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G6PD Deficiency in Syria: Identification of the *Mediterranean* Mutation Amongst Hemolytic Anemia Patients

Ghalia Aboualchamat

Associate professor at Department of biology / faculty of sciences / Damascus University / Damascus / Syria.

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Abstract: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked genetic disorder. More than 400 million individuals are affected globally. The most common clinical manifestations are neonatal jaundice and acute hemolytic anemia. Numerous mutations have been described in the *G6PD* gene, many exhibit region-specific distributions. The *G6PD Mediterranean* mutation is frequently found in the Mediterranean region. However, in Syria no previous studies were conducted on G6PD deficiency disorder on its molecular basis. The aim of this study was to screen for the *Mediterranean* mutation amongst hemolytic anemia patients, as a preliminary search to determine the frequency of *G6PD* mutations in the Syrian society. A total of 265 children with hemolytic signs presented to Children's University Hospital in Damascus, were enrolled in this study. Genomic DNA was extracted from 197 patients for genotyping, and the *Mediterranean* mutation has been determined by *PCR-RFLP* method. Our result showed that 30% of cases have a positive family history for G6PD deficiency. The Male to female ratio was 1.6:1. *Mediterranean* mutation was detected in 164 case (83%) with an allele frequency of 0.65. High prevalence of *Mediterranean* mutation in our study strongly suggests the need for nationwide screening to determine the prevalence of the deficiency in the Syrian society. Further expanded studies are needed to evaluate other mutations in the *G6PD* gene.

Keywords: Glucose-6-phosphate dehydrogenase (G6PD) Deficiency, *Mediterranean* mutation, Hemolytic anemia.

Corresponding author: (Email: dr.ghalia-aboualchamat@hotmail.com).

Introduction:

Deficiency of G6PD is a prevalent red cell enzymopathy, and is one of the most common inherited disorders with an estimated 400 million people affected globally (1). The frequency differs with respect to geographic regions and ethnicities (2). Population studies in the Middle East have shown a remarkable variation in the prevalence rates of G6PD deficiency, and a considerable differences among Arab population (3,4).

Individuals with G6PD deficiency are clinically asymptomatic but are susceptible to develop acute hemolytic episode when they are subjected to causative agents such as drugs, infections, or *fava* beans. The clinical symptoms caused by hemolysis range from minor to fatal, since it depends largely on the type and pattern of mutation causing the deficiency, as well as to the amount of dosage taken (5).

The *G6PD* X-linked gene is one of the most highly polymorphic gene of the human genome with at least 186 mutations have been described, the

majority are single-base substitutions leading to amino acid replacements (6). Remarkably most of the observed mutations in *G6PD* gene vary in recurrence rates between populations, and exhibit distinctive region-specific distributions, therefore considered as a special feature for this region (7).

The *Mediterranean* mutation (c. 563C>T, p.Ser188Phe) (NM-001042351.2 :c.563C >T) is considered the dominant molecular determinant of G6PD deficiency in many Middle Eastern countries (8,9).

No previous studies were conducted on G6PD deficiency disorder nor on its molecular basis in Syria. Since the *Mediterranean* mutation has been shown to be the specific molecular cause of most cases of G6PD deficiency in our region, the aim of this study was to screen this mutation amongst acute hemolytic anemia patients, as a preliminary search to determine the frequency of *G6PD* mutations in Syria.

Materials and Methods:

Sample study:

A total of 265 children (163 males and 102 females) with acute hemolytic signs presented to the emergency ward at Children's University Hospital in Damascus city, were enrolled in this study. Patients were suffering from hemolytic crisis such as extreme tiredness, rapid heartbeat and breathing, paleness, and dark urine. Most patients were undiagnosed cases of G6PD deficiency.

Families were informed about the study, and a written consent was taken from all patients families. Simple questionnaires were completed regarding: age, gender, family history, and recurrent hemolytic crisis. Only 197 out of the total patients agreed to give blood samples for molecular analysis.

This study was conducted as from March 2015 until June 2016, and has been approved by the ethical committee of Damascus University – Syria.

