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red cells

Molecular heterogeneity of glucose-6-phosphate dehydrogenase (G6PD) variants in Italy

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ABSTRACT

Background. Glucose-6-phosphate dehydrogenase deficiency, one of the most common human enzymatic defects, is characterized by extreme molecular and biochemical heterogeneity. The molecular bases of almost all polymorphic Italian variants have now been identified and the overall heterogeneity is lower than expected from biochemical data.

Methods. We examined 161 G6PD-deficient subjects (130 males and 31 females) originating from different parts of Italy. G6PD activity and molecular characterization were determined in all the subjects analyzed.

Results. We found the G6PD Mediterranean genotype in roughly 70%, G6PD Union and G6PD Seattle in about 6% and G6PD A- in 4% of the samples analyzed. G6PD S. Antioco and G6PD Cosenza were less frequent (1.2%), and single cases of G6PD Partenope and G6PD Tokyo were also detected.

Conclusions. We report the frequency and distribution of the most common G6PD variants in Italy. Greater molecular heterogeneity than described by others was observed, especially in Sardinia. Among the severe deficient variants, G6PD Mediterranean has a higher prevalence in Sardinia (83%) than in continental Italy (61%), as does G6PD Union (10% and 4%, respectively). G6PD Seattle and A–, associated with mild G6PD deficiency, are by contrast more frequent in continental Italy.
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Key words: G6PD variants, G6PD mutations, molecular characterization

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Glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate: NADP oxidoreductase; E.C. 1.1.1.49) deficiency, one of the most common human enzymatic defects, is characterized by broad genetic and biochemical heterogeneity. To date approximately 400 variants have been described on the basis of their biochemical properties and grouped into 5 classes according to the level of residual enzyme activity and clinical manifestations: class I, associated with chronic non-spherocytic hemolytic anemia (CNSHA); class II, severely deficient (less than 10% residual activity) associated with acute hemolytic anemia (AHA); class III, moderately deficient (10-60% residual activity); class IV, normal activity (60-150%); class V, increased activity. By contrast, only about 90 molecular defects have been identified so far as being responsible for such variants, demonstrating less genetic heterogeneity than would be expected from biochemical data.[1,2] G6PD deficiency is quite common in most Mediterranean areas including Italy. On the basis of biochemical classification, the most common variant in Italy is G6PD Mediterranean, which is associated with severe G6PD deficiency and acute hemolytic anemia (AHA) or favism, together with two other polymorphic variants with similar biochemical properties known as G6PD Cagliari and G6PD Sassari.[3] Other polymorphic class II variants such as G6PD Ferrara I have also been described among the Italian population with relatively high frequency.[4] The most common class III variants are represented by G6PD Seattle-like, G6PD A-, G6PD Matera and G6PD Ferrara II, followed by other variants known as G6PD Metaponto, G6PD Montalbano and G6PD Sibari.[3,5-7] Molecular analysis of the G6PD gene has demonstrated that different mutations are clustered in single populations: G6PD A- accounts for the majority of mutated alleles in Africa,[8] whereas G6PD Mediterranean is predominant in the Mediterranean, the Middle East, northern India and among Kurdish Jewish populations;[7,9-11] G6PD Canton is the most common variant in China.[12] The molecular bases of almost all polymorphic Italian variants have now been identified. A single point mutation 563 C→T has been determined to be responsible for the three polymorphic variants G6PD Mediterranean, Cagliari and Sassari,[13] a 202

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G→A mutation for the variants A, Matera and Ferrara I,[14,15] and a 844 G→C substitution for the G6PD Seattle-like, Ferrara II, Modena, Athens-like and Lodi variants.[13,16-19] On the other hand, different molecular abnormalities have been described as being responsible for similar biochemical variants. Recent papers published by us and by other authors have in fact described different point mutations responsible for variants with a Mediterranean-like phenotype, namely G6PD Union (1360 C→T), G6PD Cosenza (1376 G→C), G6PD S.Antioco (1342 A→G) and G6PD Partenope (1052 G→T).[7,20,21] Only G6PD Union and G6PD Mediterranean appear to be quite common and distributed worldwide since the former has also been found in southern Italian, Spanish and Oriental populations.[7,22,23]

In this paper we report the molecular characterization of the G6PD gene in a large sample of G6PD-deficient Italian subjects originating from different areas of the country. Subsequently, we evaluated the frequency of the most common G6PD mutations in Italy. The results confirm the high frequency of the G6PD Mediterranean mutation all over the country, including the islands, and a heterogeneous distribution of other mutations.

