Red Cells & Iron

Genotype and phenotype correlation in glucose-6-phosphate dehydrogenase deficiency

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Background and Objectives. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common erythrocytic enzymatic disorder in Italy and is characterized by wide clinical, biochemical and molecular variability. We studied the clinical and hematologic data from 54 G6PD-deficient, unrelated males from the Apulia region.

Design and Methods. Analyses for enzymatic activity, G6PD electrophoresis and molecular typing were performed on all subjects. Thirty-nine subjects (72.2%) showed a severe G6PD deficiency (<10% residual enzymatic activity) and 15 subjects (27.8%) a moderate deficiency (10-60% residual activity).

Results. The Mediterranean variant was found in 48.2% of cases, the Seattle variant in 33.3%, the A- variant in 7.45% and the Montalbano variant in 3.7%; the variant was not identified in four subjects. Thirty-two patients (59.2%) were asymptomatic; of these, 37.04% demonstrated acute hemolytic crises induced mainly by ingestion of fava beans and 3.7% had had neonatal jaundice. Acute hemolytic anemia was found in 53.8% of subjects with the Mediterranean variant, in 5.5% with the Seattle variant, in 100% with the A-variant and 0% with the Montalbano variant.

Interpretation and Conclusions. Enzymatic activity was shown to be a poor predictive parameter of acute hemolytic crises and was not correlated with clinical features. Subjects with Mediterranean or A- variants had a more severe clinical phenotype which was not related to enzymatic activity. The Seattle, and probably the Montalbano, variant appears to have a milder clinical expression.
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Key words: G6PD deficiency, phenotype, genotype

The enzyme glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step of the pentoso-phosphate cycle; the encoding gene is located on the long arm of the X chromosome (Xq28).[1,2] G6PD deficiency is one of the most well-known human genetic defects and has been identified in more than 400 million subjects throughout the world[3,4] and numerous reports have been published on this genetic disorder[5,6] in various geographic populations in Greece,[7] Spain,[8] Egypt,[9] Israel,[10] the Middle East,[11] Turkey,[12] Bulgaria,[13] Romania,[14] Pakistan,[15] China,[16] the United States,[17] Algeria,[18] and Arabia,[19] in both black and white races; in the latter, it is frequently associated with hemoglobin S.[20-22]

In Italy, the regions with the highest incidence of G6PD deficiency are related to those with the greatest occurrence of thalassemia[23] and, in the past, to areas in which malaria was endemic,[24,25] for example, Sardinia,[26,27] Sicily,[28] Calabria,[29-31] Basilicata,[32] Campania[33] and along the Po river.[34] In particular, the incidence of G6PD deficiency has been found to be 12-15% in Sardinia,[35,36] while that in Southern Italy and near the Po River ranges from 0.2% to 4.4%.[35-38]

Over 440 variants[24,26,29,32,39] have been classified into five classes based on the residual enzymatic activity and clinical manifestations: class I is associated with chronic non-spherocytic anemia (CNSHA), class II with severe enzymatic deficiency (REA <10%) and acute hemolytic anemia, class III with moderate deficiency (REA 10-60%), class IV with normal enzymatic activity and class V with increased enzymatic activity.[39] From the electrophoretic point of view, the enzyme can demonstrate a faster or slower mobility than the enzyme of a normal subject or have normal mobility. At present, about 130 molecular mutations have been recognized.[11,28,33,34,38-49]

Clinically, G6PD deficiency can induce manifestations such as neonatal jaundice, chronic non-spherocytic anemia, acute hemolytic anemia caused by ingestion of fava beans, drug-induced hemolysis, and hemolysis induced by infections or other etiologic agents as yet unknown;

subjects can also be fully asymptomatic during all or most of their lifetime. The clinical phenotype is a result of an interaction between molecular mutations, oxidative stress and other unknown factors.[16,19,27,33,41,46,47,50-52]

The aims of this study were to establish, in a male population with G6PD deficiency, whether a correlation exists between genotype, biochemical characteristics and clinical phenotype and to determine whether the presence of hematologic, biochemical, and molecular predictive factors could eventually condition the clinical phenotype, thus facilitating prevention and refining prognosis.

Design and Methods

Patients

This retrospective study evaluated the clinical, hematologic, biochemical (enzymatic activity and electrophoretic mobility), and molecular data from 54 G6PD-deficient, unrelated males from the Apulia region of Italy. These patients came to our attention because of reported crises of acute hemolytic anemia, favism, a family history of G6PD deficiency or occasional findings of reduced enzymatic activity. The patients' mean age was 22.48 years ± 1.83 (range: 6 months to 65 years).

