Prevalence and Genetic Characterization of Glucose-6-Phosphate Dehydrogenase Deficiency in Anemic Subjects from Uttar Pradesh, India

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is caused by one or more mutations in the G6PD gene on chromosome X. It affects approximately 400 million people worldwide. The purpose of this study was to detect the prevalence of G6PD deficiency and G6PD gene mutations in the hospital-based settings in patients referred for suspected G6PD deficiency. A qualitative fluorescent spot test and dichlorophenol-indolphenol (DCIP) test were performed. G6PD-deficient, positive samples were further processed for mutation analysis by Sanger sequencing. Out of 1,069 cases, 95 (8.8%) were detected as G6PD deficient (by DCIP test) and were sent for molecular analysis. The G6PD Mediterranean mutation (563C > T) is the most common variant among G6PD-deficient individuals followed by the Coimbra (592C→T) and Orissa (131C→G) variants. We concluded that all symptomatic patients (anemic or jaundiced) should be investigated for G6PD deficiency. Our findings will inform our population screening approach and help provide better management for G6PD-deficient patients.

Keywords

- G6PD
- gene
- mutation

Introduction

Glucose-6-phosphate dehydrogenase (G6PD), the house-keeping enzyme, catalyzes the conversion of nicotinamide adenine dinucleotide phosphate (NADP⁺) to its reduced form NADPH via hexose monophosphate (HMP) pathway. The NADPH from HMP pathway is the only source for red blood cells (RBCs) to maintain the glutathione level to protect itself from oxidative injury.¹ G6PD deficiency is the most frequent hereditary disorder in the world. Mutations in the G6PD gene cause its deficiency and enzymes have diminished ability to withstand oxidative stress leading to the destruction of RBC.² Affected individuals are mostly asymptomatic, but exposure to certain drugs, infection, and food items (e.g., Fava bean), which can produce free radicals, may trigger acute hemolysis. Other substances that can induce acute hemolysis are cosmetics such as henna (hena), and household chemicals such as naphthalene. Therefore, the effective management of

G6PD deficiency is avoiding oxidative stresses by avoiding the above-mentioned items.³

Glucose-6-phosphate dehydrogenase deficiency affects more than 400 million people worldwide with variable incidences.⁴ In India, the prevalence ranges from 2.3 to 27%, and the variation is population specific. A higher prevalence is reported among tribal groups of India.² Also, the high prevalence of G6PD deficiency correlates well with the geographic distribution of malaria, supporting the notion that G6PD deficiency carriers are protected against malarial infection.⁵

The G6PD gene, located on the distal long arm of the X chromosome, consists of 13 exons and 12 introns, spanning nearly 20 kb in total.⁶ Since the gene is located on the X chromosome, the males are always hemizygous for mutation while females can be homozygous and heterozygous. In females, homozygous, compound heterozygous, or skewed X-inactivation of normal chromosomes in the heterozygous state may produce symptoms. More than 200 G6PD gene mutations,

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which lead to amino acid substitutions, have been reported worldwide.⁷ The G6PD enzyme produced by missense variants shows variable enzyme activity, classified as class I (a severe form of deficiency) through class IV (mild or no deficiency).⁷ As per available reports, the three most commonly deficient variants are the G6PD Mediterranean mutation (563C→T), Kerala-Kalyan variant (949G→A), and Orissa variant (131C→G), which are common in the Indian population.^{8,9}

The diagnosis of G6PD deficiency can be done by a qualitative or a quantitative method. The qualitative methods such as the fluorescent spot test (FST) and dichlorophenol-indolphenol (DCIP) decolorization method are the most preferred ones and are cost-effective methods used for mass screening of suspected G6PD-deficient patients.⁴ Followed by a qualitative test, it is important to know the molecular basis of G6PD deficiency by mutational analysis for specific diagnosis and for prevention of disease in the future generations. Therefore, this study was aimed in estimating the prevalence of G6PD deficiency among suspected cohorts and in providing molecular data for better management of such cases. An improved management regime includes diet modifications and patient education, formulated at the Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, Uttar Pradesh, India, for a 3-year period. The molecular analysis was performed only in the cases with positive screening test results.

Materials and Methods

This study was performed from July 2014 to June 2017 (3 years) in the Genetic Hematology Laboratory of the Department of Medical Genetics at SGPGIMS, Lucknow, Uttar Pradesh, India. Patients who were presented with neonatal jaundice or episodes of acute hemolytic anemia or with findings of jaundice, fatigue, back pain, and chronic non-spherocytic anemia suspected of G6PD deficiency were referred to genetic hematology for G6PD investigation.