Materials and Methods

Subjects.

We examined 161 G6PD-deficient subjects, 130 males and 31 females, originating from different parts of Italy (59 from Sardinia, 45 from northern Italy and 57 from southern Italy). All of them were of Italian ancestry and most of them came to our outpatient service for G6PD activity determination because of a family history of favism, geographical origin and/or previous hemolytic crises. All of them were interviewed for this medical information.

G6PD activity and electrophoretic mobility

Blood samples were collected in acid-citrate-dextrose (ACD) or EDTA after informed consent. G6PD activity was determined in all the subjects according to the WHO and ICSH recommendations.[24,25] Electrophoretic mobility on cellulose acetate gels (Chemetron, Milan, Italy) was assessed in 115 samples according to the method of Rattazzi *et al*.[26] with minor personal modifications in three different buffer systems (Tris, Borate, Phosphate). Seventy G6PD-deficient samples were biochemically characterized in a previous study.

Detection of known mutations by polymerase chain reaction and endonuclease cleavage

DNA analysis was performed on all the specimens considered in this study. Genomic DNA was purified from leukocytes by standard methods. The coding region of the G6PD gene encompassing each nucleotide substitution was PCR-amplified using previously described primer pairs.27 The synthetic oligonucleotides were assembled on a Cyclone DNA synthesizer (Biosearch, CA, USA). The amplification reaction was performed in 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 200 µM of each dNTP, 20 pmoles of each primer in the presence of 0.5 µg of DNA and 1.0 U of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT, USA) in a final volume of 50 µL. The reactions were carried out using a DNA Thermal Cycler (Perkin Elmer) for 32 cycles as follows: denaturation at 92°C for 1 minute, annealing at 58°C for 1 minute, elongation at 72°C for 2 minutes and a final elongation at 72°C for 10 minutes. The amplified fragments were digested overnight at 37°C with 10 U of the appropriate endonucleases for the following mutations, thus creating a nucleotide substitution at the cleavage site: the CÆT mutation at nt 563 (G6PD Mediterranean) was tested with MboII, the 376 AÆG and 202 GÆA mutations (G6PD A-) with Fok I and Nla III, the 844 GÆC mutation (G6PD Seattle) and the 1376 GÆC mutation (G6PD Cosenza) with Dde I. Nla III was used for the 634 AÆG (G6PD Sibari), 854 GÆA (G6PD Montalbano) and 1347 GÆC (G6PD Cassano); Hae III was employed for the 1342 AÆG (G6PD S. Antioco) and Hinf I for the 1360 CÆT mutation (G6PD Union). Restriction fragments were analyzed on 3% agarose gel containing ethidium bromide or run on polyacrylamide mini-gels (8-10%) and silver stained. All mutations and digestion patterns of normal and mutant samples are summarized in Table 1.

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Table 1. Detection of mutations by PCR and endonuclease cleavage

G6PD variant	*Mutation*	*Amplified exons*	*Enzyme*	*Fragment size (bp)*		
				Uncut	*Cut (normal)*	*Cut (mutant)*
Class II						
Mediterranean	563 C→T	6+7	Mbo II	547	377,119, 26,25	277,119,100,26,25
S. Antioco	1342 A→G	10+11	Hae III	497	144,130,70,67,30,25,18,12,6	130,115,70,67,30,29,25,18,12,6
Cassano	1347 G→C	10+11	Nla III	497	166,115,111,110	115,111,110,105,61
Union	1360 C→T	10+11	FspI	497	452,45	497
Cosenza	1376 G→C	10+12	DdeI	733	733	548,185
Class III						
A-	202 G→A	3+4	Nla III	109	109	63,46
	376 A→G	5	Fok I	301	301	172,129
Sibari	634 A→G	6+7	Nla III	547	219,204,124	328,219
Seattle	844 G→C	8	Dde I	164	101,58,5	159,5
Montalbano	854 G→A	8	Nla III	164	82,77,5	82,40,37,5
Class I						
Tokyo	1246 G→A	10	Sty I	497	497	259,238