Enzymatic activity

Partial purification and enzymatic characterization was performed according to WHO recommendations;[53] briefly, heparinized blood samples were used for blood counting and reticulocyte counts were performed with standard methods. The erythrocytes were then hemolyzed with a buffer solution (2.7 mM Na_2EDTA pH 7.0, 0.7 mM β-mercaptoethanol) and the hemolysates were used for enzymatic dosing according to the method of Beutler,[54,55] thus permitting the calculation of the G6PD concentration after saturation with 6PGA and G6P. The increase in optical density in the kinetic reaction was measured with a spectrophotometer equipped with a 340 nm filter, after 10 min in a buffer solution at 37°C (1M Tris-HCl, 5 mM EDTA pH 8.0; 0.1 M $MgCl_2$; 2 mM NADP; 6 mM G6P, 6 mM 6PGA). Enzymatic activity was expressed in IU/g of hemoglobin. Using this method, normal G6PD enzymatic activity is 8.34±1.59 IU/g Hb. The average normal value and standard deviation of G6PD enzymatic activity in normal males in our laboratory was 8.2±1.04 (median 8, mode 8). In case of deficiency, the enzymatic activity is also reported as residual enzymatic activity (REA) compared to normal values and generally ranges between severe (less than 10% of normal values) and moderate forms (10-60% of normal). This assay was always performed with normal reticulocyte counts.

G6PD electrophoresis

The G6PD electrophoretic mobility was evaluated with electrophoresis of the hemolysates on cellulose acetate strips in pH 8.6 buffer solution (Tris-HCL, borate, EDTA). After a run at 150 V for 90 min, the strips were stained with a mixture composed of Tris-HCl (1M), G6P (10 ng/mL), NADP (5 ng/mL), $MgCl_2$ (1M), NBT (2 mg/mL), and PMS (2 ng/mL) and incubated for 10 min at 37°C in a *bain marie* in the dark.[3] On the basis of this method, an altered enzymatic state will have normal, fast or slow electrophoretic mobility.

Molecular typing

After DNA extraction with the phenol chloroform method, polymerase chain reaction (PCR) analysis was performed on the mutation-containing DNA segment. We studied the four mutations which appear to be the most frequent in Southern Italian regions: the Mediterranean, the Seattle, the A- and the Montalbano variants. The molecular features of the mutations studied are reported in Table 1. For the Mediterranean variant, a 267 bp fragment of exon VI containing the 563 C→T mutation was amplified; for the Seattle and Montalbano variants the amplified fragment was a 646 bp fragment including exons VII and VIII (844 G→C and 854 G→A at exon VIII). For the A- mutation, a 1,131 bp fragment was amplified corresponding to exons III-IV and V for recognition of the 202 G→A mutation at exon IV responsible for the enzymatic deficiency. The sequence of the primers for amplification is reported in Table 2.

For the Mediterranean variant, the amplified DNA was cleaved into two fragments of 138 bp and 116 bp. The MboII specific restriction site is created only in the presence of a mutation resulting in fragments of 138bp and 116 bp. The amplified fragment of G6PD from a normal subject does not contain this cleavage site. The Seattle mutation is in exon VIII and clears the cleavage site for the DdeI enzyme. Restriction of the normal amplified fragment yields 5 fragments of 211 bp, 157 bp, 130 bp, 101 bp and 79 bp, while the restriction pattern of the mutated sequence produces 4 fragments of 211 bp, 180 bp 157 bp and 130 bp. The mutation therefore produces a new 180 bp band resulting from the lack of cleavage of the 101 bp and 79 bp bands.

The 202 G→A mutation in exon IV, responsible for variation A-, is recognized by the enzyme NlaIII; in a normal subject, the restriction pattern is the following: 419 bp, 346 bp, 178 bp, 102 bp and 86 bp. In the presence of the mutation, the 346 bp fragment is cleaved into two fragments of 223 bp and 123 bp. In the Mon-

Table 1. Location of point mutations in four different G6PD variants.

Exon	G6PD variant	Nucleotide change	Amino acid substitution
Mediterranean	6	563 C → T	188 Ser → Phe
A-	4	202 G → A	68 Val → Met
Seattle	8	844 G → C	282 Asp → His
Montalbano	8	854 G → A	285 Arg → His

Table 2. Primers used for PCR amplification.

Primers	Sequence	Variant
R6	5'GGTGAGGCTCCTGAGTACCA3'	Mediterranean
L1	5'AGCTGTGATCCTCACTCCCC3'	Mediterranean
R8	5'TCACAGATGGGCCTGCGACA3'	Seattle and Montalban
GdL-7	5'CCACAGAGGCCCAAGGTCAG3'	Seattle and Montalban
L3	5'CGTGTCCCCAGCCACTTCTA3'	A–
R5	5'CACGCTCATAGAGTGGTGGG3'	A–

Table 3. Electrophoretic mobility and DNA analysis of 54 subjects subgrouped into WHO classes II and III on the basis of enzyme activity.

