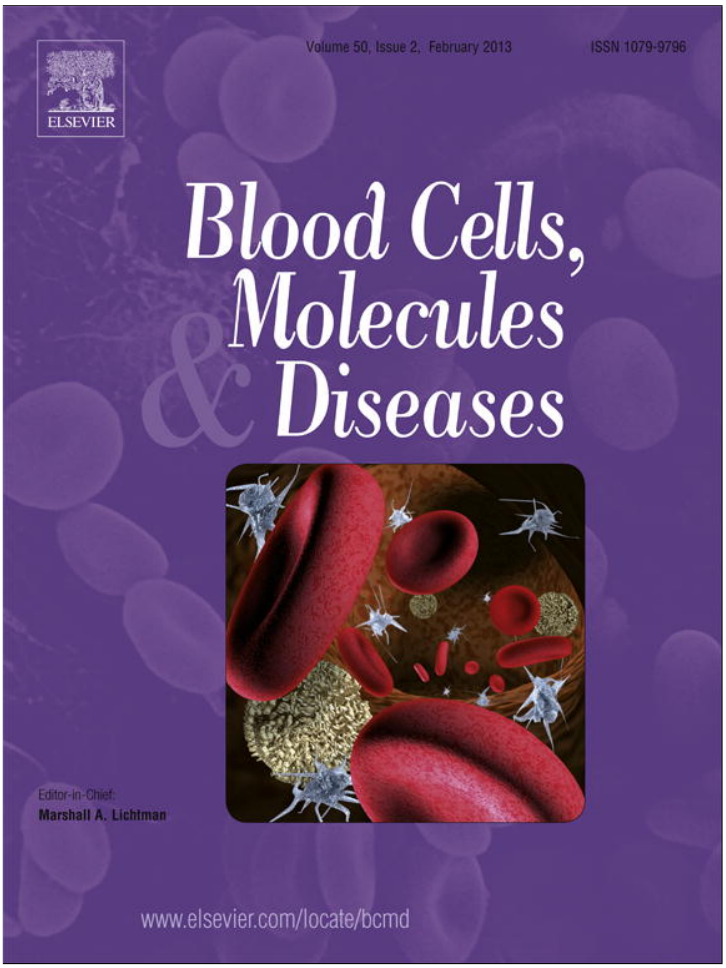

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Analysis of the genetic variants of glucose-6-phosphate dehydrogenase in inhabitants of the 4th Nile cataract region in Sudan

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ABSTRACT

Malaria is one of the most common diseases in the African population. Genetic variance in glucose dehydrogenase 6-phosphate (G6PD) in humans determines the response to malaria exposure.
In this study, we aimed to analyze the frequency of two single-nucleotide polymorphisms (G202A and A376G) present in two local tribes of Sudanese Arabs from the region of the 4th Nile cataract in Sudan, the *Shagia* and *Manasir*.
The polymorphisms in G6PD were analyzed in 217 individuals (126 representatives of the *Shagia* tribe and 91 of the *Manasir* tribe). Real-time PCR and RFLP-PCR were utilized to analyze significant differences in the prevalence of alleles and genotypes.
The *202A* G6P allele frequency was 0.7%, whereas the *G202* variant was found in 93.3% of cases. The *AA*, *GA*, and *GG* genotype frequencies for the A376G G6PD codon among the *Shagia* were 88, 11.1, and 0.9%, respectively; this is similar to the distribution among *Manasir* tribe representatives (94.5, 3.3, and 2.2%, respectively; OR 3.44 [0.85–16.17], p = 0.6). Notably, in north-eastern Sudan the G6PD B (202G/376A) compound genotype frequency was 90.3%, whereas the G6PD A variant (202G/376G) was found in 1.4% of that population. Identification of the G6PD A− variant (202A/376G) in the isolated *Shagia* tribe provides important information regarding the tribal ancestry.
Taken together, the data presented in this study suggest that the *Shagia* tribe was still nomadic between 4000 and 12,000 years ago. Moreover, the lack of G6PD A− genotype among ethnically diverse *Monasir* tribesmen indicates a separation of the *Shagia* from the other tribes in the region of the 4th Nile cataract in Sudan.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD or G6PDH) reduces intracellular oxidative stress in erythrocytes. All mutations that cause G6PD deficiency are localized to the long arm of the X chromosome on band Xq28. The G6PD gene spans approximately 18.5 kilo bases [42]. G6PD is one the most widely studied genes, and has been shown to be associated with malaria susceptibility, favism, various types of anemia, and other disorders. Currently, over 140 mutations have been discovered that result in significant biochemical variability of G6P dehydrogenase [6, 8, 12, 29, 37]. The *G6PD A*$^{-}$ mutations are located in exon 4 (c.202G>A, p.Val68>Met) and *G6PD A*$^{+}$ mutations are found in exon 5 (c.376A>G, p.Asn126Asp).

