Suez Canal University Medical Journal Vol. 26 (1), 2023
Pages 100-106

Assessment of the Frequency of the G6PD Mediterranean Gene Mutation 563 C→T(rs5030868) in Children attending Suez Canal University Hospital

Ahmed A. Elhadidy[1*], Alaa ELdin S. Abdelhamid[1], Fadia M. Attia[1], Gehan Ibrahim[1], Marwa A. Ibrahim[2]

[1]*Department of Clinical Pathology, Faculty of Medicine, Suez Canal University, Egypt*
[2]*Department of Pediatrics, Faculty of Medicine, Suez Canal University, Egypt*

Abstract

Background: **Glucose-6-phosphate dehydrogenase (G6PD) deficiency is known to be the most common enzymopathy in the world affecting ~7% of the global population. The G6PD gene, located in the long arm of chromosome X (Xq28), consists of 13 exons and 12 introns encoding 515 amino acids. Over 200 G6PD mutations have been reported worldwide, where 140 mutations or combined mutations are found to be hematologically important.** ***Aim:*** **To assess the frequency of the G6PD Mediterranean (Med) gene mutation 563 C→T in children attending Suez Canal University Hospital and its relation to G6PD silent gene mutation 1311 C→T.** ***Subjects and Methods:*** **This study was conducted on 50 G6PD deficiency patients who were previously diagnosed and confirmed by quantification of G6PD enzyme activity, 44 of them were males while 6 patients were females. The control group was 20 apparently healthy children with matched age and sex with the patients' group. Each of the study groups was tested for the following: complete blood count, G6PD enzyme activity quantification, and testing the presence or absence of the G6PD Med gene mutation and G6PD silent gene mutation using real-time PCR.** ***Results:*** **The current study shows the incidence of G6PD Med gene mutation in 26 patients (52%) and that 96.2% of G6PD patients with G6PD Med gene mutation had G6PD silent gene mutation.** ***Conclusion:*** **The current study shows the incidence of G6PD Med gene mutation in 52% of patients and that 96.2% of G6PD patients with Med Gene mutation had G6PD silent gene mutation. The presence of either G6PD Med gene mutation or both G6PD Med gene mutation and G6PD silent gene mutation together were insignificant in the prediction of G6PD status whether diseased or not. G6PD deficiency could be a consequence of a complex multifactorial mechanism probably related to both environmental factors and genetic modifiers.**

Keywords: G6PD- Mediterranean- Anemia- Mutation

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is known to be the most common enzymopathy in the world affecting ~7% of the global population. Elella et al. in Egypt screened 2782 newborns for G6PD deficiency where 4.3% of them were affected with male to female ratio 3.2:1[(1,2)]. The G6PD deficiency causes multiple abnormalities that span from asymptomatic individuals to patients presented with

[*]*Corresponding Author: dr.ahabobakr@gmail.com*

neonatal jaundice, acute episodes of hemolysis elicited by exogenous agents (acute infections, drugs, or certain types of food) or chronic non-spherocytic hemolytic anemia[3]. One of the essential functions of G6PD is producing a reduced form of nicotinamide adenine dinucleotide phosphate which is necessary for reductive biosynthetic reactions as well as for the stability of catalase and safeguarding the reduced form of glutathione. Catalase and glutathione are needed for the detoxification of H_2O_2. The cell protection from this free radical hinges on G6PD activity since the red blood cells lack other enzymes that produce NADPH[4]. The G6PD gene, located in the long arm of chromosome X (Xq28), consists of 13 exons and 12 introns encoding 515 amino acids. Over 200 G6PD mutations have been reported worldwide, where 140 mutations or combined mutations are found to be hematologically important[5]. The World Health Organization classifies G6PD genetic variants into five classes, the first three of which are deficiency states. Class I: severe deficiency (<10% activity) with chronic non spherocytic hemolytic anemia, Class II: severe deficiency (<10% activity) with intermittent hemolysis ,Class III: moderate deficiency (10-60% activity) and hemolysis with stressors only, Class IV: non-deficient variant with no clinical sequelae and Class V: increased enzyme activity with no clinical sequelae[3,6]. The occurrence of different gene mutations has been categorized in certain populations and it was found that Class II 563 C→T mutation is common in Mediterranean populations .This variant is characterized by <10% of normal G6PD activity, making it a severe form of the disease[7]. The Mediterranean variant is due to a point mutation (C→T) at nucleotide 563, leading to the substitution of serine to phenylalanine at amino acid position 188 in G6PD[8]. An additional G6PD silent mutation at nucleotide 1311 is often detected in patients from the Mediterranean region and Middle East. Many studies revealed high frequency of G6PD silent gene mutation (1311C→T) in patients having G6PD Med mutation [9]. Different studies were conducted in Egypt to identify the most prevalent G6PD mutation[9,10] but the results were variable; therefore, different molecular studies should be performed covering different areas of Egypt due to the complexity and the different ethnic origins of the Egyptian population. Therefore, this study was conducted to identify the prevalence of the G6PD Med gene mutation and silent gene mutation among the G6PD deficient children in Ismailia city.