PCR single-strand conformation polymorphism (SSCP) analysis

In samples negative for the mutations tested by endonuclease cleavage, the entire coding region of the G6PD gene was PCR-amplified in eight different fragments using pairs of primers previously described.27 All the amplified exons were then submitted to non-radioisotopic SSCP analysis. Briefly, 3 μL of the amplified product were added to 27 μL of 92% formamide, 20 mM EDTA and 0.05% bromophenol blue, denatured at 98°C for 10 minutes and quenched in a dry ice/ethanol bath for 2 minutes. Ten μL of this mixture were loaded on 6-8% polyacrylamide mini-gel (0.75 mm x 6 cm x 8 cm; acrylamide:bis 38:1.5), run at 150 volts and silver stained as previously described.28

DNA sequencing

A method based on the avidin-biotin system was used to obtain single-strand DNA. The sequence reaction was performed according to the Sanger dideoxy termination method with the Auto Read Sequencing Kit (Pharmacia LKB, Uppsala, Sweden) coupled to the Automated Laser Fluorescent DNA Sequencer (Pharmacia LKB).29,30

Results

G6PD assay and electrophoretic mobility

Among the 130 males tested for G6PD activity, 102 (78.5%) showed a class II variant (G6PD activity: 0.19±0.18 IU/gHb, mean ± SD), 27 (20.8%) were carriers of class III variants (activity: 1.91±0.93 IU/gHb, mean ± SD) and one was a class I variant associated with CNSHA. The 31 women displayed G6PD activity ranging from 0.23 to 5.2 IU/g Hb (2.45±1.27 IU/gHb, mean ± SD) and resulting in wide dispersion according to differential X chromosome inactivation. One hundred and fifteen out of the 130 male samples were subjected to electrophoretic analysis on crude RBC lysates: 80 (70%) were electrophoretically normal (79 contained in class II, 1 in class III), 16 (14%) were slow (3 contained in class II, 12 in class III, 1 in class I), and 19 (16%) were fast variants (16 in class II, 3 in class III) (Table 2).

Molecular analysis of the class I variant

The single G6PD-deficient sample associated with CNSHA and showing slow enzyme mobility was submitted to non-radioactive SSCP. The fragment encompassing exons 10 and 11 displayed abnormal electrophoretic mobility and was then sequenced. From this analysis we found a 1246 G→A mutation already classified as G6PD Tokyo. This case was described separately by our group.[28]

Molecular analysis of class II variants

All 102 DNA samples from male subjects with severe G6PD deficiency not associated with CNSHA (class II) were first screened by PCR-RE analysis for the 563 C→T mutation (G6PD Mediterranean), which is the most common Italian mutation included in WHO class II. This mutation was identified in 81 out of the 102 (79.4%) subjects. Electrophoresis carried out on the protein from these samples revealed 74 normal, 4 *fast* and 3 *slow* enzyme variants. The 21 male subjects negative for the G6PD Mediterranean mutation were then tested using the PCR-RE method for all other known molecular defects already described in the Italian population. We found 2 samples with G6PD A-, 8 with G6PD Union and 2 with G6PD Cosenza. The electrophoretic behavior of these enzymes showed that all 12 samples were *fast*. SSCP analysis of the remaining 9 samples showed two abnormal patterns. The sequence analysis revealed two new molecular abnormalities recently published and named by us as G6PD S. Antioco (2 cases) and G6PD Partenope (1 case) that do not modify the electrophoretic mobility of the enzyme.[20] Six samples (5.9%) with low G6PD activity remained uncharacterized at the molecular level; 2 of them showed normal elec-

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Table 2. Electrophoretic mobility and DNA analysis of 114 male subjects subgrouped into WHO classes II and III on the basis of enzyme activity.

	Class II Electrophoretic mobility				Class III Electrophoretic mobility				
G6PD variant	Normal*	Slow°	Fast#	ND@	Normal*	Slow°	Fast#	ND@	TOTAL
Mediterranean	74	3	4	–	–	–	1	6	88
Union	–	–	8	–	–	–	–	1	9
Seattle	–	–	–	–	–	7	–	–	7
A⁻	–	–	2	–	–	–	2	–	4
Cosenza	–	–	2	–	–	–	–	–	2
S. Antioco	2	–	–	–	–	–	–	–	2
Partenope	1	–	–	–	–	–	–	–	1
Unknown	2	–	–	4	1	5	–	4	16
TOTAL	79	3	16	4	1	12	3	11	129

*Number of samples submitted to electrophoretic analysis: class II, 98 samples; class III, 16 samples. *Total normal samples = 80/114; °Total slow samples = 15/114; #Total fast samples = 19/114; @Total undetermined samples = 15.*

trophoretic mobility and 4 were not analyzed (Table 2).