Based on the polymorphisms in positions 202 and 376 of the *G6PD gene*, the three main variants of the phenotype are denoted: B, A, and A− [1]. The most frequent B variant, (202G/376A), is associated with normal enzymatic G6PD activity and is present in the human population worldwide [36, 35]. Other variants of this gene, G6PD A− (202A/376G) and G6PD A (202G/376G or 202A/376A), are characterized by reduction of enzymatic activity and are specific to Sub-Saharan Africa, whereas the G6PD-Mediterranean variant (Med, C563T) is present in Southern Europe, the Middle East, India, Papua New Guinean, and Iran [2, 3, 34, 36, 38, 39, 43]. It is noteworthy that variant A− is coded by missense mutations in G202A and A376G positions [4, 6–8,15, 40, 41]; this potentially also occurs with other mutations such as G680T, T968C, and C1159T [4, 5, 22].

There is a direct association between malaria incidence and frequency of certain G6PD gene variants. In patients with an A phenotype,

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protection against malaria is incomplete as G6PD enzymatic activity is considerably reduced (20%) compared to wild type. G6PD A− variant is associated with a 50% decrease in the risk of malaria development among heterozygous women and hemizygous males [16]. It has been suggested that this haplotype appeared late in the course of African continent evolution (approximately 4000–12,000 years ago), whereas the G6PD A variant is potentially even older (130,000–174,000 years ago) [37].

Reduced G6PD activity is closely associated with resistance to malaria in endemic areas, and this is reflected in the quantity and distribution of genetic variants of this gene [9, 31]. Specific gene variants of G6DP were examined in different parts of the world relative to areas that exhibited high endemicity and malaria prevalence. Reports on the evolution of the human genome suggest that humans have evolved defensive mechanisms against infection by means of the protective variance of the G6PD gene, predominantly in regions exhibiting malaria prevalence [12, 36]. However, in previous reports, limited data have been generated concerning G6PD gene polymorphism distribution in the area of Northern Sudan, particularly in region of the 4th cataract in the Nile valley, the location in which the *Shagia* and *Manasir* tribes reside. These tribes constitute unique populations in terms of geographical location as they inhabit the isolated land at the interface between two deserts. This study contributes to the knowledge regarding the spread of G6PD SNPs G202A and A376G in Africa.

Materials and methods

Subjects

A total of 217 individuals (101 women and 116 men; median age 30 years) were investigated. Genetic material (buccal swabs) was collected from 126 individuals of the *Shagia* tribe (71 women and 55 men; mean age 23.9 ± 17.3, median age 20) and from 91 representatives of the *Manasir* tribe (46 women and 45 men; mean age 42 ± 19 years, median age 41.3) who consented to participate in the study. Both tribes inhabit the 4th Nile cataract.

The *Manasir* tribe inhabits a region approximately 80 km from the *Shagia* villages, and the *Manasir* are less isolated. The *Manasir* represent a population with a mixture of other tribal groups, such as *Beniamer*, *Ababda*, *Foraui*, and *Kesinger*.

This study was conducted in accordance to the Helsinki Declaration and to local legislation. Patient informed consent was a prerequisite for participation in the study. Institutional ethical clearance from the Ethics Committee of Pomeranian Medical University was obtained.

Genotyping

Buccal swabs for subsequent DNA extraction were collected from all individuals who consented to participate in the study. The swabs were carefully dried in a separate manner in order to avoid contamination. For DNA extraction, BuccalAmp DNA Extraction Kit (Epicentre, Madison, USA) was utilized.