After obtaining consent for participation, 3 mL venous blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube from anemic patients with suspected G6PD deficiency. This study was approved by our institutional ethical committee, SGPGIMS, Lucknow, Uttar Pradesh, India.

Glucose-6-phosphate dehydrogenase Diagnosis

About 1 mL blood was used for G6PD qualitative screening by a modified FST developed by Butler and Mitchell⁴ and DCIP decolorizing test (DCIP Bernstein test).

In DCIP test, NADP reduces the dye DCIP into a colorless state through the action of G6PD. Decolorization rate and degree are proportional to the G6PD activity in RBCs. Phenazine methosulfate (PMS) is used as an electron carrier between NADPH and 2,6-DCIP in test. To a test tube containing 50 μ L of PMS, 25 μ L of G6P, and 25 μ L of NADP, 20 μ L of whole blood and 400 μ L of DCIP dye solution were added, followed by addition of 1 mL of distilled water. This mixture was overlaid by liquid paraffin and incubated at 37°C for 10 minutes. The color of the mixture changes from blue to red in the presence of G6PD deficiency within 10 minutes (► Fig. 1).⁴

Glucose-6-phosphate dehydrogenase-deficient samples were evaluated by the fluorescence test following the method of Butler and Mitchell. Presence of fluorescence of reduced NADPH under ultraviolet light indicate the presence of G6PD. In this test, 2 mL of whole blood was added to 100 μ L of the screening mixture containing 2 mL G6P anhydrous (0.01 mol/L), 2 mL NADP (7.5 mmol/L), 2 mL glutathione disulfide oxidized (8.0 mmol/L), 4 mL saponin (1% v/v), 6 mL Tris-HCl buffer, pH 7.8 (750 mmol/L), and 4 mL deionized water to prepare 20 mL of solution. The solution was stored in a dark reagent bottle at 4°C and kept stable for several months. A spot of this mixture was collected on Whatman 1 filter paper, allowed to air dry at room temperature for 5 minutes, and examined for fluorescence with an ultraviolet lamp in a dark room. A distinct fluorescence indicated normal G6PD activity, while no fluorescence was observed with G6PD-deficient blood (less than 25% of normal).⁴

In the DCIP decolorization method, the presence of G6PD in red cells is detected by the decolorization of the dye within a specified time. It has an advantage over the FST in that the heterozygotes can be easily detected and a lot of samples can be processed together at the same time.¹⁰ We have followed decolorization time up to 20 minutes and the females (obligate carriers) have shown decolorization beyond 20 minutes (as a partial activity of enzyme is present). In our study, no female was found to have G6PD deficiency or

Fig. 1 Dichlorophenol-indolphenol decolorizing test (DCIP Bernstein test) for screening glucose-6-phosphate dehydrogenase (G6PD) deficiency. Normal sample (A–D and F) showing red color with normal G6PD enzyme activity and blue color (E) belongs to a G6PD-deficient sample.

Fig. 2 Sequencing results of common three mutations found in the present study population. Glucose-6-phosphate dehydrogenase (G6PD) Mediterranean mutation (A, wild-type; B, Mutant), G6PD Coimbra mutation (C, wild-type; D, Mutant), and G6PD Orissa (E, wild-type; F, Mutant).

was an obligate carrier. DCIP test is the method of choice when screening large sample size.

As Brewer's test allows only semiquantitative evaluation of an intermediate susceptibility of G6PD enzyme and a limited number of samples can be performed in one batch, we used FST and DCIP test for G6PD diagnosis.

Positive samples from both the FST and DCIP tests were further processed for mutation analysis. The remaining 2 mL of blood in the EDTA tube was used for the estimation of hematological parameters using an automated red cell counter (Sysmex 21), and genomic DNA was extracted by phenol-chloroform method as described by Poncz et al¹¹ in 1983.

DNA was quantified using NanoDrop and the quality was checked on 0.8% agarose gel.

