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Original Article

Fine Mapping of Glucose 6 Phosphate Dehydrogenase (G6PD) Deficiency in a Rural Malaria Area of South West Odisha Using the Clinical, Hematological and Molecular Approach

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Abstract. ***Introduction:*** **The aim of the study was to enumerate the clinical, hematological, and molecular spectrum of G6PD deficiency in malaria endemic regions of south west Odisha.**
Methods: **Diagnosis of G6PD deficiency was made by using the Di-chloroindophenol Dye test in two south west districts (Kalahandi and Rayagada) of Odisha State. Demographic and clinical history was taken from each individual using a pre-structured questionnaire. Molecular characterization of G6PD deficiency was done using PCR-RFLP and Sanger sequencing.**
Results: **A total of 1981 individuals were screened; among them, 59 (2.97%) individuals were G6PD deficient. The analysis revealed that G6PD deficiency was more among males (4.0%) as compared to females (2.3%). Prevalence of G6PD deficiency was significantly higher among tribal populations (4.8%) as compared to non-tribal populations (2.4%) (p=0.012, OR=2.014, 95%CI=1.206-3.365). Twenty four individuals with G6PD deficiency had mild to moderate anemia, whereas 26 G6PD deficient individuals had a history of malaria infection. Among them, 3 (11.5%) required blood transfusion during treatment. Molecular analysis revealed G6PD Orissa as the most common (88%) mutation in the studied cohort. G6PD Kaiping (n=3), G6PD Coimbra (n=2) and G6PD Union (n=1) were also noted in this cohort.**
Conclusion: **The cumulative prevalence of G6PD deficiency in the present study is below the estimated national prevalence. G6PD deficiency was higher among tribes as compared to non-tribes. Clinical significance for G6PD deficiency was noted only in malaria infected individuals. Rare G6PD Kaiping and G6PD Union variants were also present.**

Keywords: G6PD; Malaria; Molecular spectrum; Tribes.

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Introduction. Glucose 6 Phosphate Dehydrogenase (G6PD) deficiency is the most common inherited red cell enzymopathy in humans. It is estimated that 4.9% of the global population has G6PD deficiency

accounting for more than 400 million people around the world.[1] It is more prevalent in the areas where malaria is endemic or has been endemic especially in Africa, Asia, Europe and the Mediterranean region.[1] The distribution of G6PD deficiency in India is very heterogeneous and the prevalence of G6PD deficiency varies 0.3 to 30.7% per cent.[2] According to an estimate around 390000 children are born with G6PD deficiency each year in India.[3]

The clinical spectrum of G6PD deficiency is quite heterogeneous, ranging from mostly asymptomatic individuals to patients having neonatal jaundice, acute hemolytic anemia when exposed to exogenous agents (acute infections, drugs or fava beans) and chronic non-spherocytic hemolytic anemia.[4]

It is now well accepted that G6PD deficiency protects individuals against severe *Plasmodium falciparum* malaria infection. Malaria parasites require optimum RBC redox status for their survival, replication, and development,[5] which is diminished in G6PD deficient RBCs. Individuals with diminished G6PD enzyme activity, when infected with *P. falciparum,* develop less severe symptoms than individuals having regular G6PD enzyme activity. Ironically to this protective mechanism, G6PD deficient individual's RBCs are prone to destruction by hemolytic agents, as antimalarial, causing significant impediments in malaria treatment. Primaquine is a frequently used drug in combination therapy for the treatment of *P. falciparum* and *P. vivax*. This drug induces serious hemolytic events in G6PD deficient individuals.[6,7] As a result, G6PD deficiency imposes an obstacle to the success of both malaria elimination and the National health program. This warrants the need to undertake systematic studies on G6PD deficiency in the Indian population, especially in malaria endemic regions. In India, Odisha contributes to the highest number of cases and deaths due to malaria.[8] In Odisha, out of the 30 districts, 10 southern districts contributed around 64% of deaths due to malaria.[9] Although a number of prevalence studies of G6PD deficiency in Odisha have been carried out, however, in these studies and the methodologies used were variable.[10-12] In addition, the clinical manifestations and molecular spectrum of these deficient cases were not well documented.