Genotyping was conducted according to the method published by Mombo [26]. The PCR was performed using the following primers for G6PD G202A: 5′-TTACAGCTGTGCCCTGCCCT-3′ and 5′-AGGGCAAC GGCAAGCCTTAC-3′ and for G6PD A376G: 5′-AGGGCAACGGCAAGC CTTAC-3′ and 5′-CTGCGTTTTCTCCGCCAATC-3′. The reactions were conducted in a total volume of 20 μL that contained 40 ng of template DNA, 4 pM of each primer, 1×PCR buffer [10 mM Tris–HCl, 50 mM KCl, 0.08% Nonidet P40] (MBI Fermentas), 1.5 mM $MgCl_2$ Analysis of the genetic variants of glucose-6-phosphate dehydrogenase in inhabitants of the 4th Nile cataract region in SudandNTP (MBI Fermentas), and 0.5 U of Taq polymerase (MBI Fermentas). Amplification was performed with initial denaturation at 67 °C for G202A G6PD and 54 °C for A375G G6PD for 5 min, and then 37 cycles for G202A G6PD and 35 cycles for A375G G6PD of denaturation at 94 °C for 20 s, annealing at 53 °C for 40 s, and extension at 72 °C for 40 s. The final incubation at 72 °C was extended by 8 min. The G202A G6PD amplified fragments (364 bp) were digested with the restriction endonuclease Hin 1 II [Nla III]. For the RFLP assays, an aliquot of 16 μL of PCR product was incubated overnight at 37 °C with 2 μL Hin 2 II [Nla III] and 2 μL buffer. The sizes of the restriction fragments were 303 bp and 61 bp for the normal allele (202G), while the mutant allele (202A) remained uncleaved and exhibited a band of 180 bp and 123 bp and 61 bp (Fig. 1).

The G376A G6PD amplified fragments (585 bp) were digested with the restriction endonuclease BseGI [FokI]. An aliquot of 16 μL of PCR product with 2 μL BseGI and 2 μL buffer was incubated overnight at 55 °C. The sizes of the restriction fragments were 402 bp and 183 bp for the normal allele (376A), whereas the mutant allele (376G) remained uncleaved, with bands of 285, 183, and 117 bp. The fragments were separated using 3% agarose gel (G202A G6PD) or 2% agarose gel (G376A G6PD) (Fig. 2).

PCR-RFLP results were confirmed by real-Time PCR analysis. Oligonucleotide primers and TaqMan probes for polymorphisms: G202A and A375G (rs1050828 and rs1050829, respectively) were designed and synthesized by Applied Biosystems (ID: C_2228686_20, C_2228694_20). The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.2.

Statistical analysis

Statistical analysis was performed with StatView® program using the chi^2 Pearsons and chi^2 NW, Fisher test. $P<0.05$ was considered to indicate a statistically significant result.

Results

In the G202A G6PD gene polymorphisms, one female heterozygous for the *GA* genotype was found in the *Shagia* tribe, whereas all the remaining cases were *GG* homozygotes. As a result, the *GA* genotype frequency among the *Shagia* tribe cohort was 0.8%. Among *Monasir* individuals, no cases with *202A* mutation were identified. The frequency of the *GA* genotype, including the individuals of both tribes, was 0.46%.

For the A376G G6PD variant the following genotype distribution was found: 88% *AA*, 11.1% *GA*, and 0.9% *GG* among the *Shagia* individuals, and 94.5% *AA*, 3.3% *GA*, and 2.2% *GG* among *Manasir* representatives. The genotype distributions did not differ significantly between the groups (OR 3.44 [0.85–16.17], p = 0.6). Among *Shagia* women the 376A allele was found in 90.1% of cases whereas the *376G* variant was present in 9.9% of the individuals investigated. This pattern of distribution was similar to the pattern observed among *Manasir* females (96.6% for the

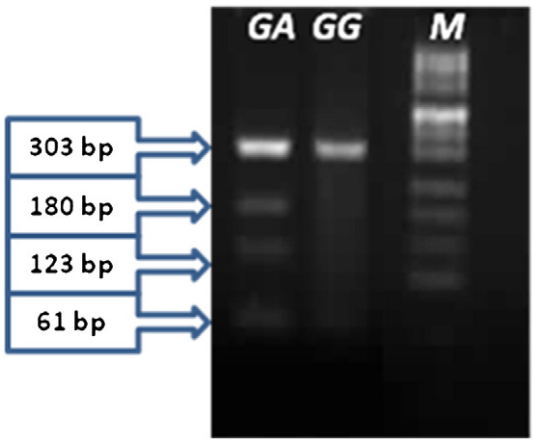

Fig. 1. Identification of G202A mutation in the G6PD gene. Electrophoretic separation in 3% agarose gel of restriction fragments after amplicon digestion with Hin 1 II. M, DNA size marker pUC Mix Marker 8; GA, heterozygote; *GG*, homozygote.