Patients and Methods

Research design

The study is an observational, case-control study. Children were recruited from the outpatient pediatric hematology clinic of the Suez Canal university hospital, Ismailia. Lab tests were performed at the clinical pathology department of the Suez Canal university hospital, Ismailia.

Study population

The study included 50 children (<18-year old) who were diagnosed with G6PD deficiency anemia. Twenty apparently healthy age and gender-matched children who attended Suez Canal university hospital for regular check-up served as a control group. All groups were enrolled to participate in the study and the children's parents signed study consent. The study excluded patients with hemolytic anemias other than G6PD deficiency (e.g., thalassemia) and those who had a history of G6PD attack or blood transfusion 3 months prior to the time of sampling.

Methods

Data collection

A) Descriptive data: Age, sex, date of last hemolytic attack.

B) Laboratory assessment:

Four mL of venous blood was collected from each child as follows:

- Two ml of venous blood in EDTA tube for complete blood count (CBC) and quantitative measurement of G6PD enzyme activity using the direct enzyme activity assay spectrophotometry.
- Two mL of venous blood in EDTA tube for molecular assessment: DNA isolation and genotyping of G6PD Med gene mutation (563 C→T) (rs5030868) and G6PD silent gene mutation (1311 C→T) (rs22300037).

Hematological assessment

Complete blood count (CBC) using automated hematology cell counter (Sysmex XN-550 fully automated hematology cell counter).

Quantitative measurement of G6PD enzyme activity using the direct enzyme activity assay spectrophotometry.

According to the instructions supplied by kit provided by ARENA-BioScien quantitative assessment of G6PD enzyme activity, G6PD in RBCs is released by lysing agent present in the reagent. The G6PD released from the red cells catalyzes the Glucose-6-phosphate with reduction of NADP to NADPH. The rate of reduction of NADP to NADPH is measured as an increase in absorbance at 340 nm produced in the reaction catalyzed by the enzyme which is proportional to the G6PD activity in the sample used[11]. The procedures provided in the manual with the kit were followed. The absorbance was taken 2 min after reaction mixture was added with blood and final value was calculated by multiplying absorbance (ΔA) with factor 4778 in Human Semi-automated analyzer divided by hemoglobin concentration of patients. The normal value of the G6PD activity at 37 °C is 8.7 ± 1.7 IU/g hemoglobin[12].

The molecular confirmation of the presence or absence of the G6PD Med gene mutation (563 C→T) and G6PD silent mutation at position (1311 C→T) by real-time PCR

Genotyping

Genomic DNA was extracted from 200 µL peripheral whole blood sample using commercially available Spin-column technique. The QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA, USA) purified genomic DNA from peripheral blood leukocytes.