The present study was thus planned to document the clinical and hematological manifestations in G6PD deficient individuals in the malaria endemic south west region of Odisha and to evaluate the underlying mutation spectrum in G6PD deficient individuals.

Material and Methods.

Study area. This prospective cross-sectional study was conducted in one hospital setting and 3 community settings from south west villages of Odisha. Patients attending Biswanathpur (19.1607°N, 84.7727°E) community health center, Kalahandi, (ii) Residents of Bissamcuttack town (19.5088°N, 83.5044°E), Rayagada (iii) residents of village Paiko-Dakuluguda (19.5842°N, 83.5394°E), Rayagada and (iv) residents of village Kachapaju (19.4984°N, 83.6295°E), Rayagada, Odisha (**Figure 1**) were included.

Sample collection. After the prior approval, scientific advisory committee and Institutional ethical committees (IEC registration number: ECR/734/Inst/MP/2015, project approval ID: 201701), a detailed demographic and clinical history of all recruited individuals were taken in predesigned structured proforma. All individuals were briefed on the purpose of the study and written informed consent was obtained from adult individuals or from parent/guardian in case of minors prior to obtaining samples. A total of 1981 individuals were recruited for the study and 1-2 mL of peripheral blood was drawn in EDTA vial under aseptic conditions from all the recruited individuals.

Laboratory analysis. G6PD deficiency status was determined at field site using standard Dichloroindophenol (DCIP) decolorizing method in all

Figure 1. Map showing Kalahandi and Rayagada districts (study area) in Odisha state, India.

samples. All the samples were stored at 4°C and transported to the laboratory under cold chain. G6PD enzyme activity was measured in all deficient patients, as described earlier.[13]

Complete blood count was done using an automated cell counter (Sysmex KX-21, Transasia, Japan). Individuals were grouped according to the severity of anemia on the basis of age specific cut off values for hemoglobin levels.[14]

Genomic DNA was extracted from all the G6PD deficient samples by standard salting out method. Three common mutations (G6PD Orissa, G6PD Mediterranean, and G6PD Kerala-Kalyan) in G6PD deficient individuals were identified using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described earlier.[10] In remaining samples, all the 13 exons of the G6PD gene were amplified using the PCR. PCR product was purified using exonuclease 1 and Shrimp alkaline phosphatase restriction enzymes and sequenced using the Sanger sequencing method on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).

Statistical analysis. All data were entered in Microsoft Excel for Windows and analyzed on IBM SPSS (IBM Corp release 2017; IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to check the normality of the continuous variables. Data for the continuous variables were given as the mean ± standard deviation or median (25-75 percentiles). Categorical or discrete variables were presented as numbers (%). Fuzzy tool was used to select the age, gender, and tribe matched controls for comparison from a large cohort of G6PD healthy individuals (for **Table 3**). Fisher's exact test was used to calculate the significance difference in the frequency of discrete variables in G6PD deficient and normal individuals. Depending upon the normality, the Mann Whitney U test or independent sample student "t" test was applied to see the significant difference in continuous variables in G6PD deficient and normal individuals. P <0.05 was considered significant.

Results. A total of 773 males and 1208 females were enrolled in the study. The median age of the studied subjects was 23 years (13-35 years). Out of 1981 subjects, 59 (2.97%) were G6PD deficient (**Table 1**). The prevalence of G6PD deficiency varies from 0.5% to 9.6% in different studied cohorts (**Figure 2**). Prevalence was significantly higher among males (31/773, 4.0%) as compared to females (28/1180, 2.3%) (p = 0.041).

The median G6PD enzyme activity was 0.585 IU/g Hb (0.33-2.65 IU/g Hb) (Normal range = 6.75-11.95 IU/g Hb for adults). Median G6PD enzyme activity in deficient females was 2.17 IU/g Hb (0.72-3.78 IU/g Hb) and it was significantly higher than those in deficient males (median 0.37, 0.29-0.55 IU/g Hb) (p<0.0001) (**Figure 3**). Four hundred seventy-seven individuals in the study were from tribal communities (24.1%), mainly Kondh and Dongaria Kondh. G6PD deficiency was significantly higher in tribes (4.8%) as compared to non-tribes (2.4%) (p = 0.007, OR=2.066, 95% CI=1.212-3.523).