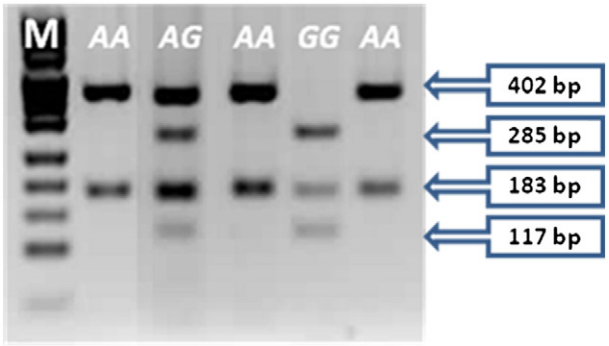

Fig. 2. Identification of A375G mutation in the G6PD gene. Electrophoretic separation in 2% agarose gel of restriction fragments after amplicon digestion with BseGI. M, DNA size marker pUC Mix Marker 8; *AA*, homozygote; *AG*, heterozygote; *GG*, homozygote.

376A and 3.3% for the *376G* allele, OR 3.17 [0.82–14.35], p = 0.063). Similarly, among *Shagia* and *Manasir* men, allele frequencies did not differ significantly (98.2% for the *376A* and 1.8% for *376G* among *Shagia* versus 95.65% (376A) and 4.35% (376G) among *Manasir* tribesmen, OR 0.4 [0.04–4.64], p = 0.6).

Based on the SNPs investigated, the G6PD phenotypes were assigned (Table 1). The G6PD B (202G/376A) phenotype was found among *Shagia* and *Manasir* representatives with the frequency of 90.3%. In 1.4% of cases, phenotype G6PD A (202G/376G) was present. In the remaining 8.3% of cases, it was impossible to unanimously assign the phenotype.

Discussion

G6PD B is highly prevalent worldwide; however, variants of this gene, especially those associated with decreased enzymatic activity, are characteristically found in certain geographic regions [6, 18, 36]. In this study, we investigated the tribes of the north-western part of Africa, and the G6PD B (202G/376A) haplotype was identified in 90.3% of cases and G6PD A (202G/376G) in 1.4% of cases. Additionally, one *GA* heterozygote was found with probable G6PD A− phenotype in the *Shagia* tribe. These results are in concordance with published reports and contribute to the current knowledge on the distribution of the G6PD variants in north-eastern Sudan. According to Tishkoff et al., G6PD variant B is found in 53.6–81.5% of inhabitants of sub-Saharan Africa (*Sierra Leone*, *Mende*, *Temne*, *Ghana*, *Fante*, *Ga*, *Cameroon*, and *Bakaka*) whereas other haplotypes are significantly less frequent (G6PD A from 0.05 to 34.2%, G6PD A− from 3.3 to 1.9%) [36]. In general, the frequency of the G6PD A− haplotype in Africa ranges from 0 to 25% [17], and may also be found among African immigrants to Southern Europe and the Middle East. In Sudan, this variant is found in 7 to 9.9% of the population [22].

The spread of genetic variants associated with frequencies of low-activity alleles of glucose-6-phosphate dehydrogenase may be correlated to the selective pressure caused by *Plasmodium spp*. [33, 36]. Malaria is a threat to human life on all continents, but primarily in the tropics. In East African urbanized areas the transmission is low and varied (Dar es Salaam, Tanzania), which is the opposite of agriculture areas in which transmission is high and constant (Kilifi, Kenya) [23]. In areas of high malaria endemicity, children under five years of age are hosts of *Plasmodium*; however, morbidity is low, whereas in areas with lower endemicity, the disease affects people of all ages. This dependence is associated with greater resistance, which is not achieved in areas in which there is low or seasonal exposure to the parasite [24]. When exposure to malaria is continuous, acquired resistance to *Plasmodium falciparum* during pregnancy is decreased; however, where there is periodic exposure to malaria, more severe forms of the disease occur in pregnant women.