Mediterranean mutation (c.563C > T) NM_001042351.2:c.563C>T detection:

Genomic DNA Extraction:

Approximately 1 ml blood sample was collected in EDTA tubes from each patient for genomic DNA isolation. DNA was extracted using *GF-1* blood DNA extraction kit (Vivantis, USA) according to the manufacturer's instructions.

PCR/RFLP technique:

Polymerase chain reaction followed by restriction fragments length polymorphism technique, PCR-*RFLP* was applied to screen for *Mediterranean* mutation. PCR method was performed to amplify a fragment of 390 bp in exon 6 of the *G6PD* gene flanking mutation region, using the following pair of primers(5'GCAGCTCTGATCCTCACTCC3') as forward and (5'CGTTGGTGGAGGAACTGACC3') as reverse (10).

The PCR reaction (25 µl final volume) contained 12.5 µl Dream Taq PCR master mix 2X (Thermo Scientific, USA), 1 µl of each primer, 10.5 µl nuclease-free water, and 3 µl extracted gDNA. PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 15 s, 61°C for 30 s and 72°C for 30 s. The final extension was at 72°C for 7 min. Each PCR experiment contained a negative control (3 µl of nuclease-free water) for contamination detection.

PCR reactions were done using Eppendorf Master Cycler.

The amplified products were electrophoresed along with a 100 bp DNA ladder (GeneDirex, USA) as size standard in 2% agarose gel containing ethidium bromide, visualized and photographed using a UV transilluminator.

Each 390 bp amplified PCR product was digested using *MboII* restriction enzyme 300 U (Thermo Scientific, USA) according to the manufacturer's instructions for 1 h at 37°C, inactivation of the enzyme was at 65°C .Finally, digested products were separated by 2% agarose gel electrophoresis, using 100 bp DNA ladder (GeneDirex, USA) as size standard, visualized and photographed using a UV transilluminator. A digestion pattern of 244+119+27 bp indicates *G6PD Mediterranean* mutation, whereas the product pattern of 363+27 bp indicates a wild-type genotype.

Results:

Two hundred sixty five patients attending Children's University Hospital for medical care, were recruit in this study. The Male to female ratio was 1.6:1, their ages ranged between 8 month to 12 years old. Our data showed that most patients had no previous history of hemolytic crisis, and 30% of cases have a positive family history where one member at least has G6PD enzyme deficiency. In addition, G6PD enzyme activity in almost all patients have shown a significant decrease, with an average (45.2± 2.6) $mU/10^9$ erythrocytes, while normal values are considered as (131 ± 13) $mU/10^9$ erythrocytes as measured by RANDOX glucose-6-phosphate (G-6-PDH) dehydrogenase kit (Randox, UK). This results indicate clearly that the patients have G6PD enzyme deficiency.

On the other hand, the presence of the *G6PD Mediterranean* mutation (c.563C>T) was assessed in one hundred ninety seven cases out of the total patients at their 281 X chromosomes. The mutation was found in 164 cases (83 %) (Figures 1 and 2). The allele frequency of the *Mediterranean* mutation in this study was 0.65 (Table 1).

Table (1): Summary of *G6PD Mediterranean* mutation detected in 197 deficient patients and characteristics of patients studied.

Sample study characteristics				Mutation detection				
Age		Number (%)		Number	No. X chromosomes	Med + [a]	Allele frequency	Med - [b]
Range	8 m-12y	males	163 (61.5%)	113	113	97 hemizygote	0.86	16 wild type
mean ± SD	3.6± 2.5	females	102 (38.5%)	84	168	49 heterozygote (CT genotype) 18 homozygote (TT genotype)	0.51	17 wild type (CC genotype)
Total			265	197			0.65	

Footnote

a = positive case for *G6PD Mediterranean* mutation.

b = negative case for *G6PD Mediterranean* mutation.