	Electrophoretic mobility						
	Class II*			Class III°			Total
G6PD variant	Fast	Normal	Slow	Fast	Normal	Slow	
Mediterranean	–	23	–	–	3	–	26
Seattle	–	–	8	–	–	10	18
A–	3	–	–	1	–	1	4
Montalbano	–	2	–	–	–	–	2
Unknown	–	2	1	1	–	–	4

*Residual enzymatic activity < 10%; °residual enzymatic activity 10–60%.

talbano variant, the enzyme NlaIII yields a restriction pattern of 424 bp, 118 bp, 104 bp, 40 bp, 37 bp and 5 bp compared to a normal pattern of 424 bp, 118 bp, 104 bp, and 82 bp.

In the case of A- and Mediterranean variants, enzymatic restriction was confirmed with ASO-probes. Then, the amplified correspondent of the DNA segments containing the mutation was denatured with 1N NaOH and subjected to the dot-blot technique. Subsequently, the membrane is hybridized with specific wild-type and mutant 5' γ-^{32}P-ATP-labeled oligonucleotides. The oligonucleotide sequences corresponding to the two variants examined are the following: for the Mediterranean variant a wild type oligonucleotide 5'-ACCA-CATCTCCTCCCTGTT3' with a 58°C hybridization temperature and a 60°C washing temperature and mutant oligonucleotide 5'-AACAGGCACAAGATGTGGT3' with a 56°C hybridization temperature and a 58°C washing temperature; for the A- variant, a wild type oligonucleotide 5'AGCCACATGAATGCCCT-CC3 with a 60°C hybridization temperature and a 62°C washing temperature and mutant oligonucleotide 5'GGAGGGGCATC-CATGTGGCT3' with a 66°C hybridization temperature

and a 68°C washing temperature. The hybridization was visualized by autoradiography at 80°C for 1-2 hrs. This method permits the analysis of a large number of samples simultaneously.

Results

The biochemical and molecular data of the 54 G6PD-deficient males are summarized in Table 3. A severe enzymatic deficiency (REA < 10%) was noted in 39 subjects (72.2%) in whom the electrophoretic mobility was normal in 27, fast in three, and slow in nine. A moderate deficiency (REA 10-60%) was present in 15 subjects (27.8%) in whom the electrophoretic mobility was normal in three, fast in two and slow in 10. In the patients with zero enzymatic activity, the electrophoretic mobility was evaluated by parental studies.

Twenty-six subjects (48.2%) demonstrated the Mediterranean variant (563 C→T); of these, 23 had severe enzymatic deficiency (REA <10%) while only three subjects had moderate enzymatic deficiency (REA 10-60%). All samples with the Mediterranean variant showed normal electrophoretic mobility, in accordance with the literature.[38] The Seattle variant (844 G→C) was found in 18 subjects (33.3%); of these, 8 and 10 subjects had, respectively, severe and moderate deficiency and all demonstrated slow electrophoretic mobility. The A- variant (202 G→A) was identified in four subjects (7.45%), of whom three had severe and one moderate deficiency; all showed fast electrophoretic mobility. Two subjects (3.7%) showed the Montalbano variant (854 G→A) and both had severe enzymatic deficiency with normal electrophoretic mobility. The molecular variation was not identified in four subjects (7.4%) in whom electrophoretic mobility was heterogeneous as was the entity of the enzymatic deficiency.

The clinical features of the G6PD variants in the 54 patients are listed in Table 4. In 32 (59.2%), no clinical symptoms were noted; of these, 10 demonstrated the Mediterranean variant, 17 the Seattle variant, two the Montalbano variant and in three the molecular variant was not identified. In 20 (37.04%) patients, clinical manifestations were present in the form of acute hemolytic crises and 14 reported hemolytic anemia after ingestion of fava beans; of these, 12 subjects had the Mediterranean variant, one the A- variant and one an unknown variant. Moreover, one subject (1.86%) with the Seattle variant had had drug-induced crises (mainly acetylsalicylic acid), and five (9.26%) subjects (two with the Mediterranean and three with A- variants) showed hemolytic crises of unknown etiology. Neonatal jaundice was also observed in two patients (3.7%) with Mediterranean variant. No patient presented chronic non-spherocytic hemolytic anemia.