The greatest immune resistance does not occur where transmission is continuous, undifferentiated, and at a high level throughout the year (holoendemic). When transmission is not continuous, such as during the dry season, there is lower intensity (hyperendemic) disease. For example, the highest incidence rates are found in Kanuri (27.9%) and Bede (32.6%) in Bornu, in the Hausa (29%) in Garki, in the Nigerian savannas of Sudan and Cameroon (32%), and in Nigeria (32%) [27].

Malaria is one of the most common health problems in Sudan. Treatment is not always associated with the actual disease. The study population inhabits villages, in which fever is always identified as malaria. According to published reports, this is a valid approach in Africa [10, 11, 14, 25, 30, 32]. In 1999, the overall incidence of malaria in Khartoum, the capital of Sudan, was 2.5%; the incidence rose to 3.2% in 2000, followed by a consistent decline to less than 1% in subsequent years [28]. There are data regarding the pathogenesis of severe malaria in areas of unstable malaria transmission [25]. It has been reported that there was a reduction in the intensity of *P. falciparum* transmission over the previous ten years. The incidence of fever in Sudan is low; 1.8% is associated with *P. falciparum* [13]. The results of this study are in concordance with previous reports. According to our estimation, 1.8–9.6% of the population does not have the G6PD variant which produces resistance to malaria in Northern Sudan.

World Health Organization guidelines published in 2010 have advocated the use of parasitological diagnosis. For the *Manasir* and *Shagia* tribes, there is no comprehensive epidemiological data on *P. falciparum* infection and thus it seems reasonable, as an estimate, to infer the probable incidence of malaria based on genomic analysis (i.e., defined SNPs responsible for susceptibility to infection with malaria).

It would be interesting to expand this study by utilizing more advanced genetic markers, e.g., analyze the chromosome Y sequence and/or mtDNA. Unfortunately, the small volume of samples collected does not allow for such research, and additional collection of genetic material from the *Shagia* people living in the region of the 4th cataract is not possible due to the changes in the socioeconomic situation of the tribe (relocation due to the construction of the Merowe Dam).

Table 1
Multi-SNP association analysis of several cases of malaria and the G6PD phenotype among *Shagia* and *Manasir*.

Gender	Genotype		Haplotype	Phenotype	Shagia N (%)	Manasir N (%)	OR	p-value
Male	202G/376A	Hemizygous wild-type	A/B	G6PD B	54 (98.2)	44 (95.65)	2.45 [0.21–27.97]	0.01
	202G/376G	Hemizygous	A/A	G6PD A	1 (1.8)	2 (4.35)		
Female	202G, 202G/376A, 376A	Heterozygous wild-type	A, A/B, B	G6PD B	56 (78.9)	42 (93.3)	0.27 [0.07–0.98]	0.03
	202G, 202A/376A, 376G	Heterozygous	A, A−/B, B	G6PD B (202G/376A) G6PD A (202G/376G) G6PD A (202A/376A) G6PD A− (202A/376G)	1 (1.4)	0 (0)		
	202G, 202G/376A, 376G		A, A/B, A	G6PD B (202G/376A) G6PD A (202G/376G)	14 (19.7)	3 (6.7)		

Conclusion

This study provides data on the genetic variability of the Sudanese native tribes and provides characterization of previously unstudied ethnic groups. Results of the previous studies and genetic investigations of the *Shagia* and *Manasir* [19–21] tribes suggest that *Shagia* individuals were separated from other tribes in the region of the 4th Nile cataract. This hypothesis is consistent with the historic and sociological data, as *Shagia* do not identify themselves with the Sudanese Arabs who dominate the north part of Sudan. Because the G6PD A− (202A/376G) variant appeared from 4000–12,000 years ago, it may be inferred that at this time *Shagia* tribe was still nomadic. The identification of the single *G202A* G6PD mutation in the *Shagia* tribe, as well as the fact that it does not occur in the heterogeneous tribe of *Manasir*, indicates that a long-lasting assimilation of *Shagia* and other tribes occurred in the 4th Nile cataract region. This study provides important information for the history of the tribe.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

A. Kempinska-Podhorodecka and M. Milkiewicz designed and implemented the survey and wrote the article. O. Knap and M. Parafiniuk were responsible for selection of the patients and collection of the samples. A. Kempinska-Podhorodecka, A. Drozd, and M. Parczewski were responsible for genotyping. A. Kempinska-Podhorodecka and M. Kaczmarczyk conducted the data analysis. All authors read and approved the final manuscript.

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