Hemoglobin estimation could be performed only in 1538 samples, and it was observed that 60.7% of the subjects were anemic. Among them, 406 (26.4%) were mildly anemic, 477 (31.0%) had moderate anemia, whereas 50 (3.3%) subjects had severe anemia (**Table 2**). Severe anemia was higher in individuals selected

Table 1. Demographic details of study cohort.

Variable	G6PD Normal	G6PD Deficient	P value
Median Age (in Years)	24(13-15)	18(8-32)	0.011
Male	742(96.0%)	31(4.0%)	0.041, OR=0.578, 95% CI=0.350-0.956
Female	1180(97.7%)	28(2.3%)	
Tribe	454(95.2%)	23(4.8%)	0.012, OR=2.014, 95%CI =1.206-3.365
Non-Tribe	1468(97.6%)	36(2.4%)	

P value for difference in age was calculated using Mann Whitney U Test whereas Fisher's exact test was applied to see the difference of prevalence of G6PD deficiency among different gender and tribal status.

Table 2. Anemia status in different settings.

	Anemia				Total
	Normal	Mild	Moderate	Severe	
Hospital Setting	90(18.4)	129(26.4)	232(47.4)	38(7.8)	489
Community Setting	515(49.1)	277(26.4)	245(23.4)	12(1.1)	1049
Total	605(39.3)	406(26.4)	477(31.0)	50(3.3)	1538

Number in parenthesis () represents percentages. Chi Square for trend P <0.0001.

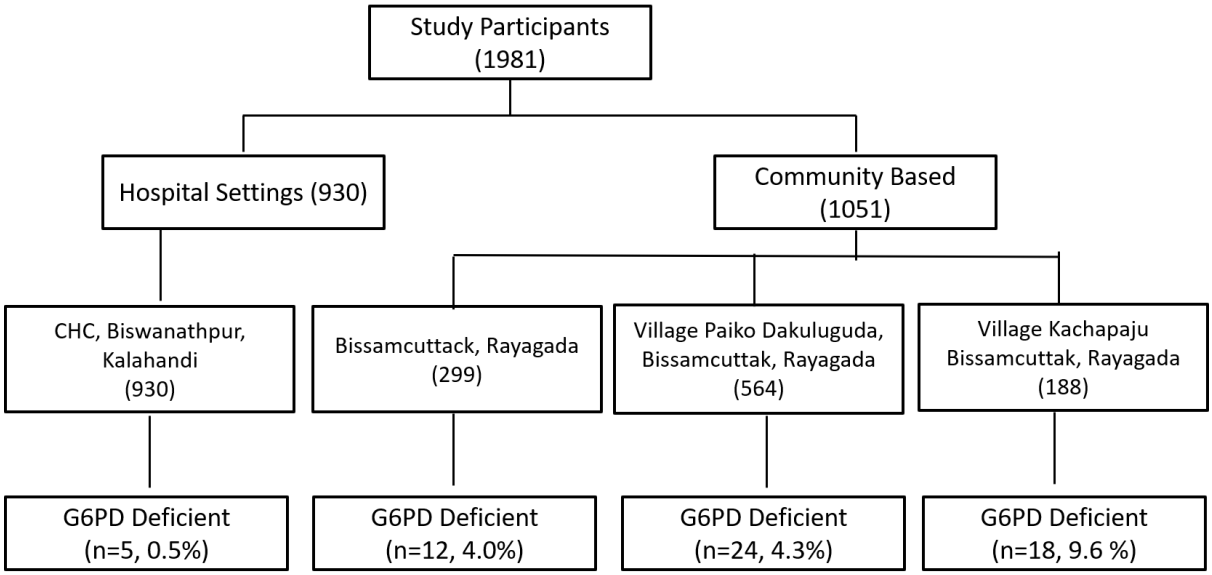

Figure 2. Flow Chart showing the study site and G6PD status in the Odisha state, India.