DNA purity and concentration

The DNA yield and purity were assessed spectrophotometrically using the Thermo Scientific NanoDrop™ Spectrophotometer (Nanodrop) *(Thermo Fisher Scientific Inc. USA).*

Real-time PCR reaction mixture preparation

In an appropriate tube, the components were combined: (TaqMan Universal Master Mix II, 2X), TaqMan genotyping assay mix (20X) and DNase-free water, then the tubes were capped and vortexed to mix the solutions. Into each column, the PCR reaction mix volume was pipetted, the columns were sealed with a MicroAmp clear adhesive film, then centrifuged briefly to spin down the contents. 1 to 10 ng of genomic DNA or control DNA were needed for each reaction in the appropriate volume. The columns were sealed using MicroAmp Optical Caps and loaded into Applied Biosystems 7500 Fast Real-Time PCR Systems. After PCR amplification, end point plate readings were performed on 7500 Fast Real-Time PCR Systems analyzer and software. Real-time PCR and melting curve analyses was performed using 7500 Fast Real-Time PCR Systems applied biosystem © USA, analyzer and software.

Step	Temp (°C)	Duration	Cycles
AmpliTaq Gold, UP Enzyme Activation	95	10 min	HOLD
Denature	95	15 sec	40
Anneal/Extend	60	1 min	

Two TaqMan® MGB probes were used for the SNP: One probe labeled with VIC® dye detected the Allele 1 sequence of G6PD Med gene mutation 563 C→T and other probe labeled with FAM™ dye detected the Allele 2 sequence of G6PD silent gene mutation.

Results

Demographic data

Tables (1 and 2) show that there were no statistically significant differences in gender and age distribution among both study groups. The mean age was 5.48 ± 2.3 and 5.98 ± 2.81 for the control and diseased groups respectively. The control group were 20 apparently healthy children with matched age and sex with the patients' group. Each of the study groups were tested for the following: complete blood count, G6PD enzyme activity quantification and testing the presence or absence of the G6PD Med gene mutation (563C→T) and G6PD silent gene mutation (1311C→T) using Realtime PCR.

Table 1: Genders' distribution among study groups

Group	Control	Diseased	p-value
Gender	n (%)	n (%)	
Male	18 (90)	44 (88)	0.812
Female	2 (10)	6 (12)	
Total	20 (100)	50 (100)	

Chi-squared test. p-value significant when < 0.05.

Table 2: Age distribution among study groups (Means and SDs)

Group	Control	Diseased	Test	p-value
Variable	Mean ± SD	Mean ± SD		
Age (years)	5.48 ± 2.3	5.98 ± 2.81	M	0.542

Mann-Whitney U test. p-value significant when < 0.05.

Molecular studies

Table (3) shows that there was a statistically significant difference between study groups regarding the distribution of G6PD Med gene mutation as 25 of the G6PD deficient males and 1 of G6PD deficient females (52%) of the sample population expressed the Med gene mutation with similar incidence reported by El Gezeiry et al.[13] which were 43.6 % but these results are slightly lower than Arnaout et al.[9] and Hafez et al.[10] which was 60 % and 62 % respectively while the highest incidence was reported by Osman et al. being 94.7 %[14]. While the current study shows the incidence is higher than those of Rizk et al., Ezz El -Deen et al. and Ramadan et al.[15,7,16] which were 28% , 23% and 33% respectively. The G6PD Med gene mutation is the most common mutation in Egypt and Asian Arab countries, with frequencies ranging from a low of 53.6% in Jordan to a high of 91.2% in Bahrain [17,18]. A recent study in Syria showed that a total of 83% of patients carry the Med mutation [19], while it is the second most common variant in some African

Arab countries such as Algeria and Tunisia with frequencies of 23% and 11.4% respectively [20,21]. The mutation decreases in frequency as we move east, though it is still present in polymorphic frequencies in Malaysia [22]. Table (4) shows that there was a statistically significant difference between study groups regarding the distribution of G6PD silent gene mutation as 68% of the G6PD patients had the G6PD silent mutation, while table (5) shows that twenty-five of G6PD patients (96.2%) having Med gene mutation are positive for G6PD silent gene mutation. This is higher than the percentages reported by Arnaout et al. [9] and Ezz El-Deen et al. [7] being 77.4% and 28% respectively and near to that reported by Hafez et al. [10]. The comparison of G6PD mutations distribution found that the frequency of each variant varied between different studies, and this could be mainly attributed to the difference of patients' recruitment sites as this study was at Ismailia while other studies were at Cairo, Mansoura and Alexandria cites which may show different ethnicities. Also, there is a variability of samples' size among different studies for example Rizk et al. [15] included 21 G6PD male patients while Arnaout et al. [9] included 50 patients. The other possible cause is the difference of the methods used to detect the presence or absence of both the G6PD Med gene mutation and silent gene mutation. Most of them used PCR-RFLP technique while this study used PCR-real time.

