype data would greatly improve the ability to determine G6PD deficiency status in an individual and increase our understanding of G6PD deficiency in Ethiopia. Future studies that examine G6PD deficiency in the general population that include genetic and phenotypic approaches are required.

Our study highlights two very interesting features of Ethiopia relevant to understanding host–malaria parasite coevolution with implications for malaria control. We see a high number of *P. vivax* cases, which is not observed most anywhere else in Africa, and we see unique diversity in the *G6PD* gene. With the sizable proportion of *P. vivax* infections and the low number of G6PD A– variants, our results indicate an interesting scenario to study the role of inherited red blood cell disorder protection against malaria and how *P. vivax* is shaped by host genetics. The role that the lone A376G (makes the G6PD A+ variant) mutation plays in malaria protection is the subject of debate and should be further explored in Ethiopia. Understanding these details are relevant to fine-tuning population-specific malaria control efforts.

CONCLUSIONS

In conclusion, G6PD A376G mutation and three uncharacterized mutations were detected in this study of malaria patients in Ethiopia, whereas the G6PD G202A mutation and C563T mutants were absent. We also observed a mutation that has not been observed anywhere else, which provides support for the need for further investigation into the G6PD variation in Ethiopia. With most of the patients in this study infected by *P. vivax*, these data add to the knowledge gap on the relationship between G6PD deficiency and *P. vivax* infection. Further studies that include G6PD phenotype data are needed to confirm the results observed here. The presented data contribute to the growing knowledgebase on G6PD deficiency in Ethiopia, which will aid decision-making on the extent of primaquine use in Ethiopia.

Received July 12, 2017. Accepted for publication September 8, 2017.

Published online October 30, 2017.

Acknowledgments: We would like to thank the participants of this study in Jimma town for providing the blood samples and the clinical staff for their assistance in collecting the samples. We would also like to thank Drs. Ronald Clouse, Mindy Shi, and Jia Wen for helpful discussions on the study design and analysis.

Financial support: This work was supported by the SIDA and NORAD core grant to the Armauer Hansen Research Institute, a UNC Charlotte Multicultural Postdoctoral Fellowship from Academic Affairs. Research reported in this publication was supported by a UNC Research Opportunities Initiative grant to UNC Charlotte, NC State University, and UNC-Chapel Hill.

Authors' addresses: Tamar E. Carter, Karen Lopez, Victoria Bonnell, Lambodhar Damodaran, and Daniel A. Janies, Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, NC, E-mails: tcarte47@uncc.edu, klopez1@uncc.edu, vbonnell@uncc.edu, ldamodar@uncc.edu, and djanies@uncc.edu. Seleshi Kebede Mekonnen, Armauer Hansen Research Institute, Addis Ababa, Ethiopia, Jimma University, Jimma, Ethiopia, E-mail: se.kebede@gmail.com. Abraham Aseffa, Armauer Hansen Research Institute, Addis Ababa, Ethiopia, E-mail: aseffaa@gmail.com.

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