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Molecular Surveying of the Common Variants of Glucose 6-Phosphate Dehydrogenase Gene in Deficient Patients in Two Neighboring Provinces, Kerman and Yazd in Central Part of Iran

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Abstract

Glucose 6-phoshphate dehydrogenase is X-chromosome linked that expressed in all tissues. This is the first enzyme of pentose phosphate pathway were 5-carbon sugar Ribose and NADPH were synthesized by coupled oxidation /reduction reactions and this enzyme is a highly polymorphic enzyme in humans. G6PD deficiency are shown to be the cause of haemolytic effect of Fava beans and primaquine. It soon became apparent that G6PD deficiency was a widespread genetic defect and hereditary deficiency of G6PD turned out to be among the most common genetic disorders, affecting more than 400 million people worldwide. G6PD deficiency is actually the most common clinically important enzyme defect. In this study, we have analyzed peripheral blood samples of 119 patients with a history of favism in Kerman and Yazd in central part of Iran. DNA was extracted from leukocyte and analyzed for four known G6PD mutations (Mediterranean, Chatham, Cosenza and $A^{-(202)}$) by PCR-RFLP technique. The results showed that Mediterranean mutation at nt563(C-A) is the most predominant mutation in this area 63.5% (Kerman: 63%, Yazd: 64%) and 1.68% of patients had Chatham mutations but none of samples was found to have Cosenza and $A^{-(202)}$ mutations. In this paper, we also try to document other disorders such as Malaria in mentioned patients.

Keywords: G6PD; Kerman; Yazd; Mediterranean; Chatham

Introduction

Glucose- 6-phosphate dehydrogenase (G6PD) is an essential enzyme to cell growth. It is a limiting enzyme of the Pentose Phosphate Pathway of carbohydrate metabolism. This enzyme is involved in the conversion

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of glucose-6-phosphate to 6-phospho gluconate, with production of NADPH and ribose-5-posphate. NADPH is required for various biosynthetic pathways as well as for the stability of catalyze and preservation and regeneration of the reduced form of glutathione (GSH) [1-2, 8]. G6PD deficiency is the most common human enzymopathy, affecting more than 400 million people worldwide. It may result in neonatal jaundice; drug induced haemolytic anaemia, favism and chronic-none spherotic haemolytic anaemia. A clinical manifestation of G6PD deficiency closely related to drug induced haemolytic anaemia induced by 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. Presumably, some other factors, such as genetic and metabolism of the active ingredients in the beans, are involved. The vast majority cases of favism occur in individuals with severely defect (class 2) variants of G6PD (e.g. Mediterranean and Cosenza) and also have been observed in class 3 variants (e.g. Chatham) [3-6,8].

The geographic distribution of G6PD deficiency led to the suggestion that G6PD polymorphisms confer resistance to infection to *falciparum* malaria. Epidemiologic investigations indicate that the highest gene frequencies are present amongst populations living in low lying areas in which the incidence of malaria is high. The high frequency of G6PD in other areas suggested that additional factors may be involved. In Iran, the highest incidence of G6PD deficiency has been found in sought village Choreb (22.8%) with high frequency of *P. vivax* and *P. falciparum* antigen. The frequency of G6PD amongst Iranian Zoroastrians has been increased following their immigration to Bambaei and Gorjeat because of malaria selection [8,9].

The G6PD gene contains 13 exons and is located on the X-chromosome (Xq28) [10]. More than 130 different mutations in the G6PD gene have been found to be the primary defect of about 177 variants of red cell G6PD deficiency [8,11]. Most of the G6PD deficiency patients are of Africa, Middle Eastern and southern Asia ancestry. One of the most common G6PD variants is the Mediterranean (563 C>T) [9]. It has been observed in several countries such as Saudi Arabia 47% [12], Bahrain [13], Oman 75%, Iraq 92%, joudan, Lebanon and one case in Iran in 1990 [14], Turkey 80% [15], Pakistan 76% [16], Egypt 52.6% [17], Greece [18], Italy 80% [19] and Spain [20]. The frequency of this variant of G6PD is 0.70 among Kurdish Jews, probably the highest incidence of G6PD deficiency in any population [21].

The aim of this study was the molecular analysis of G6PD patients for G6PD mutations for the first time in the central areas of Iran such as Kerman and Yazd provinces [7-8, 24]. G6PD deficiency is diagnosed more frequently in the two mentioned neighboring provinces, also in this area, malaria is one of the common disorders which limited in patient with G6PD, because the geographic distribution of G6PDdeficiency led to the suggestion that G6PD polymorphism confer resistance to infection to malaria.