Table 3: SNP 563's distribution among study groups

Group		Control	Diseased	p-value
Variable	*Attribute*	n (%)	n (%)	
Genotype	*Affected male*	0 (0)	25 (50)	**0.001**
	Not affected male	18 (90)	19 (38)	
	Heterozygous female	0 (0)	1 (2)	
	Not affected female	2 (10)	5 (10)	
	Total	20 (100)	50 (100)	

Chi-squared test. p-value significant when < 0.05.

Table 4: SNP 1311's distribution among study groups

Group		Control	Diseased	p-value
Variable	Attribute	n (%)	n (%)	
Genotype	Affected male	5 (25)	33 (66)	**0.006**
	Not affected male	13 (65)	11 (22)	
	Heterozygous female	0 (0)	1 (2)	
	Not affected female	2 (10)	5 (10)	
	Total	20 (100)	50 (100)	

Chi-squared test. p-value significant when < 0.05.

McDonagh et al. [23] reported that 1311T haplotype is found in populations of Mediterranean and Middle Eastern countries, but not in Asian individuals with the Mediterranean variant, suggesting the Mediterranean variant 563 might arise independently in the two geographical locations. Also, Krzelj et al. [24] stated that most individuals with the G6PD Med gene mutation originating from the Med basin possess the G6PD 1311 silent polymorphism. Table (6) shows that presence of either Med gene mutation or both G6PD Med gene mutation and silent mutation

together were insignificant in the prediction of G6PD status either diseased or not, but the presence of G6PD silent mutation is a risk factor for developing G6PD deficiency disease. These findings suggest that there are other factors that assist SNP 563 positivity in G6PD status, and that the Med mutation is not the only mutation that cause G6PD deficiency, and this could be a consequence of a complex multifactorial mechanism probably related to both environmental factors and genetic modifiers such as infection, medications, and dietary pattern of G6PD deficient individuals and the X- chromosome inactivation pattern among heterozygous females.

Table 5: SNP 1311 association with SNP 563

SNP 563	Negative	Positive	p-value
SNP 1113	n (%)	n (%)	
Negative	30 (68.2)	1 (3.8)	<0.001
Positive	14 (31.8)	25 (96.2)	
Total	44 (100)	26 (100)	

Chi-squared test. p-value significant when < 0.05.

Table 6: G6PD's disease prediction models

Model	Variable	Beta	p-value	OR	95% CI OR
1	SNP 563	21.021	0.998	1346229053.965	-
	Constant	0.182	0.547	1.200	
2	SNP 1311	1.852	0.002	6.375	1.971 - 20.615
	Constant	0.065	0.857	1.067	-
3	Both	20.980	0.998	1292379891.435	-
	Constant	0.223	0.457	1.250	-

Chi-squared test. p-value significant when < 0.05.

Conclusion

The current study shows the incidence of G6PD Med gene mutation in 52% of patients and that 96.2% of G6PD patients with Med Gene mutation had G6PD silent gene mutation. The presence of either G6PD Med gene mutation or both G6PD Med gene mutation and G6PD silent gene mutation together were insignificant in the prediction of G6PD status whether diseased or not.

References

1. Elella SA, Tawfik M, Barseem N, et al. Prevalence of glucose-6-phosphate dehydrogenase deficiency in neonates in Egypt. Ann Saudi Med 2017;37 (5):362–5.
2. Parsanathan R, Jain SK. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is linked with cardiovascular disease. Hypertens Res. 2020;43 (6) :582–4.
3. Cappellini MD, Fiorelli G. Glucose-6-phosphate dehydrogenase deficiency. Lancet. 2008;371(9606):64–74.
4. Mohamed MM, AU-RE-H. Molecular Characterization of New Variants of Glucose-6-phosphate Dehydrogenase Deficiency Gene Isolated in Western Province of Saudi Arabia Causing Hemolytic Anemia. Pakistan J Biol Sci. 2006;9(9):1605–16.
5. Jamwal M, Sharma P, Das R. Laboratory Approach to Hemolytic Anemia. Indian J Pediatr 2019; 87 (1):66–74.

