

Molecular Identification of the Most Prevalent Mutations of the Glucose-6-Phosphate Dehydrogenase (G6PD) Gene in Deficient Egyptian Patients

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Abstract

Introduction

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is a common human enzyme deficiency. Molecular abnormalities data from Egypt are scarce, and have not been extensively investigated. To fill this gap, we investigated the frequency of the presence of certain known G6PD mutations among Egyptian patients with G6PD-deficiency.

Methods

DNA was extracted from 50 G6PD-deficient unrelated male subjects. We have analyzed the G6PD gene mutations in those with a history of favism by using the appropriate PCR-restriction enzyme digestion technique (PCR/RFLP analysis).

Results

The G6PD Mediterranean mutation was found in 16 patients (32%). The African A-variant (202 G→A & 376 A→G) were detected in 5 (10%) G6PD-deficient patients, the Chatham variant was detected in 2 (4%) of the patients and the Aures variant was not detected in any of the patients. Enzymatic activity was shown to be a poor predictive parameter of acute hemolytic crisis and was not correlated with clinical features.

Conclusion

The findings suggest that gene flow from the Indian subcontinent, sub-Saharan African, and other parts of the Mediterranean may have contributed to the observed G6PD mutations seen in the Egyptian population. The PCR-RFLP technique can be used for rapid molecular screening of the

to individual variability. As a result, cases can be misdiagnosed, but in patients who have other mutations in the G6PD gene, these should be subjected to direct sequencing, in an attempt to fully characterize their genotypes and to search for other novel mutations.

Keywords: G6PD; Glucose-6-Phosphate Dehydrogenase; Hemolytic Anemia; Acute Hemolytic Crisis (AHC); Chatham; Mediterranean; Aures; Favism

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Introduction

Although many other Red Blood Cell (RBC) enzyme deficiencies are now known [1-3], Glucose-6-Phosphate Dehydrogenase (G6PD) enzyme deficiency still remains the most common of all clinically significant enzyme effects, not only in hematology, but in human biology as a whole. A variety of drugs and infections cause hemolytic anemia in persons with the deficiency, and non hematologic sequelae have been claimed as well. Using classic biochemical techniques, enormous apparent

diversity of mutations causing G6PD deficiency have been documented in hundreds of publications. The distribution of the deficiency, in different populations, has been investigated extensively, and gene frequencies of over 0.5 have been observed in some ethnic groups. With the advances made possible by the cloning of G6PD cDNA and gene [4, 5] has come a better understanding of the diversity that exists.

G6PD deficiency (OMIM#305900) is a highly common X-linked genetic disorder. It is the most common enzymopathy in humans. More than 400 million people worldwide are G6PD deficient. Different populations have different types of mutations but, within a specific population, common mutations are usually shared. The most common of these variants are the G6PD Mediterranean and African A-variant (202 G→A & 376A→G). G6PD Chatham and Aures were found at lower frequencies in nearby ethnic populations [6]. Different populations express this deficiency where it is prevalent in the Mediterranean region, the Middle East, Africa, and South Asia [7, 8].

The housekeeping enzyme G6PD catalyzes the first step in the pentose phosphate pathway (conversion of glucose-6-phosphate to 6-phosphoglucono lactone) with the concomitant reduction of NADP⁺. This pathway is the sole pathway in RBCs to produce nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is essential for maintaining an adequate intracellular level of reduced forms of glutathione (GSH) and other sulphydryl groups. By preserving and regenerating reduced forms of glutathione, as well as promoting the stability of catalase, NADPH plays a major role in the stability of cells to withstand oxidative stress. In RBCs, since G6PD is the only source of NADPH, defense against oxidative damage is dependent on its activity [9-11]. G6PD deficiency causes reduced NADPH and hence increased oxidative damage where the membranes of RBCs are affected leading to acute hemolysis [12]. Acute hemolysis results from increased oxidative stress, for example: oxidative drugs, infections, metabolic conditions and fava bean ingestion. Other common clinical manifestations include neonatal jaundice which might lead to kernicterus (mental retardation), and in severe cases fatal chronic non-spherocytic hemolytic anemia [7].