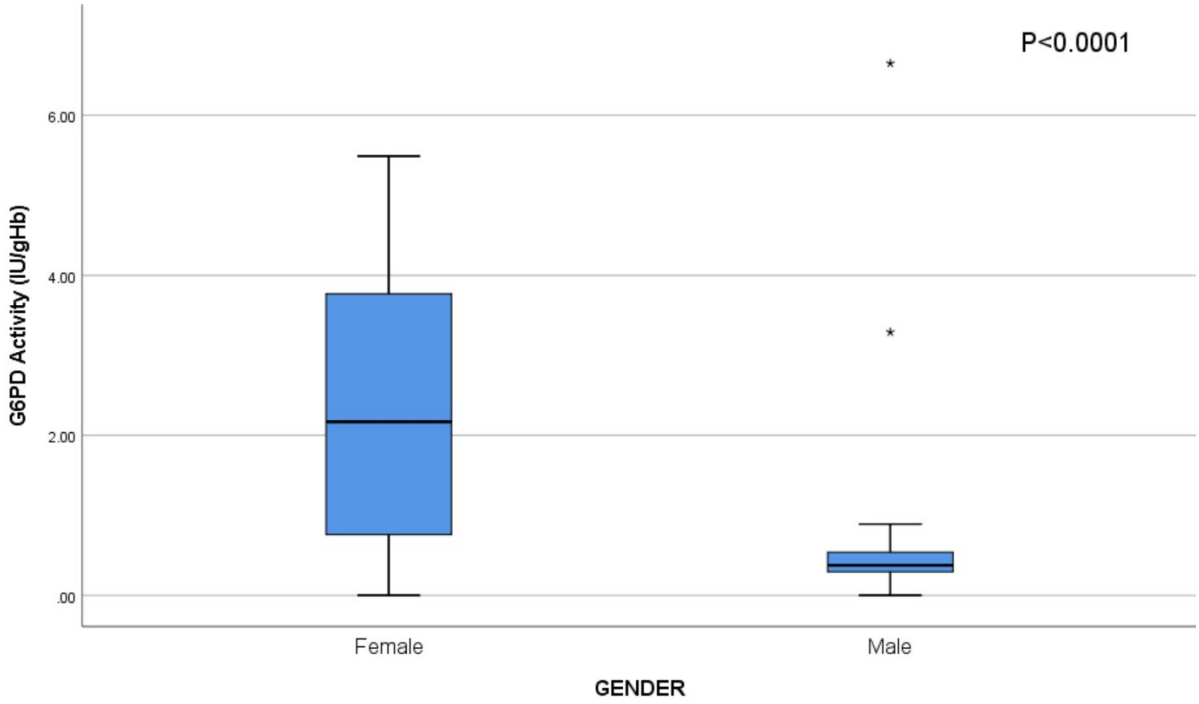

Figure 3. Boxplot showing G6PD activity among different gender.

from hospital settings as compared to those from community settings (p <0.001). Twenty-four individuals had both G6PD deficiency and anemia out of which only 3 individuals had a moderate degree of anemia (Hb range 7-9.9 gm/dL). None of these individuals had severe anemia (Hb<7.0 gm/dL).

Communities in Odisha state are reported to suffer from various hemoglobinopathies. Therefore, hemoglobin electrophoresis was performed to ascertain whether the screened individuals also carried any hemoglobin disorder. We observed the high prevalence of sickle cell disease (1.7% 32/1897) and trait (18.5%, 350/1897). Beta thalassemia trait was observed in only 0.9% (18/1897) individuals. No beta thalassemia major was encountered. Electrophoresis could not be performed on 84 samples due to low sample volume. Only one individual had both G6PD deficiency and sickle cell disease and showed severe hemolytic disease. Eight sickle cell trait individuals also had G6PD deficiency. All these 8 individuals had no history of blood transfusion.

The presence of malaria was tested using the bivalent rapid diagnostic kit. Two hundred fifty-four samples were positive for malaria; among them, 238 were positive for *P. falciparum* (Pf), 15 for *P. vivax* (Pv) infection. One patient had a mixed infection of both Pf and Pv. Two Pf positive females were also found to be G6PD deficient. No male individual with G6PD deficiency was found positive for Pf or Pv infection.

A total of 843 (42.6%) individuals had a prior history of malaria; however, due to lack of previous

medical records, the type of parasite infection could not be ascertained. Out of these 843 individuals, only 26 [12 male and 14 female] had G6PD deficiency. No association of malaria with G6PD deficiency was observed (p = 0.792). Out of these 26 G6PD deficient individuals, three developed severe anemia during malaria treatment and required blood transfusion. There was a significant association with the need for blood transfusion during malaria treatment and G6PD deficiency (p=0.026, OR=3.816, 95%CI=1.079-13.496).