Materials and Methods

Totally, 119 unrelated boys and girls, from some hospital in Kerman and Yazd provinces were referred following acute anaemia triggered by the ingestion of fava beans. All patients were diagnosed as G6PD deficient using the fluorescent spot test [22]. In the majority of our cases, G6PD deficiency was associated with jaundice, dark or even black urine, abdominal or back pain, acute anaemia. In additional, in all patients, the G6PD deficiency was associated with favism. Patients' profiles is shown in Table 1. Informed consent was obtained for all patients. Genomic DNA were extracted by the salting out and kit (Cinnagen) methods from peripheral blood leukocytes. All 119 sample were screened for common known G6PD mutations using restriction enzyme analysis of the appropriate PCR-amplified exons [11,23-24]. This was carried out to identify G6PD Mediterranean *(MboII)*, G6PD Chatham *(BstXI)*, G6PD Cosenza *(Eco811)* and G6PD $A^{-(202)}$

Table 1. Patients' data on some demographic and clinical features

Variable		n (%)
Age (year)	<=7	31 (26.1)
	8-12	30 (25.2)
	13-21	29 (24.4)
	≥22	29 (24.4)
Sex	Male	49 (41.2)
	Female	70 (58.8)
Anemia	Present	25 (21.0)
	Absent	94 (79.0)
Infection	Present	28 (23.5)
	Absent	91 (76.5)
Ethnicity	Fars	90 (75.6)
	Kord	3 (2.5)
	Lor	9 (7.6)
	Tork	2 (1.7)
	Arab	7 (5.9)
	Others	8 (6.7)

(NlaIII). Restriction endonuclease digestion were carried out overnight at 55°C for *BstXI* and 37°C for *MboII, NlaIII* and *Eco811*, using five unit of enzyme. Samples were analyzed for Mediterranean, Chatham, Cosenza and $A^{-(202)}$ by PCR-RFLP method as described [25-26]. The DNA region from the G6PD gene containing each point mutation was selectively amplified by PCR using specific oligonucleotide primers followed by digestion with restriction enzymes. Cinnagen Taq DNA polymerase was used. Oligonucleotide were Mediterranean (nt563 C>T) (Forward: 5′-CCCGGAAGAGGAAATCCAGGGGGT-3′; Reverse: 5′-GAAGAGTAGCCCTCGAGGGTGACT-3′), Chatham (nt1003G>A) (Forward: 5′-CAAGGAGCC CATTCTCTCCCTT-3′; Reverse: 5′-TTCTCC ACATAGAGGACGACGGCTAAAGT-3′), Cosenza (Forward: 5′-GCAGCCAGTGGGATCAGCAAG-3′; Reverse: 5′-GGCAAGGAGGGTGGCGG TGG-3′) and $A^{-(202)}$ (Forward: 5′-GTGGCTGTTCCGGGATGGC CTTCTG-3′; Reverse: 5′-CTTGAAGAAGGGCTCA CTCTGTTTG-3′). PCR conditions were: Mediterranean (30 cycles: one cycle consists of 1 min for each of following temperatures 94°, 61° and 72° C), Chatham (30 cycles 95°, 65° and 72° C, each temperature 1 min), Cosenza (30 cycles 95°, 64° and 72° C, each temperature 1 min) and A^- (202 G>A) (30 cycles 95°, 61° and 72° C, each temperature 1 min).

Statistical Analysis

Statistical analysis was carried out using the SPSS 11.5 software program. The clinical features and demographic factors of subjects were compared by means of kruskal-wallis test or χ^2 test, as appropriate. All hypothesis testing was two tailed and P-value less than 0.05 was considered statistically significant.

Results

All of the 119 patients obtained in this study were diagnosed as G6PD deficient by the fluorescent spot test. DNA samples were investigated for four mutations: Mediterranean Chatham, Cosenza and $A^{-(202)}$ by PCR-RFLP method. Results were analyzed on 12% polyacrylamide gel electrophoresis (PAGE). DNA samples of Kerman (64) and Yazd (55) were analyzed for Mediterranean (C563 T, Ser188Phe). After *MboII* digestion of the PCR product (a 583 pb fragment including exon 6 and 7), the normal samples showed four fragments (24, 60, 120 and 379bp) on acrilamide gel. Mutant fragments of 276 and 103 bp were seen in place of the normal fragment of 379 bp. We found the G6PD Mediterranean (Gd-Med) genotype in 63.5% (75 cases of 119 subjects, 63% for Kerman and 64% for Yazd) (Fig. 1). The 44 remaining samples were then examined for Chatham (G1003A, Ala 335Thr), Cosenza (G1367C, Arg459 Pro) and A^-(G202A, val>Met). Two samples out of 55 samples from Yazd province (3.63%) and none samples from Kerman province (0%) have the Chatham mutation (Fig. 2) and none of the 42 remaining samples showed the Cosenza, and $A^{-(202)}$ mutations (Figs. 3 and 4). Therefore, from all of the 119, only 1.68% showed Chatham mutation. The remaining samples require SSCP and sequencing for other possible mutations.

