ORIGINAL ARTICLE

Mediterranean Glucose-6-Phosphate Dehydrogenase ($G6PD^{C563T}$) Mutation Among Jordanian Females with Acute Hemolytic Crisis

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ABSTRACT

Objective: To evaluate the $G6PD^{C563T}$ Mediterranean mutation among Jordanian females who were admitted to Princess Rahma Teaching Hospital (PRTH) with/or previous history of favism.

Study Design: A descriptive study.

Place and Duration of Study: Jordanian University of Science and Technology and PRTH, from October 2003 to October 2004.

Methodology: After obtaining approval from the Ethics Committee of Jordanian University of Science and Technology, a total of 32 females were included in this study. Samples from 15 healthy individual females were used as a negative control. Blood samples from these patients were collected and analyzed by allele-specific polymerase chain reaction (AS-PCR) to determine the $G6PD^{C563T}$ mutation.

Results: Twenty one out of 32 patients were found to be $G6PD^{C563T}$ Mediterranean mutation (65.6%) positive. Three out of 21 patients were homozygous and remaining 18 were heterozygous for $G6PD^{C563T}$ Mediterranean mutation. Eleven (34.4%) out of 32 patients were found to be negative for $G6PD^{C563T}$ mutation indicating the presence of other G6PD mutations in the study sample.

Conclusion: $G6PD^{C563T}$ Mediterranean mutation accounted for 65.6% of the study sample with favism in the North of Jordan. There is likely to be another G6PD deficiency variant implicated in acute hemolytic crisis (favism).

Key words: *Favism. G6PD deficiency. Polymerase chain reaction. Hemolysis. Jordan.*

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) distributed in all human body cells where it plays a role in the metabolism of glucose and maintenance of reduced glutathione (GSH) level.[1] G6PD catalyses the first step in the pentose phosphate pathway (PPP) of glycolysis in which glucose-6-phosphate is oxidized into 6-phospho-gluconolactone, concomitantly converting nicotinamide adenine dinucleotide phosphate (NADP) into reduced form (NADPH), which is the major source of reducing agent essential for protection of erythrocytes against reactive oxidation species (ROS).[2]

G6PD is the only enzyme that produces NADPH in erythrocytes, since the erythrocytes do not have nucleus and mitochondria, thus the only source for NADPH and most of GSH generation is from activity of G6PD which is the only defense against oxidant stress in human erythrocytes.[3] The physiological significance of the enzyme is implicated in various cell functions and its deficiency causes hemolytic anemia and neonatal jaundice.[4]

The gene for G6PD is located on the X-chromosome in Xq28 region, and it spans more than 20 Kb with 12 exons and 13 introns. Most of the mutations are single-base changes that result in an amino acid substitution. The defect is fully expressed in affected hemizygous males and homozygous females. Heterozygous females are carrier to the defect because of the random inactivation of one X-chromosome in each female body cell. Therefore, heterozygous females have two types of RBCs, G6PD normal and G6PD deficient RBCs.[1,2] In a population with high gene frequency and high consanguineous marriage the prevalence rate of affected homozygous females will be high and hemolysis will just be as severe as in the hemizygous males, and heterozygous females are likely to be affected because of variable inactivation of the X-chromosome.[5-8]

Favism, which results from ingestion of fava beans in G6PD deficient individual, is characterized by acute hemolysis, hemoglobinuria, anemia, and jaundice. Fava beans have unusually high amount of oxidants, which G6PD deficient are unable to tackle because of decreased GSH. Therefore, these oxidants cause stress

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Received August 31, 2010; accepted October 25, 2010.

Table I: Sequence and properties of primers used.

Primer name and sequence	Length (nt)	*G+C%	**Tm (°C)	Reference
G6PD C563T forward normal (FN) 5'CCG GCT GTC CAA CCA CAT ATC 3'	21	57.14%	72.79	Maffi D., 2002[8]
G6PD C563T forward mutant (FM) 5'CCG GCT GTC CAA CCA CAT ATT 3'	21	52.38%	70.83	Maffi D., 2002[8]
G6PD C563T reverse (R) 5'CCA GCC TCC CAG GCG AGA 3'	18	72.22%	73.61	Maffi D., 2002[8]

*(G+C %): Guanine + Cytocine %; **Tm: melting temperature.

