Identification of Mediterranean mutation in Egyptian favism patients

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Abstract. – OBJECTIVE: Identify and screen the G6PD Mediterranean mutation in favism patients by applying Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR).

PATIENTS AND METHODS: A total of 114 unrelated Egyptians patients were included in the present study; their ages ranged between (2-9) years with male to female ratio 4.5:1. G6PD activity was determined qualitatively from red cell hemolysate during attack. The G6PD Mediterranean mutation in patients has been identified by ARMS-PCR.

RESULTS: G6PD deficiency was detected in 87.7%, (n=100). The frequency of G6PD Mediterranean mutation was (94.7%), (n=108). The association between G6PD deficiency and Mediterranean mutation was a highly significant.

CONCLUSIONS: Glucose-6-phosphate dehydrogenase Mediterranean mutation is one of the most common mutations causing G6PD deficiency among Egyptian children with favism.

Key Words:
G6PD-Deficiency, Favism, Mediterranean mutation, ARMS PCR.

Introduction

Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway of carbohydrate metabolism. G6PD deficiency is the most common human enzymopathy, affecting more than 400 million people worldwide. It may result in neonatal jaundice; drug induced hemolytic anemia, favism and chronic-non spherocytic hemolytic anemia. A clinical symptom of G6PD deficiency closely linked to drug induced haemolysis is the hemolytic anemia resulting from the ingestion of the fava bean. Patients with favism are always G6PD deficient, but not all G6PD deficient individuals develop haemolysis when they ingest fava beans. It is assumed that some other factors, such as genetic and metabolism of the active ingredients in the beans, which causes oxidative damage in red blood cells, are involved. A recent study has shown increased oxidative stress parameters and less antioxidant defense in favic patients compared to normal individuals. The vast majority of cases of favism occur in individuals with severely deficient (class 2) variants of G6PD (e.g. Mediterranean and Cosenza) as well as class 3 variants (e.g. Chatham).

The gene for G6PD containing 13 exons is located on the X-chromosome (Xq28). The active enzyme is composed of different proportions of two or four identical subunits of 515 amino acids with a GC-rich promoter typically of many housekeeping genes; each monomer has a molecular weight of 59 kDa. Glucose-6-phosphate dehydrogenase (G6PD) is a polymorphic enzyme encoded by the extremely X-linked (Xq28) genes of human and expressed in several tissues in the body. There is a wide variation in the activity of G6PD in the individual red blood cells and, therefore, a number of cases can escape from the correct diagnosis. The most important source of error is the presence of population of young red cells with high reticulocytic number showing an increase in G6PD activity, leading to false results. This condition is common after hemolytic attack, in patients affected by congenital non-spherical cell hemolytic anemia (CNSHA), and in cases of unbalanced X chromosome is disabled in heterozygous females. For all must be completed by the previous reasons, and DNA analysis for a definitive diagnosis.

The frequency of G6PD deficiency varies widely among world populations. The prevalence of G6PD deficiency in Egypt has been re-
Mutation Analysis

DNA Extraction

Genomic DNA was extracted (according to manufacturer’s instructions) from the peripheral blood using column method by Gene Jet™ Whole Blood Genomic DNA Purification Mini Kit (Cat No= #K0781, #K0782) (Waltham, MA, USA).

Detection of G6PD Mediterranean Mutation (563 C—-T) by (ARMS) PCR

Two forward allele-specific primers, within exon 6 of the G6PD gene, were designed as follows: ARMS M (5`CCG GCT GT C CAA CCA CAT ATT 3`) complementary to the mutant allele and ARMS N (5`CCG G CT GT C C AA CCA CAT ATC 3`) complementary to the normal one. These primers contained a specific base at their 3’ terminus: C for normal DNA (563 C) and T for mutant DNA (563 T). An additional mismatch (C—-T) nucleotide upstream from 3’ end was included. The J primer (5’CCA GCC TTC CAG GAG AGA 3’)11, used as the reverse primer. The amplification product was 442-bp DNA fragment.

DNA samples were amplified simultaneously in two parallel reactions with each primer set: (1) ARMS M and J to amplify the mutant allele; (2) ARMS N and J for the normal allele. The amplification mixture (50 µl) contained 25 µl of PCR Master Mix (Maxima™ Hot Start PCR k1051 containing TaqDNA polymerase in reaction buffer (Thermo Fisher Scientific, Wilmington, MA, USA), MgCl2, and dNTPs), 2 µl (20 pmole/µl) of either Primer M or Primer N, 2 µl (20 pmole/µl) of the Primer J, 2 µl (20 pmole/µl) of each forward and reverse internal control primers, 5 µl of Template DNA, and sterile distilled water (12 µl) to a final volume of 50 µl.