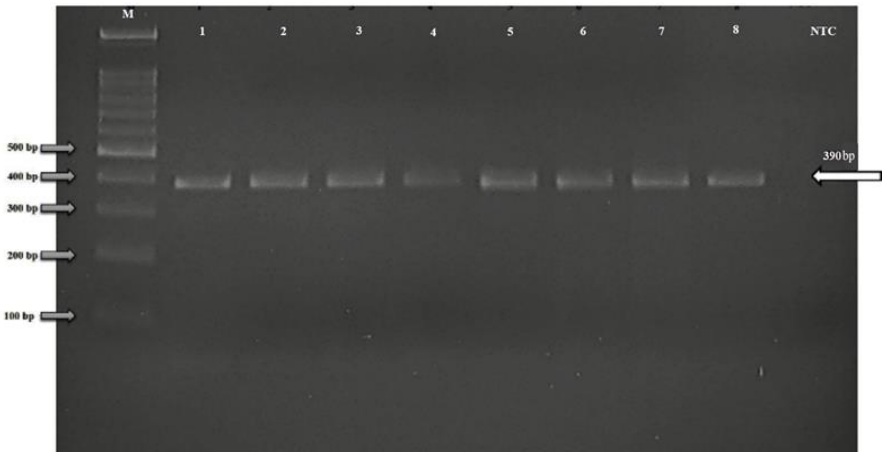

Figure (1): Agarose gel electrophoresis of the PCR products. Lanes1–8 a single PCR fragment of 390 bp. M, molecular marker (100 bp). NTC: No template control for contamination detection.

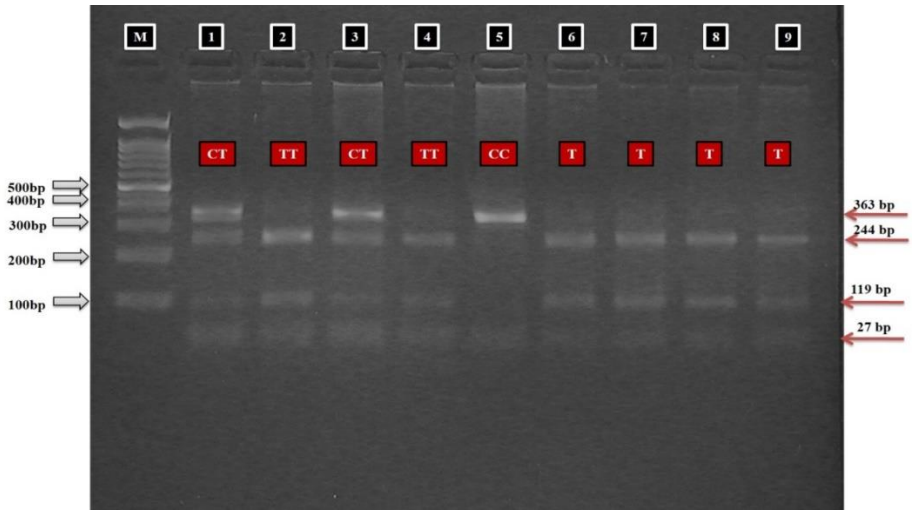

Figure (2): Agarose gel electrophoresis of the *PCR –RFLP* products after digestion with *Mbo II* in some G6PD deficient subjects. **Lanes 1, 3:** heterozygote females for *G6PD Mediterranean* mutation (c.563C > T). Lanes **2, 4:** homozygote females for *G6PD Mediterranean* mutation. **Lane 5:** wild type female. Lanes 6-9: hemizygote male cases positive for *G6PD Mediterranean* mutation. M: molecular marker (100 bp).

Discussion:

G6PD deficiency has high degree of heterogeneity for regional and global prevalence estimates. The prevalence rates of G6PD deficiency are of public health importance, especially for improving neonatal health and in the distribution of various medications particularly, in malaria-endemic areas where G6PD deficiency is most prevalent (11).

The prevalence of G6PD deficiency in the Arab world has been estimated to range from 1 to 65% (3). As for Syria only two different reports conducted on Syrian individuals living outside Syria estimating the frequency of G6PD deficiency to be 2.9% (4, 12). No available data assessing its actual frequency in all regions of Syria, for G6PD deficiency is not routinely screened amongst newborns in our country. However, our data showed that 30% of studied cases have a positive

family history for favism or hemolytic anemia, suggesting that G6PD deficiency is prevalent and there is an urgent need to estimate its frequency amongst Syrians.