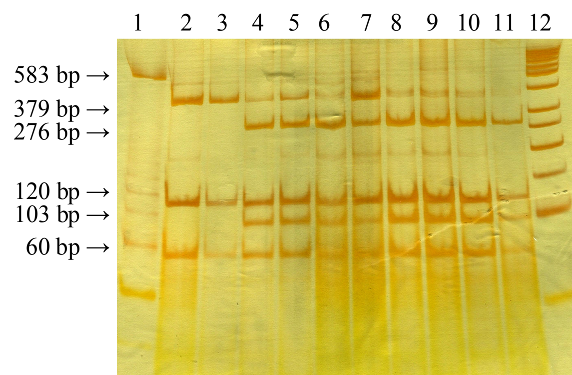


Figure 1. Restriction digestion analysis of PCR products related to G6PD Mediterranean mutation with MboII enzyme on 12% acrylamide gel from left to right. Arrows show restriction site in normal and deficient patients. Lane 1: PCR product; Lane 2: normal sample; Lane 3: positive control; Lanes 4-11: G6PD Mediterranean mutation; Lane 12: Molecular weight (ladder) of DNA (100bp ladder).

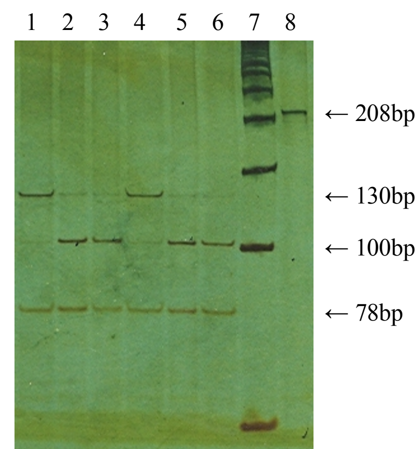


Figure 2. Restriction digestion analysis of PCR products related to G6PD Chatham mutation with *BstXI* enzyme on 12% acrylamide gel from. Arrows show restriction site in normal and deficient patients. Lanes 2, 3: a sample with G6PD Chatham mutation; Lanes 5, 6: G6PD Chatham positive control; Lane 7: 50bp DNA size marker; Lane 8: PCR product.

Demographic and clinical features such as anemia and infections are summarized in Table 1 from 119 Iranian subjects with G6PD variants. In comparison for clinical features and demographic factors such as age, sex and ethnicity between variants of G6PD we could not detect any significant difference (p>20).

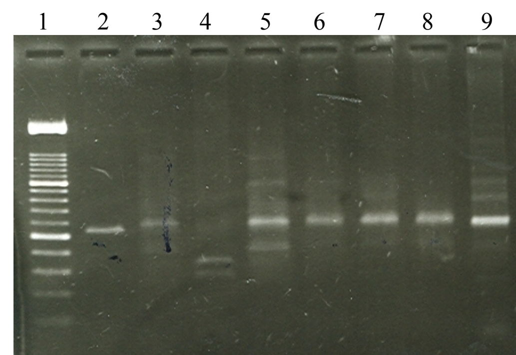
Discussion

In this study, we showed that at least two different types of mutations are responsible for G6PD deficiency in central part of Iran. The prevalence of G6PD varies greatly overall the world. In Asian populations, G6PD deficiency is known to exist with a relatively high frequency. According to the report of World Health Organization (WHO), 2.9% of the world populations are G6PD-deficient and Iran is in moderately high incidence area (10%-15%). Therefore, there are higher rates of G6PD deficiency (8.65%-16.4%) in northern part [Mazandaran and Gilan provinces] and 12%-19.3% in the southern part of Iran [7,26]. In this survey, we found that the most common G6PD deficient variant in Kerman and Yazd provinces in central part of Iran is the G6PD Mediterranean, which accounts for 63.5% of the 119 analyzed samples. Similar to other part of Iran (Table 2), favism in majority of the people in these provinces is most probably simply due to Gd-Med, which also may suggest a common origin for the population in Iran and the Mediterranean population. Its frequency reached 91.2% in Kermanshah province [28].

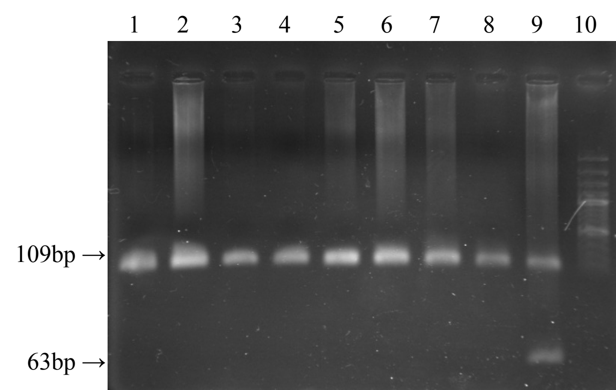
The data show that the Chatham mutation is the next mutation after the Mediterranean mutation in Kerman and Yazd provinces of Iran and accounts only for 1.68% of the total. It is classified as class III variant with milder clinical manifestations. It is also observed in Mediterranean areas, especially in Italy. G6PD Chatham was first reported in an Indian boy living in London and subsequently was detected in Algeria, Philippine, Japan, Spain, Italy, Brazil, Oman, Indonesia, Kuwait, and Malaysia [8,29-31].