The computerized thermocycler was programmed for the following conditions: an initial heat denaturation step at 95°C for 4 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Each PCR run had a blank without genomic DNA, as a negative control. The amplification products were evaluated by electrophoresis on 2% agarose gel containing ethidium bromide, at 150 V for 20 minutes.

Statistical Analysis

Data were exported into a Microsoft Excel worksheet. Statistical analysis was conducted using SPSS 14 software (SPSS Inc., Chicago, IL,
A preliminary analysis was done on the data provided, using the classical measures (e.g., mean, standard deviation (SD), frequencies, and percentages). Chi square test was used to estimate differences in qualitative variables. A p value of ≤ 0.05 was considered statistically significant, and a p value of ≤ 0.01 was considered highly statistically significant.

**Results**

The studied group included 114 G6PD-deficient pediatric patients. Their ages ranged between (2-9) years; with a mean of (3.99 ± 1.2). The male to female ratio was 4.5:1 (Table I).

G6PD deficiency was detected in 100 patients (87.7%), while the other patients were clinically normal (12.3%). As for control group they were all clinically normal.

The G6PD Med (563 C/T) mutation was detected in 108 patients (94.7%). Among the 108 Mediterranean positive patients, there were 94 homozygote patients (91 homozygote male and 3 homozygote female) and 14 heterozygote female (Table II).

In control group G6PD Mediterranean mutation was found in 2 heterozygote females (1.7%) and the other 118 children were normal (98.3%) (Figure 1).

There was highly statistically significant distribution for Mediterranean mutation between patient and control groups according to Chi square test (Table III).

Also in the present study it was found that there was a highly significant correlation (Figure 2) between G6PD deficiency and Mediterranean mutation (Table IV).

Patients’ profiles are shown in (Table V) and data were expressed by Mean ± SD.

**Discussion**

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common hereditary disorder in humans and is found in people of Mediterranean, South-east Asian and African descent. The common clinical manifestations of G6PD deficiency are neonatal jaundice and acute hemolytic anemia triggered by certain drugs, infections or ingestion of fava beans. G6PD deficiency is a heterogeneous enzyme abnormality.

It was found that in the present study, G6PD deficiency was detected in 100 patients (87.7%) while the other patients were clinically normal.
(12.3%). However, subjects of control group were all clinically normal. This may be due to the fact that there is a wide individual variability in G6PD activity in RBCs. Therefore, a number of cases can escape the correct diagnosis. The most important source of error is the presence of a young red cell population with a high reticulocytic number (8.02 ± 1.11, \( p < 0.001 \)) (Table V) showing an increase in G6PD activity giving rise to false-normal results. Furthermore, patients with favism are always G6PD deficient, but not all G6PD-deficient individuals develop hemolysis when they ingest fava beans.

As biochemical characterization has lost its significance as a means of identifying G6PD variants; therefore, current molecular techniques are required to elucidate a structure function relationship. Moreover, these molecular techniques are needed to confirm the molecular homogeneity of G6PD deficiency.

One hundred and fourteen G6PD-deficient unrelated patients were included in this study with a male to female ratio of 4.5:1 (Table I). This could be attributed to the fact that the G6PD deficiency is a sex-linked disease. Thus, a female would present clinically only if she were 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 would make the expression of the G6PD deficiency more likely.

The prevalence of G6PD Mediterranean mutation among the G6PD-deficient children in the present study was (94.7%). In other previous studies, prevalence in Egypt (60%) was reported by Hafez et al. However, Rizk et al. reported a lower frequency of such a mutation (28.6%) among G6PD-deficient Egyptian children. In consistent with the present study, other authors found a high prevalence of G6PD Mediterranean mutation among glucose-6-phosphate dehydrogenase deficient patients, as found by El-Gezeiry et al (43.6%), while Arnaout et al stated (60%).

Table III. Highly statistically significant distribution for Mediterranean mutation between patient and control groups according to Chi-Square test.

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>df</th>
<th>Asymp. Sig. (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-Square</td>
<td>204.142a</td>
<td>2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>264.135</td>
<td>2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Linear-by-Linear Association</td>
<td>195.968</td>
<td>1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>234</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a: 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.79.
The increased frequency of the G6PD Mediterranean mutation among the Egyptian G6PD-deficient patients in the present study relative to the studies in Egypt could be attributed to the larger scale of this study and the restriction of this investigation to patients presenting with favism, contrary to the other studies including more clinical variants. Therefore, different molecular researches should be performed covering the various regions of Egypt due to the complexity and the different ethnic origins of the Egyptian population. Another important factor contributing to such a difference in the prevalence was the use of a more sensitive and specific technique (ARMS-PCR) in the current study.