The G6PD gene is a 13 exon gene which is present on the q-arm of the X chromosome (Xq28), the gene codes for 515 amino acids which weigh 59 KDa. To date, about 186 mutations

have been identified: 159 (85.4%) are single nucleotide substitutions (missense variants), 15 (8.0%) are multiple mutations (two or more substitutions), 10 (5.3%) are deletions, and 2 (1.0%) mutations affecting the introns [12]. The WHO has classified G6PD deficiency according to the residual enzyme activity which relates to the clinical manifestations as well as the mutation position in the protein. Class I is severe deficiency (<1% or non-detectable activity); this class manifests Chronic Non-Spherocytic Hemolytic Anemia (CNSHA). Until now, 68 different mutations causing CNSHA have been identified; the majority is found in exons 6, 10 and 13. Class II mutations have severe deficiency with residual G6PD activity less than 10%. Class II patients suffer from acute hemolysis with no CNSHA, for example the Mediterranean variant (c.563C/T). Class III have moderate deficiency with residual G6PD activity from 10%-60% which causes occasional hemolysis, for example the African variant (A-) (c. 202 G/A & c.376A/G). Class IV (60 -100% activity) and Class V activity (>100% activity) show no clinical symptoms [7].

G6PD deficiency is inherited as an X-linked recessive condition, incidence and transmission of such disease is greatly increased if the incidence of consanguineous marriage is also high. The percentage of first cousin marriages among all marriages has been reported to be 11.4% in Egypt, 30% in rural areas in the Islamic Republic of Iran, 29.2% in Iraq, 32% in Jordan, 30.2% in Kuwait, 17.3% among Muslim Lebanese and 7.9% among Christian Lebanese, 37.1% in Pakistan, 31.4% in Saudi Arabia and 30% in the UAE [13]. G6PD deficiency is a sex linked disease and thus, the female could be presented only if she is homozygous for the mutant gene, or heterozygous with the preferential expression of the G6PD deficient gene and X-inactivation of the normal gene, or the presence of an enhancer gene that makes the expression of the G6PD deficiency more likely [14].

The aim of this study was to determine the incidence of the G6PD Mediterranean variant, the African A-variant (202 G→A & 376 A→G), and the G6PD Chatham and Aures variants in fifty G6PD deficient Egyptian male patients and analyze the relative frequency of these mutations. Then, to correlate between the clinical features of G6PD deficient patients and their respective G6PD gene mutations.

Subjects and Methods

Sample Collection

Blood samples were collected from 50 G6PD-deficient patients, who were attending the outpatient Hematology Clinic of Benha Governmental University Hospital in Egypt, and 25 normal age with sex matched controls, in the period from 2010 to 2011. The patient group included 50 males patients based on medical history, clinical and biochemical examination after careful exclusion of other hemolytic blood disorders. Samples were analyzed in the Molecular Genetics and Enzymology lab. In the National Research Center in Egypt (NRC). Informed consent according to the Ethical Committee in the National Research Centre and the Institutional Review Board (IRB) of the American University in Cairo (AUC) was taken for participating in the study. Collection of samples ensured minimal risk to human subjects and confidentiality of all data is preserved. All the patients being studied, were subjected to recording of their medical history, which included history of present illness, clinical findings including age, sex, initial complaint, antenatal history (pregnancy complications, and maternal drug intake), natal history (gestational age, mode of delivery) and postnatal history of patients with emphasis on neonatal hyperbilirubinemia, hemoglobinuria, and previous acute hemolytic attacks.

Methods

Laboratory investigations

Routine hematological evaluation (complete blood picture (CBC), hemoglobin level, percentages of reticulocytes, and hematocrit), total and direct serum bilirubin levels, and G6PD enzyme activity were determined for all the subjects in the study. At the time of the routine laboratory investigations (between attack), 5ml of venous blood were obtained from each patient, by a sterile venipuncture, and then divided between 2 sterile EDTA vacutainer tubes. One vacutainer tube was used for performing CBC, reticulocytic count, in addition to quantitative G6PD enzyme level assay. The second tube was used for performing DNA extraction and PCR. Clinical conditions and severity of G6PD enzyme deficiency, of the studied groups, were correlated with their corresponding G6PD gene mutations to detect the severity of prevalent genotypes. The red cell G6PD activity, expressed as units per gram of haemoglobin (U/gHb), was determined by an enzymatic colorimetric assay for the quantitative determination of G6PD deficiency using a commercial kit (Greiner, Germany, catalogue No. 143,000). The

assay was performed according to the instructions included in the kit and the activity was finally calculated using the following equation: $G6PD (U/g Hb) = \Delta A/min \times 4777.8 / Hb (g/dl)$. On the basis of frequency distribution of activity levels, the critical level for diagnosing G6PD-deficiency was considered 6.4 U/gHb [15-16].