Primers were synthesized by Alpha DNA, 5584A Sherbrooke St. W. Montreal, Quebec H4A 1W3.

on the red blood cells and cause severe acute hemolytic anemia.[3-9] Its occurrence is common in the Middle East and the Mediterranean region especially during spring.

G6PD deficiency affects all races and severity of the deficiency varies significantly between racial groups because of different variants of the G6PD enzyme. G6PD Mediterranean (G6PD^{C563T}) is the most common G6PD deficient variant in many parts of the world especially in Southern Europe, Middle East, India and South East Asia.[10] This mutation is characterized by very low enzyme activity in erythrocytes (0-10%). In the G6PD Mediterranean individuals there is greater enzyme instability as the half-life is only about 8 days compared to 62 days of the normal G6PD enzyme. G6PD Mediterranean mutation result from C→T transition at nucleotide 563 that causes a serine-to-phenylalanine substitution at amino acid 188.[11] The prevalence of G6PD deficiency in North Jordan Valley and Irbid were reported to be 5.5% and 4.62% respectively,[12] but there are no studies to show the prevalence of G6PD variants among females with acute hemolytic anemia.

The aim of this study was to evaluate the G6PD-Mediterranean C563T mutation among females patients either admitted with acute hemolytic crisis or with previous history of favism in the north of Jordan by using allele specific polymerase chain reaction(AS-PCR).

METHODOLOGY

This descriptive study was conducted from October 2003 to October 2004 at Jordan University of Science and Technology and Princess Rahma Teaching Hospital, Jordan. After obtaining approval from ethics committee of Jordanian University of Science and Technology, a total of 32 females presenting with favism or having past history of favism were included in this study. Samples from 15 healthy individual females were used as a negative control. AS-PCR was used to detect the presence of G6PD^{C563T} mutation.

Five milliliter of blood was collected in EDTA tubes (5 mL).

Statistical analysis was performed by calculating the percentage of positive cases for the G6PD-Mediterranean C563T mutation in the sample.

Genomic DNA was extracted from whole blood using Promega DNA purification kit (Promega, USA) according to protocol in the kit. AS-PCR was carried out for detection of G6PD^{C563T}. This method is based on using sequence specific primers to differentiate between normal and mutant variants. Two forward allele-specific primers and a reverse primer for G6PD^{C563T} mutation within exon 6 of the G6PD gene were used, out of two forward primers, one was the wild type primer (hybridizes to the normal allele) and the other was the mutant type primer (hybridizes to the mutant allele). The properties and sequences for each primer are shown in Table I.

The normal (N) and the mutant (M) amplification mixtures reaction for G6PD^{C563T} mutation was performed by mixing the following components: first tube contained 2.5 µl of PCR buffer (1 x buffer), 1.5 µl of $MgCl_2$, 0.5 µl of dNTP (0.2 mM), 0.2 µl of Taq DNA Polymerase (1 unit), 2 µl forward normal primer (1 mM), 2 µl of reverse primer (1 mM), 2 µl of sample DNA and adjusted to 25 µl as a final volume using distill nuclease free water while the second PCR tube contained 2.5 µl of PCR buffer (1 x buffer), 1.5 µl of $MgCl_2$, 0.5 µl of dNTP (0.2 mM), 0.2 µl of Taq DNA Polymerase (1 unit), 2 µl forward mutant primer (1 mM), 2 µl of reverse primer (1 mM), 2 µl of sample DNA and adjust to 25 µl as a final volume using distill nuclease free water. The N and the M reactions amplification conditions were preceded by i-Cycler thermal treatment (BioRad i-cycler, USA) as follow: at first denaturation cycle at 96°C for 10 minutes, then 30 cycles with denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, elongation at 72°C for 3 minutes and a final extension cycle at 72°C for 10 minutes.

RESULTS

A PCR product of 442-bp was amplified using forward mutant - reverse FM-R and forward normal-reverse FN-R primers sets representing either mutant or normal G6PD as shown in Figure 1. PCR for G6PD homozygous females gave an amplification product only with the FM-R mutant primer set (Figure 1, lanes: 3, 6, and 9); while DNA from normal females gave a PCR product only with the FN-R normal primers set (Figure 1, lanes: 1, 5 and 7). Heterozygous deficient females gave a PCR product with both sets of primers (Figure 1, lanes: 2, 4, and 8).