Sanger Sequencing

Genetic analysis was performed by direct sequencing of the *G6PD* gene for exon 2, 6, and 8. Polymerase chain reaction (PCR) contained genomic DNA (100 ng), PCR forward primer (5 pmol), PCR reverse primer (5 pmol), dNTPs (10 mM), 0.5 U Taq DNA polymerase (Qiagen, 5 U/μL), and 10× PCR buffer (Bio Labs) containing 1.5 mM MgCl₂ of 25 μL total volume. Sequencing of PCR products was performed by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City,

California, United States), and products were resolved on the ABI 3130XL Genetic Analyzer (Applied Biosystems). Sequence electropherograms were analyzed by using Finch TV.

Results

A total of 1,069 anemic patients were enrolled in the study (–Table 1). Of the 1,069 subjects, 95 (8.8%) were found to be G6PD deficient. In the DCIP test, we have followed decolorization time of up to 20 minutes to identify obligate carriers (females) as decolorization can be seen due to partial enzyme activity, but no female was found to be G6PD deficient and an obligate carrier. The hematological parameters of G6PD-deficient patients and other anemic individuals are presented in –Table 2. Various studies showing the prevalence of G6PD deficiency in India are presented in –Table 3.

To find out mutation in deficient subjects ($n = 95$), Sanger sequencing was performed for exon 2, 6, and 8 of the *G6PD* gene. Three variants, including Mediterraneanc.563C > T (p. Ser188Phe), G6PD Coimbrac.592C > T (p.Arg198Cys), and G6PD Orissa c.131C > G (p.Ala44Gly), were identified among these 95 deficient subjects (–Fig. 2). The most common variant was the Mediterranean mutation 563C > T with a frequency of 92% (87/95), followed by Coimbrac.592C > T (4%, 4/95) and G6PD Orissa c.131C > G (4%, 4/95).

Table 1 Demographic parameters of study population

Parameter	Values
Age	Newborn–50 y
Gender	
Male	449 (42%)
Female	620 (58%)

Table 2 Reference values to describe the G6PD activity profile for study population

Hematological parameter	Patients with G6PD deficiency ($n = 95$) Mean $\pm$ SD	Patients without G6PD deficiency ($n = 974$) Mean $\pm$ SD	p-Value
Hb (g/dL)	7.16 $\pm$ 2.4	9.2 $\pm$ 2.1	< 0.0001
Hematocrit (%)	32.76 $\pm$ 6.99	34.1 $\pm$ 4.33	NS ^a
MCV (fL)	56.02 $\pm$ 7.99	69.2 $\pm$ 3.59	< 0.0001
MCH (pg/cell)	19.13 $\pm$ 2.9	23.3 $\pm$ 3.24	NS ^a
MCHC (g/dL)	26.08 $\pm$ 1.63	34.6 $\pm$ 2.64	NS ^a

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NS, not significant; SD, standard deviation.

^aThe p-values are not statistically significant.

Table 3 Various studies showing prevalence of G6PD deficiency in India

Name of author	Year	Place	Prevalence %
Baxi et al ²⁴	1961	Mumbai	13.64
Swaroop et al ²⁵	1961	Kolkata	5
Baxi et al ²⁶	1963	Mumbai	15.7
Meera Khan ²⁷	1964	Andhra Pradesh	4.7
Khanduja et al ²⁸	1966	Delhi	2.76
Deshmukh and Sharma ²⁹	1968	Aurangabad	6
Da Costa et al ³⁰	1969	Mumbai	5.1
Inamdar and Pohowalla ³¹	1969	Indore	6.2
Kalra et al ³²	1973	Agra	5
Agarwal et al ³³	1975	Lucknow	1.7
Sharma et al ³⁴	1975	Rohtak	0.6
Praharaj et al ³⁵	1977	Orissa	11
Anand et al ³⁶	1981	Rajasthan	12
Choubisa et al ³⁷	1987	Udaipur, Rajasthan	4.59
Verma et al ³⁸	1990	–	3.9
Choubisa ³⁹	1991	Dungarpur, Rajasthan	11.25
Pant et al ⁴⁰	1992	Kheda, Gujarat	5.9
Thakur and Verma ⁴¹	1992	Bastar	25
Ramadevi et al ⁴²	1994	South India	7.8
Reddy et al ⁴³	1995	Central India	4.57
Kaeda et al ⁴⁴	1995	Baiga, Madhya Pradesh	4.2
Joshi et al ⁴⁵	2001	Surat, Gujarat	9.73
Shankar Kumar ⁴⁶	2003	Dhule, Maharashtra	9
Balgir et al ⁴⁷	2004	Orissa	4.3–17.4
Sukumar et al ⁴⁸	2004	Mumbai	10.5
Gupte et al ⁴⁹	2005	Surat, Gujarat	22
Pao et al ⁵⁰	2005	–	2.0
Nishank et al ⁵¹	2008	Bhubaneswar, Orissa	6.4
Choubisa ⁵²	2009	Sirohi, Rajasthan	15.21
Shanthala Devi et al ⁵³	2010	South and West India	0.8
Rai and Kumar ⁵⁴	2012	Jaunpur	10
Rai and Kumar ⁵⁵	2014	Jaunpur	8.5
Singh et al ⁵⁶	2014	Madhya Pradesh	3.7
Mukherjee et al ¹	2015	Country scenario	2.3–27.0
Goyal et al ⁵⁷	2015	North India (New Delhi)	1.5
Moinuddin et al ⁵⁸	2017	Bengaluru	0.40
Present study	2017	Lucknow	8.8