Molecular Investigation

Genomic DNA was extracted from peripheral blood leukocytes samples of all subjects in the study using Genomic DNA Extraction and purification kit techniques (Qiagen kit) catalogue number 51304. The DNA region from the G6PD gene encompassing each point mutation was selectively amplified by PCR using specific oligonucleotide primers (Table 1), followed by digestion with the suitable restriction enzymes (Table 1). For the Mediterranean variant, a 267 bp fragment was amplified corresponding to exon VI for recognition of the 563 CT mutation (substitution of Cytosine for thymine at nucleotide 563, leads to substitution of Serine by Phenylalanine at amino acid position 188). For the A-variant, a 1,131 bp fragment was amplified corresponding to exon III-V for recognition of the 202 GA mutations (substitution of guanine for adenine at nucleotide 202, leads to substitution of Valine by Methionine at amino acid position 68). For the A-variant, a 295bp fragment was amplified corresponding to exon V for recognition of the 376 A→G mutations (substitution of adenine for guanine at nucleotide 376, leads to substitution of Asparagine for Aspartic at amino acid position 126). For the Chatham mutation, a 208 bp fragment was amplified corresponding to exon IX for recognition of the 1003 G→A mutation (substitution of adenine for guanine at nucleotide 1003, leads to substitution of alanine by threonine at amino acid position 335). For the Aures mutation, a 353 bp fragment was amplified corresponding to exon III for recognition of the 143 T→C mutation (substitution of thymine for Cytosine at nucleotide 143, leads to substitution of Isoleucine by Threonine at amino acid position 481). Polymerase chain reaction assays were performed in 50 µL PCR reaction mix containing 1X GeneAmp PCR buffer, 200 mM each of deoxynucleotide triphosphate, 25 pmol of each primer, 0.5 mg genomic DNA, and 1.25 U of AmpliTaq DNA polymerase. Tubes were heated to 95°C for 5 minutes, then 32 PCR cycles started as follows: 94°C for 1 minute, 56°C or 57°C for 1 minute (Table 1), and 72°C for 1 minute, followed by a final extension step of 72°C for 5 minutes. Ten microliters of the amplified fragments were digested

overnight at 37°C using the appropriate endonuclease (New England Biolabs, Beverly, Mass). The digestion products were tested on a 3% agarose gel containing ethidium bromide. Digestion patterns of normal and mutant samples are summarized in Table 1.

Statistical Analysis

The present study began with a preliminary analysis of the data provided, using the classical measures e.g. mean, standard deviation, frequency and percentage. Then, a comparative analysis of quantitative variables was carried out between the study groups. This has been performed using student t-test for independent samples when normally distributed and the Mann Whitney U-test for independent samples when not normally distributed. As for the categorical data, Chi square-test has been applied. A probability value (p-value) ≤ 0.05 was considered statistically significant and a p-value ≤ 0.01 was considered highly statistically significant.

Results

The patient group included 50 males whose mean age was 6.77 ± 4.76 (ranging from 3 months to 21 years). Descriptive data analysis of the control and G6PD-deficient patients groups is shown in Table 2. Statistical comparison of the results of different laboratory assays between the control group and the G6PD-deficient patients showed that the mean Hb level, Hct (%), MCV and MCH was lower in G6PD deficient patients compared to the control group, and this difference was statistically highly significant ($p < 0.01$). Reticulocytic count was higher in G6PD deficient patients compared to the control group and this difference was statistically highly significant ($p < 0.01$). Also the mean quantitative G6PD enzyme assay was lower in G6PD-deficient patients compared to the control group and this difference was highly statistically significant ($p < 0.01$). Consanguinity was noticed in 4 out of the 50 cases (8%), while negative consanguinity was noticed in 46 out of the 50 cases (92%). The presence of other affected members in the family was observed in 29 out of the 50 cases (58%). Also, there were 21 out of the 50 cases (42%) with no prior family history. Quantitative G6PD enzyme assay showed that 66% of the patients had moderate deficiency, while the other 34% had marked enzyme deficiency. All the studied patients fulfilled the criteria of class II or class III of the WHO classification of G6PD enzyme deficiency. Clinically only 2 patients (4%) among the studied group were asymptomatic, while 48 patients (96 %) came