None of the G6PD deficient subjects had splenomegaly or hepatomegaly at the time of screening. Twenty-two per cent of G6PD deficient individuals had a history of anemia, fever, and joint pain. Four individuals also had a previous history of blood transfusion. To compare the association of G6PD deficiency with clinical symptoms, age, gender, and tribe, matched equal subjects were randomly selected from people having regular G6PD enzyme activity. **Table 3** shows the association of G6PD deficiency with a clinical history or symptoms and it was observed that there was no significant difference in the frequency of pallor, fatigue, history of anemia, fever, joint pains or body pain in G6PD deficient as compared to normal individuals. Although the G6PD deficient individuals more frequently reported a prior history of blood transfusion than the not affected subjects, yet it did not reach statistical significance (p=0.679). Red cell indices like mean hemoglobin, mean corpuscular volume and mean corpuscular hemoglobin levels were also similar in both the groups (**Table 4**).

Molecular characterization of the G6PD gene could not be done in 8 samples due to low sample quantity. G6PD Orissa (C.131C>G, p.Ala44Gly) (Class III variant) was found to be the most common mutation and was seen in 45 (88.2%) subjects. Other commonly known mutations like G6PD Mediterranean (Class II variant), G6PD Kerala Kalyan (Class III) were not encountered.

On the other hand, G6PD Coimbra (C.592C>T, p.Arg198Cys)(Class II) was observed in 2 children of the Kondh tribe, one of whom also had a history of malaria infection. Both children had no history of blood transfusion or anemia.

Rare G6PD mutant variant G6PD Kaiping (c.1388G>A, p.Arg463His) (**Figura 4a**) was observed in 3 individuals. One of them (20 year old male) belonged to Porja Tribe. Clinical evaluation revealed pallor, prominent lymph node, and purpuric spot. He also had a prior history of jaundice. G6PD enzyme activity was 0.31 IU/g Hb. The second individual belonged to Kondh tribal community (32 years old). He had no history of jaundice or anemia, and his general health condition was good. The G6PD enzyme activity was 0.48 IU/g Hb. The third individual was a 55 years old female non-tribe with the G6PD enzyme activity of 2.34 IU/g Hb. She had a history of malaria with severe anemia and blood transfusion. Furthermore, rare variant, G6PD Union (C.1360C>T, p.Arg454Cys) (**Figure 4b**), was identified in a 21 year old non-tribal woman in the heterozygous state. In this case, the G6PD enzyme activity was 3.3 IU/g Hb. This woman also reported suffering from malaria earlier.

Table 3. Distribution of clinical parameters between age, gender and tribal status matched G6PD deficient and normal subjects.

Variable	G6PD Normal	G6PD Deficient	P value
Pallor	10(16.9)	5(8.5)	0.269
Fatigue	5(8.5)	4(6.8)	1.000
H/O Anemia	10(16.9)	13(22.0)	0.643
H/O Fever	18(30.5)	13(22.0)	0.403
H/O blood Transfusion	2(3.4)	4(6.8)	0.679
H/O Joint Pain	9(15.3)	13(22.0)	0.479
H/O Body Pain	8(13.6)	8(13.6)	1.000
H/O Back Pain	13(22.0)	11(18.6)	0.820
H/O Malaria	27(45.8)	26(44.1)	1.000

Number in parenthesis () represents percentages, P value was calculated using Fisher's Exact Test.

Table 4. Hematological profile of age, gender and tribal status matched G6PD deficient and normal subjects.

Variable	G6PD Normal	G6PD Deficient	P value
Hemoglobin (Hb)	12.0±2.2	12.1±1.9	0.772
Hematocrit (HCT)	38.6±6.6	38.5±5.9	0.914
Mean Cell Volume (MCV)	77.5±9.1	79.3±8.9	0.331
Mean Cell hemoglobin (MCH)	24.1±3.4	25.0±3.4	0.167

P value was calculated using the independent sample student "t" test.