Molecular analysis of class III variants

All 27 male subjects with enzyme activity exceeding 10% of the normal level were screened for G6PD Seattle (844 G→C), which is the most common Italian class III G6PD mutation. This mutation was found in 7 of them (26%). All 7 of these samples showed, as expected, *slow* enzyme mobility. The remaining 20 subjects were submitted to PCR-RE analysis to search for other known molecular abnormalities: the G6PD A– mutation was detected in 2 of them (7.4%, electrophoretically *fast*); surprisingly, 7 others (26%) displayed the G6PD Mediterranean mutation and one (3.7%) the G6PD Union mutation. Unfortunately, only one of the subjects carrying the Mediterranean mutation was available for electrophoretic mobility determination, which revealed *fast* enzyme. Ten samples (37%) remained uncharacterized at the molecular level. G6PD Sibari and G6PD Montalbano, contained in class III, were screened in these 10 samples but proved to be negative, and SSCP analysis did not display any abnormal pattern in the entire coding region. Of these 10 samples, five demonstrated slow electrophoretic mobility, 1 was normal and 4 were not determined (Table 2).

Molecular analysis of female subjects

Since G6PD-deficient females could have not been grouped according to the WHO classification because of G6PD activity variations due to X chromosome inactivation, all the aforementioned mutations were tested in our 31 samples. All the females were heterozygotes except for one homozygous subject (32 mutated alleles expected). The G6PD Mediterranean mutation was found in 23 out of the 31 females studied. This molecular defect displayed heterozygosity in 22 subjects and homozygosity in 1 (G6PD activity = 0.23 IU/gHb), resulting in 24 G6PD Mediterranean alleles out of 32 mutated alleles (allele frequency = 75%). G6PD Union was detected in one sample (allele frequency = 3.1%), G6PD Seattle in 3 (allele frequency = 9.4%) and G6PD A– in 2 (allele frequency = 6.2%). Two samples remained uncharacterized (Table 3).

Frequency of G6PD mutations

Considering the whole sample, representative of the Italian population, the frequency of the G6PD Mediterranean mutation was 69% and that of G6PD Union and G6PD Seattle was 6.2%. Considering the geographical origin of the G6PD-deficient subjects analyzed in this study, some differences were observed in the frequency of the diverse G6PD mutations.

The frequency of the G6PD Mediterranean mutation was 83% in Sardinia, 63% in southern Italy and 58% in northern Italy. The frequency of G6PD Union was 10% in Sardinia and only 3.9% in continental Italy. By contrast, G6PD A– was never detected in Sardinia but its frequency was 7% and 4.4% in southern and northern Italy respectively. The frequency of G6PD Seattle was 1.7% in Sardinia and 8.8% in the rest of Italy (7% in the South, 11% in the North).

Some other rare G6PD variants seemed to be detectable only in restricted areas, such as G6PD S. Antioco in southern Sardinia and G6PD Partenope in Naples. Moreover G6PD Cosenza was found in two male subjects, respectively from Sardinia and from southern Italy. The frequency and geographic distribution of all these G6PD molecular defects are shown in Table 3.

Discussion

This paper reports the frequency of the most common molecular variants of G6PD in Italy. Previous works reported the frequency and distribution of molecular G6PD variants in restricted Italian areas,[7,18,31,32] but a large-scale study comparing subjects originating from different geographic areas had not yet been performed. Molecular analysis of the G6PD coding region of the 161 subjects divided into subgroups according to the WHO classification confirmed a high frequency of G6PD Mediterranean genotypes among the Italian population: G6PD Mediterranean was found in roughly 70% of the total number of mutated alleles considered in this study, followed by G6PD Seattle and G6PD Union (6.2%), G6PD A– (3.7%), G6PD Cosenza

Table 3. Allele frequency of the most common G6PD mutations in Sardinia and continental Italy.