in crisis. Out of those who came in crisis, 40 patients (80%) had the crisis following ingestion of fava beans, 5 patients (10%) had a history of drug intake and in 3 patients (6%) the crisis was induced by multiple other reasons e.g. infections, diabetic ketoacidosis, etc.. History of blood transfusion in the studied group revealed that 25 patients (50%) had no history of previous blood transfusion whereas 25 patients (50%) had received blood. Twenty patients (40%) had a history of single blood transfusion and 5 patients (10%) had a history of blood transfusion twice. The Mediterranean variant was detected in 32 % of the patients, there were 16 hemizygous males. Five patients (10%) had the two point mutations (202 G→A) and (376 A→G), which together give rise to what is called the African A-variant. The Chatham variant was detected in 4% of the patients; there were 2 positive hemizygous males. The Aures variant was not detected in any of the patients. The other 27 patients (54%) showed mutations other than the Mediterranean mutation, African A-(202 G→A & 376 A→G), and Chatham and Aures mutations (Figures 1-3). Among patients having the G6PD Mediterranean mutation, 56.25 % had moderate enzymatic deficiency i.e. belonging to class III enzyme deficiency; whereas 43.75% had severe enzymatic deficiency i.e. belonging to class II enzyme deficiency. Among patients having the G6PD African A-(202 G→A & 376 A→G) variant, 80% had moderate enzymatic deficiency i.e. belonging to class III enzyme deficiency, whereas 20 % had severe enzymatic deficiency i.e. belonging to class II enzyme deficiency. Among patients having the G6PD Chatham mutation, 100 % had moderate enzymatic deficiency i.e. belonging to class III enzyme deficiency. In the group of patients having unidentified mutation, 66.66% had moderate enzymatic deficiency i.e. belonging to class III enzyme deficiency, whereas 33.33% had severe enzymatic deficiency i.e. belonging to class II enzyme deficiency. In the 33 patients with moderate enzyme deficiency, who were designated class III enzyme deficiency, nine patients (27.27%) had the Mediterranean variant, 4 patients (12.12%), had the African A-(202 G→A & 376 A→G) variant two patients (6.06%) had the Chatham variant and 18 patients (54.54%) were with unidentified mutation. In the 17 patients with marked enzyme deficiency, who were designated class II enzyme deficiency, 7 patients (41.17%) had the Mediterranean variant, 9 patients (52.94%) were with unidentified mutation and one patient (5.88%) had the African-(202 G→A & 376 A→G) variant. The clinical features of the patients having the

Mediterranean variant were as follows: 75% had Acute Hemolytic Crisis (AHC) following fava beans ingestion, 18.75% presented with AHC after drug intake and finally in 6.25%, the crisis was due to other reasons. Within patients with the African A-(202 G→A & 376 A→G) variant 80% had AHC following fava beans ingestion and 20% presented with AHC after drug intake. Patients with the Chatham variant 100% had AHC following fava beans ingestion. Patients with unknown mutation 7.4% were asymptomatic, 81.48% had AHC following fava beans ingestion, 3.7% presented with AHC after drug intake and finally in 7.4%, the crisis was due to other reasons. It has been reported that 2 G6PD-deficient patients (4%) who presented with no clinical symptoms were with unidentified mutation. In the 40 G6PD-deficient patients who presented with clinical manifestations in the form of acute hemolytic crisis (AHC) after fava beans ingestion; 12 patients had the Mediterranean variant (30%), 4 patients had the African A-(202 G→A & 376 A→G) variant (10%), two patients had the Chatham variant (5%) and 22 patients were with unidentified mutation (55%). In the 5 G6PD-deficient patients who presented with clinical manifestations in the form of AHC after drug intake; three patients had the Mediterranean variant (60%), one patient was African A-(202 G→A & 376 A→G) variant (20%) and one patient was with unidentified mutation (20%). Moreover, the 3 G6PD deficient patients (10%) who presented with AHC secondary to multiple reasons; only one demonstrated the Mediterranean variant (33.33%) and four patients were with unidentified mutation (66.66%).

Discussion

In the present study we have shown that 16 patients (32%) had the G6PD Mediterranean variant and 5 patients (10%) had the two point mutations (202 G→A) and (376 A→G), which together give rise to what is called the African A- variant. The Chatham variant was detected in 2 patients (4%), there were no Aures variant patients in the studied sample. The inability to find Aures in the study does not necessarily imply that these phenotypes are absent in our population, they probably could be found if a larger scale study was performed.

The Mediterranean 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 [15,16], 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 [17,18]. The Mediterranean mutation is also the most frequent mutation among Iraqi Kurds (87.8%), Turkey (80%), Iran (66.2%-91.2%), Southern Europe (69-77%) and the Indian subcontinent (60.4-79.6%). The mutation decreases in frequency as we move east, though it is still present in polymorphic frequencies in Malaysia [19]. The African A-(202 G→A & 376 A→G) variant is the most frequent variant in some African Arab countries such as Tunisia and Algeria with rates of 63.6% and 46% respectively [17,18]. It is the second most common variant in some Asian Arab countries with rates of 5.8 & -16.7%. Reports from Turkey and Iran suggest rates of 2% and 0-1.35% respectively [20, 21].