The frequencies of Mediterranean G6PD mutation among deficient individuals ranged between 71.4% and 84% for Eastern Saudi Arabia. In Iraq according to Helmi et al. stated that the occurrence of the mutation was 54.8%. While a higher prevalence of 87.8% in the Kurdish population of Northern Iraq was reported according to Al-Allawi et al. In a recent work in Baghdad, G6PD Mediterranean mutation was detected in 74.3% of deficient males. Frequency of G6PD deficiency varied worldwide possibly due to the patient selection criteria, the ethnic and geographical differences.

Furthermore, in this report few cases of favism patients (12.3%) have shown normal G6PD enzyme activity, were found to have G6PD Mediterranean mutation, this could be due to the false negative qualitative test that could occur when blood is obtained just after an acute hemolytic action. This could be explained by destruction of severely enzyme deficient RBCs during the hemolytic attack leaving normal RBCs behind. These patients were found to be heterozygote females (Table II), where in heterozygote females, red cell mosaicism arising from random X-chromosome inactivation results in two populations of G6PD-deficient and G6PD-normal cells. The proportions of these two cell types can vary greatly, ranging from completely normal activity to complete deficiency.

On the other hand, six cases have shown to have enzymatic deficiency, but found to have no G6PD Mediterranean mutation. Hence, the absence of this mutation among the six cases of G6PD deficiency diagnosed qualitatively may necessitate molecular workup for detection of other gene mutations.

**Correlation is significant at the 0.01 level (2-tailed).**

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### Table IV: Significant correlation between G6PD deficiency and Mediterranean mutation.

<table>
<thead>
<tr>
<th></th>
<th>G6PD</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson Correlation</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>114</td>
</tr>
<tr>
<td>Polymorphism</td>
<td>Pearson Correlation</td>
<td>0.494**</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>114</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level (2-tailed).**

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### Table V: Clinical manifestations of patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control subjects mean ± SD</th>
<th>Favism patients mean ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dl)</td>
<td>12.85 ± 1.72</td>
<td>3.88 ± 1.25</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N</td>
<td>120</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Conjugated bilirubin (g/dl)</td>
<td>0.28 ± 0.01</td>
<td>0.13 ± 0.039</td>
<td>0.05</td>
</tr>
<tr>
<td>N</td>
<td>120</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes count (%)</td>
<td>0.90 ± 0.60</td>
<td>8.02 ± 1.11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N</td>
<td>120</td>
<td>114</td>
<td></td>
</tr>
</tbody>
</table>
It was found that 2 female cases of control group were found to have the G6PD Mediterranean mutation (heterozygote), this may be due to X-inactivation of the mutant gene, or presence of an enhancer gene would make the expression of the G6PD deficiency less likely 23.

There was statistically significant distribution for Mediterranean mutation between patient and control groups according to Chi square test (Table III). Also, it was found that there were a significant correlation between G6PD deficiency and Mediterranean mutation (Figure 2, Table IV). Thus, it could be concluded that Mediterranean mutation is frequently distributed between Egyptians Favism patients. Sukumar et al 24 reported that G6PD Mediterranean mutation was associated with more severe clinical manifestation. This was identical with our findings, where in our study the favism patients have shown to have decreased conjugated bilirubin (0.13 ± 0.039, p < 0.05) and decreased hemoglobin levels, (3.88 ± 1.25, p < 0.001) compared to controls (0.28 ± 0.01) and (12.85 ± 1.72), respectively (Table V). These clinical manifestations of favism present distinctively with sudden onset of severe hemolytic anemia within 24 to 48 h ingestion of the fava beans 25.

**Conclusions**

The G6PD Mediterranean mutation is one of the most common mutations causing G6PD deficiency in Egyptian favism disease. Further molecular characterization of the genetic variants of G6PD is required to fully cover its molecular pattern in Egyptian patients. Biochemical characterization has lost its significance as a means of identifying variants. Fully characterized biochemical variants, therefore, need to be reanalyzed using molecular techniques to elucidate a structure-function relationship. Identification of mutations that cause pathology is of interest not only in the understanding of genetic disease but also for the information that can be provided concerning the corresponding normal function of the respective gene product.

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**References**


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