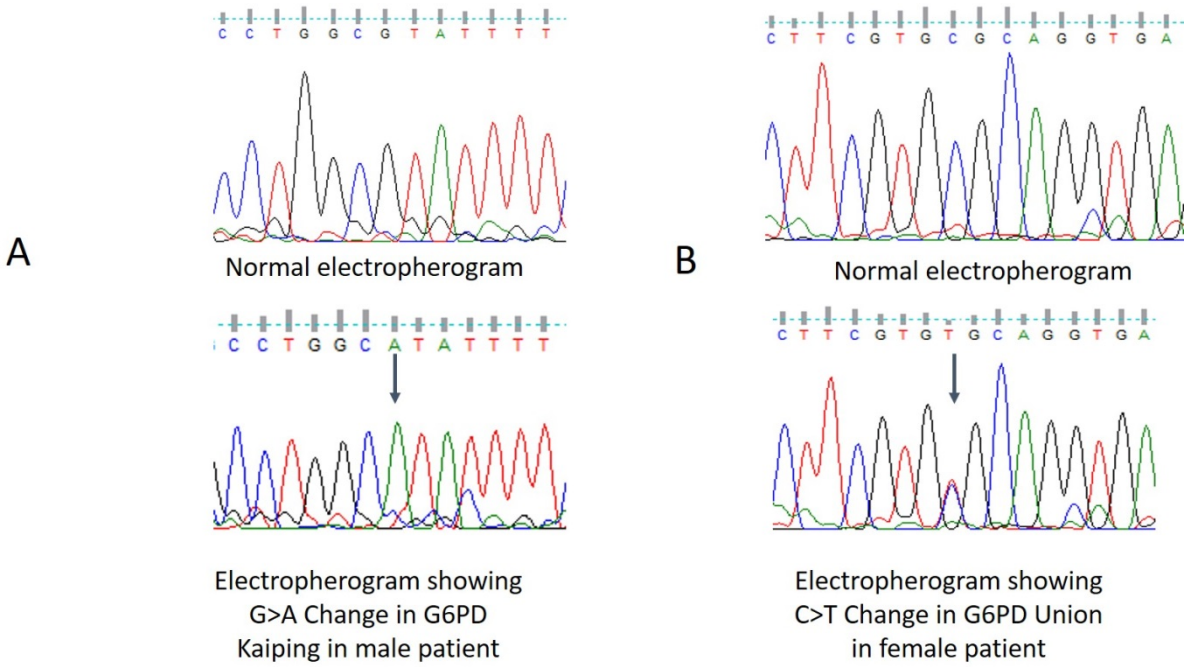

Figure 4. Electropherograms of the G6PD Kaiping and G6PD Union variants.

Discussion. The Indian population comprises numerous tribal communities, each with common physical, cultural, and genetic traits. Due to the high degree of endogamy and consanguineous marriages, hereditary diseases are common among tribal communities leading to a high degree of morbidity and mortality. The Odisha state in India is recognized as a malaria hyper-endemic area and G6PD deficiency is a common inherited red cell enzyme disorder in this region.[10] The various tribal communities inhabiting Odisha state have been screened for G6PD deficiency from time to time and prevalence of G6PD deficiency ranges from 0.3 to 30.7% in different tribes.[2,10,11,15] Recently Kumar et al. carried out a meta-analysis and reported an 8.5% prevalence of G6PD deficiency in the Indian population.[2] In the present studies, a 2.97% prevalence for G6PD deficiency was observed. However, the prevalence of G6PD deficiency varies from 0.5% to 9.6% in different studied cohorts. The prevalence of G6PD deficiency was higher in the community-based cohort, whereas in the hospital setting a very low prevalence of G6PD deficiency was observed. This suggests that for the identification of accurate estimates of prevalence, community-based screening is the best approach. Furthermore, the distribution of G6PD deficiency in the community-based cohort was also heterogeneous. The cline witnessed may be due to migration and isolation rather than to the effect on selection. The observed prevalence of G6PD deficiency in tribals was 4.82% and 2.39% in non-tribal communities.