G6PD Chatham is now recognized as one of the common variants worldwide. Studies from other Arab countries show variable rates of 10.1% in Kuwait, 3.6% in Jordan and 1% in Algeria [17, 19, 22], while it is encountered in 8.7% of Iraqi Kurds, 4% in Turkey and 2.2-27% in Iran [22, 23], and in addition to Mediterranean countries, it has also been reported in polymorphic frequencies in Spain, India and Malaysia [21-23, 24]. G6PD Aures was first described in an Algerian and later found to account for 7% [17]. Thereafter, reports on this variant have appeared from several Arab countries including Tunisia, Jordan, Kuwait, UAE and Western Saudi Arabia with frequencies of 4.5%, 3.6%, 3.0%, 16.7% and 17% respectively [17, 18, 22, 25].

The population prevalence of G6PD deficiency in Egypt has been reported to be 5.9%, which is relatively higher than some other Mediterranean countries such as Greece (3.1%), Cyprus (5.1%), Italy (1-2%), Spain (1%), Turkey (1.2%), Algeria (5.4%) and Libya (4.7%) [26]. The term Middle East has come to be applied to the lands around the southern and eastern shores of the Mediterranean Sea, extending from Morocco to the Arabian Peninsula and Iran and sometimes beyond [27]. G6PD deficiency was first established to be the genetic basis of favism in 1958 in subjects from Sardinia. In the following years, the Mediterranean G6PD became the prototype of a polymorphic, severely deficient variant, underlying not only favism but also neonatal jaundice and acute hemolytic anaemia triggered by drugs or by infection [28]. The name Mediterranean seems appropriate because G6PD-deficiency, with similar clinical and biochemical characteristics, was reported in Greece, Southern Italy, Spain, Egypt, Israel, Lebanon and Turkey and also in neighbouring countries not bordering the Mediterranean Sea, such as Bulgaria, Romania, and

Pakistan. However, in many of these countries other variants were also reported. These findings suggest that the G6PD Mediterranean mutation might be quite ancient and that, whatever its primary origin, it might have spread in the Mediterranean area along with Greek civilization [29]. The molecular basis of G6PD Mediterranean was first shown to be a single point mutation resulting in a serine-to-phenylalanine replacement at amino acid position 188 in a subject from Calabria. Another salient mutation, at amino acid position 437, was reported to be regularly present in G6PD-Mediterranean subjects from Sardinia and Southern Italy [30].

Compared to other countries in the Middle East, high frequencies of G6PD deficiency have been reported in most countries of the region. Among males, prevalence for G6PD deficiency have been reported to be 39.8% in Saudi Arabia, 30% in Syria, 29% in Oman, 18% in Bahrain, 11.6% in Iran, 11% in UAE, 6.2% in Yemen, 6.1% in Iraq, 5.5% in Kuwait, 3.6% in Jordan and 2.1% in Lebanon [31]. There is a great regional variation in the incidence of G6PD deficiency in Egypt. It has been reported that the incidence of 4.9% with variation in frequency as 5% were from cities, 5.7% from Lower Egypt, 4.7% from Upper Egypt and 3.9% unclassified [32]. The incidence of 1.4% in Aswanese, and 2% in Nubians has also been documented.

In Egypt, [33] two studies reported the frequency of the G6PD Mediterranean mutation among two groups of 50 and 21 children with G6PD deficiency, 31 of the studied cases (62%) had the mutation, while 6 of the studied cases (28.6%) had the Mediterranean mutation respectively [34]. The difference in incidence of the G6PD Mediterranean mutation among the Egyptian G6PD deficient patients in the present study, relative to the previous studies, could be attributed to the continuous changes in the Egyptian population which is characterized by a dynamic state of migration inside and outside Egypt. These changes must affect the changes in the incidence of different variants and necessitate their continuous assay on a larger scale study.