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

Discussion

The G6PD deficiency disorder has a wide spread distribution in the world and is more common in malaria endemic regions of tropical Africa, the Mediterranean, the Middle East, and the Indian subcontinent.^{12,13} The frequency of the deficiency is highest among the populations of sub-Saharan Africa, where the prevalence peaks around 30% in several areas, but is absent from parts of southern Africa and communities in the Horn of Africa. China and India, for instance, were estimated to be home to 41.3% of all G6PD-deficient males across the whole malaria endemic region globally. The high allele frequencies seen in Africa meant that 28.0% of the overall deficient population was in sub-Saharan Africa, while only 4.5% of the global population burden was in the Americas and 67.5% across Asia.¹⁴

In India, regional-wide variation in the incidence of G6PD deficiency ranges from 2.3 to 27.7%.² Biochemical screening followed by mutational profiling of G6PD deficiency among the diverse Indian population is being focused. The three most common mutations, Mediterranean, Orissa, and Kerala-Kalyan, account for most of the G6PD deficiency cases reported in the Indian subcontinent.^{6,15}

This study was conducted with the aim of finding out G6PD gene mutations among cases referred to the SGPGIMS for anemia. In the first step, we performed G6PD screening by FST and DCIP test to eliminate the other causes of anemia. We observed 8.8% cases as G6PD deficient among the referred cases of anemia (1,069). Among 95 deficient cases, G6PD Mediterranean (92%) was found as the most prevalent mutation followed by Coimbra (4%) and Orissa (4%).² The Coimbra and Orissa mutations are equally prevalent. In contrast to the reports, we did not find Kerala-Kalyan mutation in our study cohort.

The G6PD Mediterranean c.563C > T (p.Ser188Phe) is located on exon 6 of the *G6PD* gene and is the most common variant that leads to complete absence of enzyme activity (class II).¹⁰ This variant is due to a substitution of (C→T) nucleotide at 563, changing serine to phenylalanine at amino acid 188.⁸ Similar to other reports, in our study the Mediterranean mutation was the most common.

The G6PD Coimbra c.592C > T (p.Arg198Cys) transition results in the substitution of cysteine for arginine. Also, it was found that the mutation is located at the end of the coenzyme domain, which may cause enzyme instability¹⁶ and falls under the category of class II mutations.

The G6PD Orissa c.131C > G (p.Ala44Gly) substitution of alanine by a glycine residue at position 44 affects one of the residues of dinucleotide-binding fingerprint, resulting in a different variant with increased Km for NADP⁺. In wild-type, the alanine residue has a methyl side chain, whereas substituted glycine has a hydrogen atom instead of methyl group and the resulting change causes relatively reduced binding affinity of G6PD enzyme to NADP, explaining moderate effect on the enzyme activity.² This mutation is highly prevalent among the tribal population of Orissa, India.¹⁷

The G6PD Mediterranean is one of the most common polymorphic variants. It is characterized by very low activity (less than 5% of normal), and is associated with favism and acute hemolytic anemia caused by infection or the ingestion of

certain drugs. One mutation placed at nucleotide (nt) 592 (G6PD Coimbra) has been identified as responsible for a phenotype similar to that of the G6PD Mediterranean. It could have been hypothesized that alterations in this part of the gene could lead to similar biochemical properties and affecting the surrounding G6P binding site.^{18,19}

Glucose-6-phosphate dehydrogenase -deficient persons are predisposed to the development of sepsis, complications related to sepsis after a severe injury, hepatitis, malaria, and drug intake. These are the main factors causing hemolytic crisis in G6PD-deficient children.^{2,20} Since malaria and G6PD deficiency share similar geographical distribution, treatment of malaria patients with antimalarial drug primaquine or other 8-aminoquinolines may be associated with potential hemolytic anemia.