Geographic area	*Alleles*	*Mediterranean*	*Union*	*Cosenza*	*S. Antioco*	*Partenope°*	*Seattle*	*A–*	*Tokyo°*	*Undefined*
Sardinia	60*	50 (83%)	6 (10%)	1 (1.65%)	2 (3.3%)	–	1 (1.65%)	–	–	–
Southern Italy	57	36 (63%)	2 (3.5%)	1 (1.75%)	–	1 (1.75%)	4 (7%)	4 (7%)	1 (1.75%)	8 (14%)
Northern Italy	45	26 (58%)	2 (4.4%)	–	–	–	5 (11%)	2 (4.4%)	–	10 (22%)
Total	162	112 (69%)	10 (6.2%)	2 (1.2%)	2 (1.2%)	1 (0.6%)	10 (6.2%)	6 (3.7%)	1 (0.6%)	18 (11.1%)

**One female was homozygous; °Not polymorphic.*

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(1.2%) and G6PD S. Antioco (1.2%). Single cases of G6PD Tokyo and G6PD Partenope were also detected. The frequency of the G6PD Mediterranean genotype rose to 80% when only subjects with severe G6PD deficiency (activity < 10% of normal, WHO class II) associated with acute hemolytic anemia (AHA) were considered. This genotype was also frequently observed (75%) in the group of female heterozygotes. When we analyzed in detail variants of WHO class III, we found 26% G6PD Seattle and 7% G6PD A–, while none of the subjects was carrier of any of these other previously described Italian class III G6PD variants (G6PD Montalbano and G6PD Sibari), thus showing greater molecular heterogeneity than expected from previous molecular and biochemical studies. Seven cases of G6PD Mediterranean and one case of G6PD Union were also observed in subjects with mild G6PD deficiency (WHO class III), suggesting to two different hypotheses: a) an overestimation of G6PD activity; b) a synergetic effect due to a second unknown mutation, leading to partial phenotypic reversion. The first hypothesis seemed to account for the majority of cases since all the biochemical properties of the enzyme were comparable with those of G6PD Mediterranean except the enzyme activity (unfortunately, we have no data on teh G6PD electrophoretic mobility of these samples). The second hypothesis was supported in at least one male subject with the nt 563 mutation who showed G6PD activity that was roughly 50% of normal and biochemical properties completely different from those of G6PD Mediterranean-like variants, resulting in normal substrate analogue utilization, normal K_mG6P, high K_mNADP and fast electrophoretic mobility (data not shown). This last observation actually seems to suggest the existence of an unknown second mutation, since none of the most common molecular abnormalities (i.e. G6PD A–) were present in the coding region. The subject with the class I variant associated with CNSHA proved to be G6PD Tokyo. To our knowledge, this molecular abnormality has not been described before in Italy, being reported only in Japan and in England.[33,34]

When we compare the frequency of the different mutations in different geographic areas, it soon becomes apparent that G6PD Mediterranean has a higher prevalence in Sardinia (83%) than in either in southern or northern Italy (63.1% and 57.8%, respectively) and this difference appears to be significant ($p<0.005$). On the other hand, the molecular heterogeneity of G6PD variants in Sardinia was higher than described by other authors, who found only the G6PD Mediterranean and G6PD Seattle genotypes.[13,32] We discovered at least five different molecular defects in Sardinia that accounted for all the subjects studied. The molecular heterogeneity observed in Sardinia is, however, much lower than that observed in continental Italy, since we were unable to characterize 18 out of 102 (17.6%) cases in the latter group. These samples are now under investigation by sequence analysis.

Finally, this paper strongly confirms the importance of a first screening method based on simple determination of the activity and electrophoretic mobility of the enzyme to test for candidate mutations. The electrophoretic mobility of G6PD, determined on cellulose acetate gels, has in fact proven to be a useful method in the search for candidate mutations among G6PD genes since the four most common molecular defects present in the Italian population display different electrophoretic behavior: G6PD Mediterranean is electrophoretically normal while G6PD Seattle is slow and G6PD Union and G6PD A– are fast. These last two varieties can also be easily distinguished by their differences in enzyme activity. This approach, together with a study of the clinical manifestations of G6PD-deficient subjects, led to rapid identification of over 85% of the molecular defects responsible for G6PD deficiency in a large Italian sample population. Some apparent discrepancies observed in a few samples among electrophoretic mobility, biochemical properties and molecular characterization could be explained in at least two different ways: first, we cannot exclude the presence of other mutations or polymorphisms which could alter the electrophoretic mobility of the enzyme; second, the presence of some artifacts due to sample storage or linkage with cofactors cannot completely be excluded either, since the enzymes were not always tested at different times and on different blood samples. These observations lead to the final conclusion that a large number of variables must be considered in the study of G6PD deficiency and caution must be used in the biochemical and molecular characterization of G6PD variants.

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