In Tunisia, it has been shown that the frequency of the Mediterranean variant is G6PD A- (46.07%), G6PD Chatham (2.04%) and G6PD Aures (1.7%), which is similar to the results of our study (32%) [35]. A previous study showed that the Mediterranean mutation was detected in (9.7%), G6PD A- in (63%), and G6PD Aures in (4.8%) [18]. In Algeria, it has been stated that the most common variants accounting for the prevalence of G6PD deficiency were G6PD A- (46%) and

Mediterranean (23%) [17]. G6PD Aures were identified in (7%) and G6PD Chatham in (1%). In Nigeria, it was reported that 100% of a homogeneous population in Nigeria had the G6PD A-mutation and no cases of G6PD Aures, Chatham or Mediterranean were identified among (89%) of the population [36]. In Jordan, it has been observed the Mediterranean mutation in (53.6%), G6PD A- in (14.2%), G6PD Chatham in (3.6%) and G6PD Aures in (3.6%) among 28 Jordan population [17]. Another study in Jordan also reported a higher incidence of the Mediterranean mutation (76.2%), a higher incidence of G6PD A- (19%), and a lower incidence of G6PD Chatham (1.6%) [37]. In Kuwait [32], a study among 89 of the population showed that the Mediterranean mutation was detected in (74.2%), G6PD A- in (12.4%), G6PD Chatham was in (10.1%), and G6PD Aures in (3%) [22]. In Saudi Arabia, analysis of G6PD deficiency mutations in 114 individuals revealed that (84%) had G6PD Mediterranean, (5.8%) had African deficient variant (G6PD A-) and no cases of G6PD Aures or G6PD Chatham were identified [38]. Another study reported a lower incidence of G6PD Mediterranean (45%), G6PD A- was observed in (20.4%), Aures in (12.2%) and G6PD Chatham in (4%) [39]. In contrast a further study reported a higher incidence of G6PD Mediterranean (89.1%), none of the samples showed G6PD A-mutation, G6PD Aures (10 %), and G6PD Chatham (0.9%) [40]. In Oman, it has been reported that G6PD Mediterranean was the most common genetic variant 75% [41]. G6PD Chatham was observed in 10% and G6PD A- in 5%. In the United Arab Emirates, a study showed that Mediterranean mutation was detected in (71.4%), G6PD A- (22%) and G6PD Aures (22%) [42]. In Iraqi, a study of 46 individuals showed that (54.8%) were Mediterranean variant and (6.4%) were A- variant [43]. Another study reported that the Mediterranean mutation was detected in (74.3%), G6PD A- in (2%), G6PD Chatham in 5.0% and G6PD Aures in none [44]. Among the Kurdish population, it has been determined that 87.8% had a Mediterranean variant and 8.7% had Chatham variant after analysis for 115 unrelated male patients. No cases of G6PD Aures or G6PD A- were identified [45]. In Iran, it has been stated that the most common variants account for the prevalence of G6PD deficiency was Mediterranean (91.2%). G6PD Chatham was observed in (7.3%) and no cases of G6PD A- or G6PD Aures were identified [46]. In addition it has been reported an incidence of Mediterranean of (85.1 %) [47]. In contrast a lower incidence of Mediterranean of (72.91%) [48] and a higher incidence of G6PD Chatham

(13-27%) have been documented among Iranians as well [49]. In India, it has been stated that G6PD Mediterranean was the commonest variant (60.4%) and G6PD Chatham were identified in (1.2%), no cases of G6PD A- or G6PD Aures were identified [25]. In Malaysia, it has been showed that, showed that Mediterranean mutation was detected in 26.7% , G6PD Chatham in (2.3%), no cases of G6PD A- or G6PD Aures were identified among (86) Malays population [50]. In addition, it has been reported a higher incidence of G6PD Mediterranean (71.3%) [51].

In Pakistan, a study which reported that G6PD Mediterranean variant was the most common genetic variant (78%) and G6PD Chatham was observed in (5%) [52]. In Turkey, [53] a study among 50 of the population reported that the Mediterranean mutation was detected in (80%), G6PD A- (2%) and similar to our results G6PD Chatham (4%). Moreover, no cases of G6PD Aures were identified [62]. In Italy, it has been showed that Mediterranean mutation was detected in 69%, G6PD A- 3.7% and no cases of G6PD Aures or G6PD Chatham were identified [54]. In addition analysis for 54 G6PD-deficient unrelated male patients reported that Mediterranean mutation was detected in (48.2%) and G6PD A- in (7.45%) [55]. in Cyprus, The molecular study of the G6PD-deficient patients indicated that the G6PD Mediterranean was the most common variant (52.6%) and Chatham were identified with the frequency of (3%) [56].

The molecular heterogeneity of G6PD-deficiency in Egypt with high incidences of the Mediterranean variant could be explained by the special geographical site that Egypt occupies, at the meeting of the 3 continents of the different ethnic groups. Furthermore, Egypt has been a crossroad of trade between the Mediterranean basin, Asia Minor, the Arabian Peninsula, India and North Central Africa. Thus, G6PD mutations from all these areas would be represented among the Egyptian population [57].