6. Organization WH. Working group glucose-6-phosphate dehydrogenase deficiency. Bull WHO. 1989;67:601–11.
7. Ezz El-Deen ZM, Hussin NF, Abdel Hamid TA, et al. G6PD Deficiency and G6PD (Mediterranean and Silent) Polymorphisms in Egyptian Infants with Neonatal Hyperbilirubinemia. Lab Med. 2013;44(3):228–34.
8. Vulliamy TJ, D'Urso M, Battistuzzi G, et al. Diverse point mutations in the human glucose-6-phosphate dehydrogenase gene cause enzyme deficiency and mild or severe hemolytic anemia. Proc Natl Acad Sci U S A. 1988 Jul;85(14):5171–5.
9. Arnaout HH, El-Gharbawy NM, Shaheen IA, et al. Incidence and Association of 563 C/T Mediterranean and the Silent 1311C/T G6PD Mutations in G6PD-deficient Egyptian Children. Lab Med. 2011;42(6):355–60.
10. Hafez M, Settin Z, El Moursy Y EBR. Gene mutations of G6PD in Egypt. Egypt J Pediatr. 2000;17–21.
11. Gautam N, Gaire B, Manandhar T, et al. Glucose 6 phosphate dehydrogenase deficiency and hemoglobinopathy in South Western Region Nepal: a boon or burden. BMC Res Notes. 2019;12(1):734.
12. Roper D, Layton M, Rees D, et al. Laboratory diagnosis of G6PD deficiency. A British Society for Haematology Guideline. Br J Haematol. 2020.
13. El Gezeiry D, El Gendy W, El Kaffash D, et al. Comparative study for the detection of 563 CT G6PD mutation using restriction enzyme assay and amplification and refractory mutation system [ARMS], Alex. J Pediatr. 2005;19(1):51–5.
14. Osman HG, Zahran FM, El-Sokkary AM, et al. Identification of Mediterranean mutation in Egyptian favism patients. Eur Rev Med Pharmacol Sci. 2014;18(19):2821–7.
15. Rizk SH, Aziz M, El-Ghany HMA, et al. Frequency of the 563^ C^→^ T Mediterranean Mutation Among a Group of Egyptian Pediatric Patients. Lab Hematol. 2000;6:127–32.
16. Ramadan A, Zeidan MAA, Kholoussi N, et al. Aperito J Cellular and Mol Biol. 2015.
17. Karadsheh NS, Moses L, Ismail SI, et al. Molecular heterogeneity of glucose-6-phosphate dehydrogenase deficiency in Jordan. Haematologica. 2005;90 (12):1693–4.
18. Mornen NAI, Shaikha SAI. Molecular homogeneity of G6PD deficiency. Bahrain Med Bull. 2004;26(4).
19. Aboualchamat G. G6PD Deficiency in Syria: Identification of the Mediterranean Mutation Amongst Hemolytic Anemia Patients. Iraqi J Biotechnol. 2019;18(2).
20. Nafa K, Reghis A, Osmani N, et al. At least five polymorphic mutants account for the prevalence of glucose-6-phosphate dehydrogenase deficiency in Algeria. Hum Genet. 1994;94(5).
21. Daoud B Ben, Mosbehi I, Préhu C, et al. Molecular characterization of erythrocyte glucose-6-phosphate dehydrogen-se deficiency in Tunisia. Pathol Biol. 2008;56(5):260–7.
22. Ainoon O, Yu YH, Amir Muhriz AL, et al. Glucose-6-phosphate dehydrogenase (G6PD) variants in Malaysian Malays. Hum Mutat. 2002;21(1):101.
23. McDonagh, Ellen M., et al. "PharmGKB summary: very important pharmacogene information for G6PD." Pharmacogenetics and genomics 22.3 (2012): 219.
24. Krželj, Vjekoslav, et al. "Prevalence of G-6-PD deficiency in the Croatian Adriatic Coast population." Archives of Medical Research 32.5 (2001): 454-457.