The lowest frequencies for G6PD Chatham have been reported in brazil (0.66%) [30].

Its frequency reached 7.3% in Kermanshah and 13.5% Southern Iran [8,23]. In Iran, other reports indicated that in Sistan and Baluchestam (2/1%), Hormozgan (8%), Gilan (9/7%), Mazandaran (27%), Golestan (26.7%) and Khorasan (12%) have relatively this variant (see also Table 2). Chatham mutation also observed in Mediterranean areas such as Italy (15-20%), Oman (10%), Philippine (13%), Spain (2%) [7,8]. The highest frequency of this mutation reported in Mazan-daran and Golestan (about 27%) states in north of Iran. Regarding the third and fourth polymorphic variants, G6PD Cosenza and $A^{-(202)}$, none of samples contained these mutations. The G6PD Cosenza was described for the first time in the Cosenza province, North of Calabria (Southern Italy, and its phenotype is associated with a severe enzyme deficiency [8,29,30, 32]. It seems that the frequency of G6PD variants is different in various regions of Iran. For example, previous reports indicated that Mediterranean mutation is the main variant accounting in southern provinces [Sistan and Baluchestam (80%), Hormozgan (79%)] and Northern provinces [Gilan (86/4%), Mazandaran (66/2%), Golestan (69%) and Khorasan (66%) [7,8,24, 25,33,34]. This variant is the highest in some countries such as Italy (85%), India (82%), Turkey (77%), Oman (75%),



Figuer 3. Restriction digestion analysis of PCR products related to G6PD Cosenza mutation with *Eco811* enzyme on 2% on agarose gel. Arrows show restriction site in normal and deficient patients. Lane 1: molecular weight marker (100bp ladder); Lanes 2, 3: PCR product; Lane 3: positive control; the other lanes had not Cosenza mutation.



Figuer 4. Restriction digestion analysis of PCR products related to $A^{-(202)}$ G6PD mutation with *NlaIII* enzyme on 2% agarose gel from. Arrows show restriction site in normal and deficient patients. Lane 1-7: patient samples, Lane 8: PCR product, Lane 9: positive control, Lane 10: Moleclar weight (100bp ladder).

Saudi Arabia (60%), Emarat (55%), But, in south of Asia countries such as China and Japan, the common mutation is G6PD Mahidol (487 G>A) [7-8,24,35-37].

Clinical and experimental evidence strongly support the view that G6PD deficiency has been selected by *P. falciparum* malaria infection in humans. The relative resistance to *P. faciparun* infection in G6PD deficient individuals has provided the selective pressure that explains the high gene frequency [23, 38-39]. Malaria has been widely prevalent for a long time in Iran. The fist scientific survey of malaria in Iran was initiated by Latychev in 1921. He studied malaria in cities of Rasht and Bandaranzali in the Gilan province, Caspian littoral areas. In that survey spleen rate was 52.7% and parasite rate was 19.2% with parasite formula (percent of each species of Plasmodia) of 56.6% *P. falciparum*, 32.4% *P. vivax*, 4.7% and 6.3% mixed [8,40,41]. It is generally accepted that the frequencies of low-acting alleles of human G6PD are highly correlated with the prevalence of malaria. This is a hypothesis that G6PD-deficient red blood cells are more tolerant to malaria parasites, *Plasmodium falciparum* [7,8]. Although the origin of the Iranian population is rather uncertain, the closer similarity of the mutational spectrum to Italian (80-84% for Mediterranean, 20% for Chatham and 1.9% for Cosenza) rather than Middle East population may indicate that these population have a common ancestral origin [7,8, 23-25, 27, 33-34].

In this study, the defect in 42 samples remains unknown. Attempts are in progress to determine the molecular basis of disease in these samples.

Table 2. Prevalance of Mediterranean, Chatham and Cosenza in north, center and south of Iran

Area s group	Provinces	Percent of mutations		
		Mediterranean	Chatham	Cosenza
Northern				
	Gilan	86.5	9.7	–
	Mazandaran	66.2	27	6.7
	Golestan	69	26.7	–
	Khorasan	66	12	–
Central				
	Yazd	64	3.6	–
	Kerman	63	–	–
	Fars	83	13.5	–
Southern				
	Sistan & Baluchestan	80	2.1	–
	Hormozgan	79	8	–

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