Egypt is not a malaria-endemic area, and as G6PD deficiency is known to be associated with malaria [58] this poses some interesting questions as to the origin of these mutations. The results would suggest that there has been significant gene flow from the Indian subcontinent, sub-Saharan African, and other parts of the Mediterranean to Egypt. Coupled with the relatively long history of Egypt, this might explain the findings of this study. However, probably the most pertinent part of this study is the clinical significance of the G6PD deficiency, and in particular favism in the population. It has been reported [32] that favism is

not only associated with G6PD Mediterranean, but may also occur in individuals with G6PD A- [59]. Our study, in characterizing the most common G6PD variant among Egyptians, is a first step in the rational management of patients with this abnormality.

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Authors' contributions

AR contributed the concept and design, part of the molecular studies, data analysis and drafting of the manuscript; AR, MAZ, NG, HMK and DAE, contributed to the collection of data, larger part of the molecular work and analysis of data; WAZ and AMA, contributed to the concept and design and part of the molecular work; AR and AMA, contributed to the collection of data, performing hematological, enzyme assays and data analysis; AR, MAZ and NG, contributed to the analysis and interpretation of results, drafting and revision of the manuscript. All authors revised and approved the final submitted version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Conclusion

This study has revealed high molecular homogeneity of G6PD deficiency among Egyptian population attributed mainly to the G6PD Mediterranean variant. Further studies including a larger number of patients, more diverse ethnic backgrounds and including screening for other deficient variants and DNA sequencing are needed to give a more comprehensive view of G6PD variants in Egypt and to uncover factor(s) contributing to heterogeneous phenotypic expression of the disease.

Figure 1: PCR amplified *FokI* digested exon V the restriction fragment in normal and mutant samples of the African A-(376 A→G) mutation Lane (1) represents size marker (ϕ X 174 DNA-Hae III digest); Lane (2) illustrates a PCR product (a 295 bp fragment of exon V) in a G6PD deficient patients, while Lanes (2, 4, 6) after *FokI* digestion of the PCR products, illustrate the normal samples of the African A-(376 A→G) mutation showing one bands (295 bp); Lanes (3, 5) after *FokI* digestion of the PCR products, illustrate the mutant samples of the African A-(376 A→G) mutation showing two bands on the agarose gel (154bp,141bp) for hemizygotes (males).

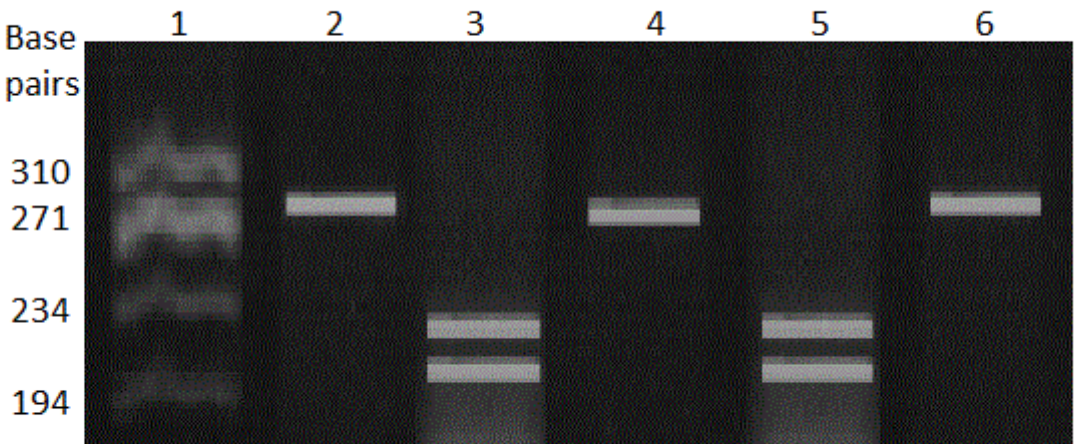


Figure 2: PCR amplified *BstXI* -digested exon IX showed the restriction fragment in normal and mutant samples of the Chatham variant Lane (1) represents size marker (ϕ X 174 DNA-Hae III digest); Lane (2) illustrates a PCR product (a 208-bp fragment of exon IX) in a G6PD deficient patient, while Lanes (3, 5, 6, 8, 9) after *BstXI* digestion of the PCR products, illustrate the normal samples of the Chatham variant showing two bands (130 and 78 bp); Lanes (4, 7) after *BstXI* digestion of the PCR products, illustrate the mutant samples of the Chatham variant showing three bands on the agarose gel (100, 78 and 30 bp) for hemizygotes (males).