On the other hand, almost all researchers conducted on G6PD deficiency patients in the Arab region emphasized that *G6PD-Mediterranean* mutation is the most frequent genotype amongst *G6PD* variants (13-16). It is believed to be a quite ancient mutation and might have spread in the Mediterranean area along with the Greek civilization and Population migration (17,18).

However, the frequency of *G6PD-Mediterranean* mutation vary greatly between societies. In our country no previous study had been performed on *G6PD* gene mutations, therefore the aim of this study was to perform the first molecular screen for *Mediterranean* mutation in a group of G6PD deficiency patients.

Our results showed that a total of 83% of patients carry the *Med* mutation; (86% amongst males, and 80% amongst females) with an allele frequency of 0.65. While in Egypt the *Med* mutation was found amongst 94.7% of favism patients (19). In Saudi Arabia the *Med* mutation was identified in 89.1% of the studied cases (10). On the other hand, in Jordan according to the study of Karadsheh and Colleagues 53.3% of male subjects carry the *Med* mutation(9). Whereas in Gaza Strip 34% of children studied carry the *G6PD* Mediterranean variant (20). These discrepancies between studies could be due to the genetic heterogeneity related to ethnical and geographical differences between societies as well as patient selection criteria, the studied sample size and the methods used in the mutation detection.

Mutations in the *G6PD* gene can destabilize the enzyme and reduce its activity levels, causing cells to be susceptible to damage from oxidative agents that triggers Red blood cell lysis and may lead to an acute hemolytic anemia (21). The *G6PD Mediterranean* mutation is a point mutation which leads to the replacement of cytosine into thymine at nucleotide 563 and thus change the amino acid to phenylalanine

(c. 563C>T, p.Ser188Phe), it is known as Class II variant, of which the enzyme efficiency drops to more than half and is associated with a spectrum of deficiency manifestations including: acute acquired hemolytic anemia, favism and neonatal hyperbilirubinaemia (22, 23).

According to the data collected from Children's University Hospital, the enzyme activity of nearly all patients revealed a substantial decrease, with an average (45.2± 2.6) $mU/10^9$ erythrocytes, this results confirm that the case of hemolytic anemia which our patients suffer from may be due to the presence of *G6PD Mediterranean* mutation leading to G6PD deficiency. However, our results showed that 17% of our patients were negative for the presence of the *Mediterranean* mutation, this could be explained if other mutations in the *G6PD* gene uncovered.

G6PD deficiency is characterized as X-linked recessive genetic disorder, where hemizygous males and homozygous females are deficient and heterozygous females are characterized as carriers, because of mosaicism. However, unlike other X-linked recessive diseases G6PD deficiency is expressed both biochemically and

clinically in heterozygous females. The abnormal cells of a female heterozygous for G6PD deficiency are just as deficient as those of a hemizygous deficient male. Although, on average heterozygotes have less severe clinical manifestations, individual heterozygotes may develop severe acute hemolytic anemia (24). In addition, heterozygous females are often classified as partial G6PD deficiency (25).

Our results showed that 49 /84 (58.3%) of females were heterozygotes for the *G6PD* Mediterranean mutation this results comes in agreement with the findings of Ainoon and Colleagues which indicate that partial enzyme deficiency is expected to be seen more commonly among female heterozygotes as a result of X inactivation, and the majorities of *G6PD* variants that cause the deficiency belong to class II variants(26).

Furthermore, homozygous deficient females are usually rare in X-linked recessive inheritance.

However, our results indicated that 21.4% (18/84) of female cases carry the *G6PD* Mediterranean mutation in its homozygous form. This may be due to the high rate of consanguineous marriages in Syria; that increases the frequency of homozygosity in the society. High prevalence of G6PD deficient females have been described previously and studies recommend the need to implement a universal screening program for this disorder (27-30). Moreover, The Male to female ratio in our study was 1.6:1, indicating a high prevalence of G6PD deficiency amongst female carriers. In conclusion, *G6PD Mediterranean* mutation is prevalent in our study. Indeed a large scale studies are needed to estimate the prevalence of mutations in the *G6PD* gene, and a nationwide newborn screening program should be considered for G6PD enzyme deficiency disorder, in the Syrian society.

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