Table 5 summarizes the relationship between the molecular variants, residual enzymatic activity, presence or absence of hemolytic crises and transfusional requirements. Of the 26 subjects with the Mediterranean variant, 14 had a clinical history of acute hemolytic crises, with severe enzymatic deficiency in 11 and moderate

deficiency in 3. Three subjects reported more than one hemolytic crisis which had been induced by ingestion of fava beans at 2 and 8 years old for the first patient and at 13-14 years for the second; the third subject had had an acute drug-induced hemolytic crisis when 28 years old. Of these 14 subjects, only 5 were submitted to blood transfusions during the hemolytic crisis as the median minimum hemoglobin level during hemolysis was 5.52±0.70 g/dL (range 4.9-6.5 g/dL). In the remaining 12 patients, the minimum hemoglobin level reached 8.30±0.86 g/dL (range 7-10 g/dL). The severity of the hemolytic crisis, therefore, was independent of the residual enzymatic activity. The only Seattle variant patient with an acute hemolytic crisis had a moderate enzymatic deficiency. With regard to the four subjects with the A- variant, all had experienced a hemolytic crisis and one patient relapsed; no transfusion was necessary. No Montalbano variant patient had hemolytic crises. The only subject with an unknown variant who had acute hemolytic crises showed a severe enzymatic deficiency and reported multiple hemolytic crises, not requiring blood transfusions, induced by fava beans.

Conclusions

The population of Apulia demonstrated a polymorphic G6PD molecular deficiency, as also found in the populations of Calabria, Campania and Sicily but unlike that in Sardinia for which a single genetic deficiency has been reported for both G6PD and thalassemia. In our study, the Mediterranean variant had an allelic frequency of 48.2 % which is similar to that in the other regions of Southern Italy, such as Campania (45%) and Sicily (45-50%), while in the Sardinian population the frequency of this variant is roughly 83%.[33,38,41] The allelic frequency of the Seattle variant in our study was 33.3%, similar to that in Campania (25.8%)[33] while in Sardinia this variation is rather rare.[38] The allelic frequency of the A- variant in Apulia was 7.4%, while in Campania it is 13%[33] and even less in the other Italian regions.[38] All other variations described in the literature are rare in this area.

In previous reports, the Mediterranean variant has been mainly found in patients with severe enzymatic deficiency and normal electrophoretic mobility. Moreover, 50% of these subjects experienced an acute hemolytic crisis induced by ingestion of fava beans. The Seattle mutation was equally demonstrated in subjects with severe and moderate enzymatic deficiencies, and in all had a slow electrophoretic mobility; it was rarely responsible for acute hemolytic crises. The majority of subjects with the A- variant showed a severe enzymatic deficiency together with a fast electrophoretic mobility and acute hemolytic crises, mainly caused by unknown agents. The Montalbano variant, even though scarcely present in our study, had a uniform behavior from biochemical and molecular points of view; in fact, our two subjects demonstrated a severe enzymatic deficiency with normal electrophoretic mobility and were not subject to acute hemolytic crises. Due to the limited number of patients,

Table 4. Clinical features of G6PD variant in 54 subjects.

G6PD variant	Asymptomatic	Hemolytic crises Triggering agent			Neonatal jaundice
		Fava beans	Drugs	Other agents	
Mediterranean	10	12	-	2	2
Seattle	17	-	1	-	-
A-	-	1	-	3	-
Montalbano	2	-	-	-	-
Unknown	3	1	-	-	-
Total (%)	32 (59.2)	20 (37.04)			2 (3.7)

Table 5. G6PD variants: WHO classes and hemolytic crises in 54 enzyme-deficient males.

	Class II*			Class III°			
G6Pd variant	Patients	Subjects with crises	Transfusion	Patients	Subjects with crises	Transfusion	Total
Mediterranean	23	11	3	3	3	2	26
Seattle	8	-	-	10	1	-	18
A-	3	3	-	1	1	-	4
Montalbano	2	-	-	-	-	-	2
Unknown	3	1	-	1	-	-	4

*Residual enzymatic activity < 10%; °residual enzymatic activity 10-60%.

these data require confirmation. Among the four patients with an unknown genotype, marked heterogeneity of biochemical characteristics was observed, as expected, with only one case of favism.

In conclusion, from our data it appears that the enzymatic activity deficiency is not a predictive parameter of the severity of the clinical condition. The Mediterranean and A– variants more generally produce a more severe clinical phenotype which is not, however, correlated to the enzymatic activity level. The clinical expression of the Seattle and Montalbano variants appears to be milder. Although it is not yet clearly understood whether genetic or extragenetic, other mechanisms must exist which offer protection from the oxidative stresses which certainly play a role in the clinical expression of G6PD deficiency.

Potential implications for clinical practice

Enzymatic activity is not a predictive parameter of the severity of the clinical conditions. The molecular mutation can characterize the clinical phenotype but is not a predictive parameter of hemolitic crises.

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