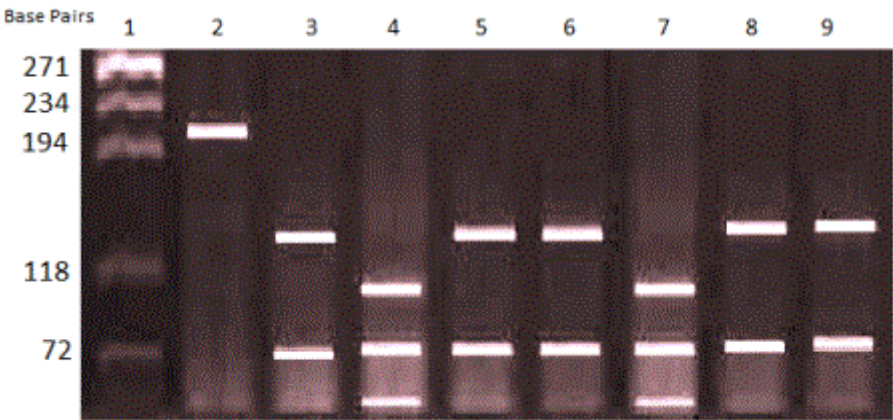


Figure 3: PCR amplified MboI -digested exon showed absence of the Aures variant Lane (1) represents size marker (ϕ X 174 DNA-Hae III digest); **Lane** (2) illustrates a PCR product (a 353 bp fragment of exon III) in a G6PD deficient patient, while **Lanes** (3 and 4) after Mbo I digestion of the PCR products, illustrate the normal samples and absence of the Aures variant showing two bands (293 bp & 60 bp) on the agarose gel.

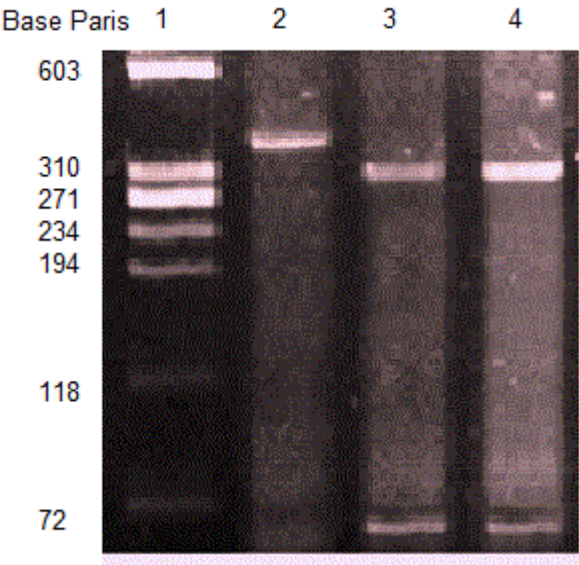


Table 1: Primer Sequences and the Polymerase Chain Reaction Restriction Enzyme Analysis Used to Amplify Targeted Exons of the G6PD mutations

			Fragment Size, Base Pairs (bp)			
Amplified Exons		*Sequence (5'-3')	G6PD variant	R.E***	PCR** size Uncut & Annealing temperature °C	normal Cut (Mutant)
VI	F	5'-GGT GAG GCT CCT GAG TAC CA-3'	Mediterranean	MboII	267	267 138 and 116
	R	5'-AGC TGT GAT CCT CAC TCC CC-3'			57	
III-V	F	5'-CGT GTC CCC AGC CAC TTC TA-3'	A- 202 G→A	NlaIII	1,131	419, 346, 178, 102 and 86 419, 223,178bp, 123,102 and 86
	R	5'-CAC GCT CAT AGA GTG GTG GG-3'			57	
V	F	5'-CTG TCT GTG TGT CTG TCT GTCC-3'	A- 376 A→G	FokI	295	295 154 ,141
	R	5'-GGC CAG CCT GGC AGG CGG GAA GG-3'			56	
IX	F	5'-CAA GGA GCC CAT TCT CTC CCT T-3'	Chatham	BstXI	208	130 and 78 100, 78 and 30
	R	5'-TTC TCC ACA TAG AGG AGG ACG GCT GCC AAA GT-3'			65	
III	F	5'-CAG CCA CTT CTA ACC ACA CAC CT-3'	Aures	MboI	353	130 and 78 353
	R	5'-CCG AAG TTG GCC ATG CTG GG-3'			56	

*Primers are listed from 5' to 3' ends and the second of each pair is the reverse or antisense sequence. F: forward, R: reverse. * **PCR indicates polymerase chain reaction

***R.E : Restriction Enzyme. The created new DNA fragments size in base pair (bp)