Our study shows that G6PD deficiency is prevalent among the anemic individuals of our study population and may be contributing to their anemia severity by making them more susceptible to hemolysis. However, there are some other causes of anemia which include iron deficiency anemia, thalassemia, sickle cell anemia, hereditary spherocytosis, pyruvate kinase deficiency, and red cell membrane disorder. The G6PD-deficient individuals become subjects of hemolysis if exposed to fava beans (which contains divicine and isouramil glycosides responsible for hemolysis), naphthalene (used for storing clothes), Zn-thiocarbamate (antifungal spray used in the fields), aniline dyes (food-coloring agents), etc.^{2,21} Several drugs can also precipitate hemolysis in G6PD-deficient subjects. These drugs can interact with hemoglobin and oxygen, leading to the intracellular formation of hydrogen peroxide (H₂O₂) and other oxidizing radicals. As these oxidants accumulate within an enzyme-deficient cell, hemoglobin and other proteins are oxidized, leading to the loss of function and cell death. A study by Elyassi et al²² shows the effects of certain anesthetic agents, such as halothane, isoflurane, ketamine, sevoflurane, propofol, diazepam, and midazolam, on enzymatic activity of G6PD. Although isoflurane, sevoflurane, diazepam, and midazolam had an inhibitory effect on G6PD activity in vitro, halothane, ketamine, and propofol had none. The most effective management strategy for the G6PD-deficient patient in case of a hemolytic crisis by any hemolytic agent is to avoid the offending agent and to monitor the patient closely.^{22,23}

Also, a severe form of deficiency of G6PD can cause jaundice at birth and chronic hemolytic anemia in later life. Thus, it is important to screen for G6PD enzyme deficiency before intervention of any kind.

As its inheritance is X-linked recessive form, females are obligate carriers while males are more commonly and severely affected. Therefore, G6PD deficiency screening is an effective way to detect the enzyme-deficient male individuals. Furthermore, extended family screening to find out obligate carriers and genetic counseling should be provided at the proper counseling center. This argument is in line with our finding that 8.8% of identified individuals are males and no female with severe form of G6PD deficiency was identified. However, it should be noted, at times, that these screening methods may not detect the heterozygous females though we extended the observation time to 20 minutes.

Conclusion

This study explored the overall incidence (8.8%) of G6PD in our hospital-based anemic referrals; however, the incidence of G6PD deficiency in the general Uttar Pradesh population is likely to be lower. So, screening and educational program for G6PD deficiency are required to identify patients at risk. The molecular analysis of deficient subjects identified a higher frequency of the Mediterranean (563C→T) mutation followed by Coimbra (592C→T) and Orissa (131C→G) mutations.

With the prevalence of 8.8% G6PD deficiency among the anemic subjects, we suggest that all anemic neonates as well as chronic/acute adult subjects should be screened for the G6PD enzyme deficiency before administration of any oxidant, anti-malarial, or antibacterial drugs that is likely to cause hemolysis. The cost of the combined DCIP and mean corpuscular volume tests would be cheaper and can be used in large scale for population screening. Lastly, there is a need of awareness program for pediatrician and other clinical practitioner to ensure cost-effective diagnosis and management of G6PD-deficient individuals, to avoid any drug-induced complications.

Since we do not have a screening test to detect obligate carrier (with higher accuracy) and the quantitative assay shows only the enzyme activity, so for definitive diagnosis on the molecular level mutation detection is the only reliable test at present. This study reveals that the most common mutations responsible for G6PD deficiency are the Mediterranean c.563C > T (p.Ser188Phe) (92%), Coimbra c.592C > T (p.Arg198Cys) (4%), and G6PD Orissa c.131C > G (p.Ala44Gly) (4%) mutations. Out of the three common mutations of G6PD deficiency, the Mediterranean c.563C > T (p.Ser188Phe) mutation was found to be of higher prevalence causing G6PD deficiency among the present study population. Therefore, screening and educational programs for G6PD deficiency are required to identify patients at risk.

Conflict of Interest

The authors report grants from ICMR, New Delhi, and UPCST, Lucknow, during the conduct of the study.

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