A total of 32 blood samples from G6PD-deficient patients with favism were tested for presence of G6PD^{C563T} Mediterranean mutation using AS-PCR. Samples from

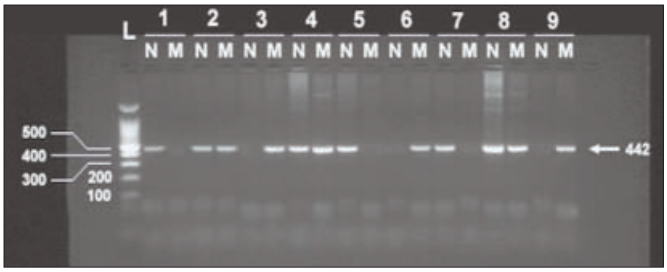

Figure 1: 2% agarose gel electrophoresis of allele-specific PCR products for G6PD B-^{C563T} mutation. L: 100-bp DNA ladder. Lanes 1, 5 and 7 Negative control (healthy individuals). Lanes 3, 6 and 9: Deficient homozygous. Lanes 2, 4, and 8 deficient heterozygous females.

15 healthy individual females were used as a negative control.

Twenty one of 32 cases were positive for G6PD^{C563T} Mediterranean mutation (65.6%). Eleven cases (34.4%) out of 32 were found to be negative for G6PD^{C563T} mutation.

Out of twenty one positive cases, 3 (14.3%) female patients were homozygous and 18 (85.7%) were heterozygous for G6PD^{C563T} Mediterranean mutation.

DISCUSSION

G6PD^{C563T} Mediterranean mutation is the most severe variant underlying favism, neonatal jaundice and acute hemolytic anemia. In Jordan it was estimated that the prevalence of G6PD deficiency in North Jordan Valley was 5.5% and in Irbid it was 4.62%.[12] In this study, the percentage of G6PD^{C563T} in the G6PD deficient patients underlying favism in North of Jordan was determined by AS-PCR method and it was found to be 65.6%. Our findings are similar to those reported in Italy, 70% of 161 patients,[13] and from Kuwait which was 72.9%.[14] This is also in agreement with that the large majority of Middle Eastern subjects with G6PD-Mediterranean phenotype have the same mutation found in Italy and that all of their 21 patients were of Italian ancestry with previous hemolytic crisis or family history of favism.[15] The prevalence is also slightly different to that reported in Greece, which was 77% and in Turkey, which was 79%.[6,17] The percentage in this study is more than that reported in Cyprus which was 52.6% and close to that reported by Mesbah-Namin *et al.* which was 66.2%.[18,19] The differences in sample size included in other reports and the difference in the population heterogeneity may be the reasons for such variations in the percentage of mutation in this study and other countries. These results were similar with those reported by Abdul Razzaq *et al.* who found that 42 of his sample patients (60%) were heterozygous, 10 (17%) of them were homozygous for G6PD deficiency.[12]

Since 11 cases (34.4%) with G6PD deficiency in this study were not carrying G6PD^{C563T} mutation so this study could not exclude the presence of other types of G6PD mutations that were associated with favism. The scope of this study was to determine the G6PD Mediterranean mutation. Therefore, the nature and extent of other mutations were not taken into consideration. It is obvious that the existence of variants other than the Mediterranean mutation is most likely to be present in this region. Thus, other types of G6PD mutations that lead to favism should be investigated such as G6PD^{G202A} African, Catham (G1003A), Cosenza (G1376C), G6PD Union (C1360T) and G6PD Antioco (A1342G) as reported by other investigator in the Mediterranean region.[13,19,20]

It is recommended that further studies involving DNA sequencing or endonuclease cleavage are required to determine the existence of G6PD mutation variants other than G6PD^{C563T} which are associated with favism.

CONCLUSION

G6PD^{C563T} mutation accounted for 65.6% of female patients with favism from North of Jordan. In this sample 34.4% of patients were likely to have other undetected G6PD mutations associated with favism.

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