G6PD deficiency was higher among males as compared to females, which is in concordance with previous studies and inheritance patterns. Since G6PD deficiency is X linked disorder, males can be G6PD deficient (hemizygous) or G6PD normal genotype, and females can be homozygous or heterozygous for G6PD mutation. Furthermore, due to random X chromosome inactivation, RBCs in heterozygous females have a mosaic pattern based on the G6PD allele expressed. The relative ratio of the two RBC populations governs the G6PD activity in females. These ratios range from a high proportion of RBCs with the normal G6PD enzyme to a high proportion of G6PD deficient RBCs.[16] May et al. reported that only 14% of heterozygous females have deficient G6PD activity, and 33.3% had intermediate activity, whereas over 50% of heterozygous females had normal G6PD enzyme activity.[17]

Individuals having malaria infection with G6PD deficiency are prone to drug induced hemolytic anemia. In the present studies, it was observed that the individuals having G6PD deficiency required blood transfusion during malaria treatment. However, due to the absence of medical records, we were unable to ascertain the information about the type of malaria infection or drug administered. Likely a recent study in Africa,[18] no clinical association between G6PD deficiency and malaria was observed, and that further confirms that G6PD deficient subjects remain asymptomatic unless perturbated by exogenous factors such as antimalarial drugs.

More than 400 G6PD variants have been described so far on the basis of their biochemical and functional characteristic. On the basis of molecular defect alone, 217 unique G6PD variants have been described to date.[18,19,20] However, of all recognized G6PD variants, only 10% variants have been characterized at the structural and functional levels. The spectrum of G6PD mutations found in India has not been studied in detail.

Around 13 mutations have been reported so far from India.[20] Based on previous reports, G6PD Mediterranean mutation (563C→T) has been identified as the most prevalent mutation followed by G6PD Kerala-Kalyan (949G→A) and G6PD Orissa (131C→G). Other mutations such as G6PD Chatham, G6PD Insuli, G6PD Coimbra, G6PD Nilgiri, G6PD Gond, G6PD Namoru, G6PD Dindori, G6PD Jammu, G6PD Porbandar, and G6PD Andhra Pradesh have been reported sporadically.[21-24] It is interesting to note that none of the individuals tested carried G6PD Mediterranean or G6PD Kerala-Kalyan mutation suggesting an extensive heterogeneity in the spectrum of mutation across different populations.

In the present studies, G6PD Kaiping (c.1388G>A, p.Arg463His) and G6PD Union (C.1360C>T, p.Arg454Cys) mutations were encountered; these mutations were hitherto not reported from any Indian population. We noted G6PD Kaiping mutation in 3 unrelated individuals belonging to 3 different ethnicities suggesting a common origin or hotspot for G6PD mutations. G6PD Kaiping has been classified as Class II variant (G6PD residual activity less than 10%). G6PD Kaiping is a common G6PD mutation in China[25] and also reported from other South East Asian countries like Vietnam,[26] Thailand,[27] and Indonesia.[28] Wang et al. in 2008[29] reported G6PD Kaiping in 2 Malaysians of Indian Origin. It is interesting to note that the mutations found by us in this endemic malaria area of India are entirely different from those found in the endemic malaria area of Burkina-Faso (Africa).[18]

On the other hand, G6PD Union (class II variant) has a worldwide distribution.[30] Rovira et al.[31] reported that G6PD Union individuals found in Spain are of gypsy origin. Gypsies belong to a tribal group that originated from the North West of India that migrated into central and Western Europe. The presence of this mutation supports the migratory link.

In the present studies, the clinical history of the individuals was obtained only through personal interviews, most of them were unable to recall the name of the antimalarial drugs which they were administered for malaria due to low literacy, as a consequence we were unable to analyze the effect of antimalarial drug in G6PD deficient individuals. This lack of information is a bias or limitation of the study.

We carried out a systematic analysis involving clinical hematological and molecular approach for the G6PD deficiency in both hospital and community settings. The cumulative frequency of G6PD was found to be low in hospital settings as compared to community settings. G6PD deficient individuals were at high risk for hemolytic anemia and needed blood transfusions during malaria treatment. The presence of various G6PD mutants, in particular, G6PD Kaiping and Union in the study area, indicates migratory link and genetic drift. However, further community-based studies need to be carried out to determine the prevalence, distribution and phenotypic correlation of these variants in different ethnic groups and in different geographical areas.

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