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Original Article

Molecular Identification of Mediterranean Mutation in Patients with Deficiency of Glucose-6-Phosphate Dehydrogenase (G6pd) in North West of Iran

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) is a highly polymorphic enzyme that is encoded by human X-linked gene.G6PD deficiency is the most common enzymopathy in human with approximately 400 million people affected worldwide. According to the previous studies, Mediterranean mutation (C563T) is one of the most common G6PD enzyme mutations in Iranian population with a prevalence range of %63 to %86/4.This study was conducted in order to determine Mediterranean mutation, through molecular method ,in the population of the North-west of Iran. In this study, by Rapid Genomic DNA Extraction (RGDE) method, from 77 blood samples of unrelated male and female patients with genetic deficiency of G6PD, DNA was extracted and after digestion by *MboII* enzymes, in order to search for Mediterranean mutation, they were analyzed by means of PCR-RFLP and sequencing methods. Of the 77 patients, 48 patients (37 male, 11 female) had a Mediterranean mutation frequency of 62/3 %. 29 patients (37/7%) had mutations other than Mediterranean type. This study revealed that G6PD is the most common mutation in Iranian North-west population. Further studies are recommended to identify the mutation type of other varieties.

Keywords: Glucose-6-phosphate dehydrogenase, mediterranean mutation, north- west of Iran.

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Introduction

The enzyme G6PD (Glucose-6-phosphate Dehydrogenase) is one of the most important enzymes in human body, which different cells in human body including red blood cells contain varying amounts of the enzyme (Vulliamy *et al.,* 1992). G6PD catalyzes the first and pace-making reaction of the pentose phosphate cycle (PPC) by which glucose6-phosphate (G6P) is oxidized to 6-phosphogluconolactone with production of NADPH (Grimes,1980; Beutler, 2001; Cappellini *et al.,* 2008; Arese *et al.,* 1990). PPC converts glucose into pentose phosphates and produces reducing power in the form of NADPH. In RBCs NADPH is essential for the protection against oxidative stress (Pandolfi *et al.,* 1995). A major role of NADPH is to maintain reduced glutathione (GSH) at a ratio greater than 500:1 over the oxidized form GSSG (Cappellini *et al.,* 2008; Arese *et al.,* 1990). GSH plays a vital role in antioxidant defense by reacting with H2O2 and organic peroxides as well as by maintaining thiol groups of Hb, other proteins and enzymes in the reduced state. H2O2 can be also detoxified by catalase which is also stabilized by tightly bound NADPH (Gaetani *et al.,* 1996; Scott *et al.,* 1993). The major clinical manifestations of G6PD deficiency are Acute Hemolytic Anemia, Neonatal Jaundice, Mental Retardation, Chronic Renal Failure, Chronic nonspherocytic Hemolytic Anemia. in children or adults with G6PD deficiency using of some substances such as Anti-malarialdrugs, oxidizing materials, or fava beans (Vicia faba) causes severe hemolytic attacks which can be life-threatening (Beutler *et al.,* 1994; Malluh *et al.,* 1992).

The gene encoding G6PD is located on the long arm of the X chromosome (Xq28) and consists of 13 exons and 12 introns, spanning approximately 18 kb. The coding sequencing of the gene is 1,545 bp long, coding for the 515 amino acids of the G6PD primary sequence (Arese *et al.,* 2012).

Due to the location of genes, heredity of G6PD is a X-linked pattern (Mortazavi *et al.,* 2006). Hence, the defective gene in men is fully out broken. In females due to having two X chromosomes, different forms of homozygote and heterozygote are visible (Beutler, 1994).

Glucose-6-phosphate dehydrogenase enzyme deficiency is one of the most frequent human genetic disorders so that more than 400 million people are affected worldwide (Ruwende *et al.,* 1995). However; its prevalence in different populations varies from 1 to 65% (Mason *et al.,* 2007).

Although Iranian population consists of different ethnic groups, but the overall incidence of G6PD deficiency in Iranian population is estimated around 10%-14.9% (Noori-Daloii *et al.,* 2007). Some studies were carried out on the molecular basis of G6PD deficiency in Iran and showed that the Mediterranean mutation have the highest frequency in Gilan, Mazandaran, Golestan (Noori-Daloii *et al.,* 2006b). Khorasan, Hormozgan, Sistan&Baluchestan, Yazd and Kerman (Noori-Daloii *et al.,* 2007).

In most cases displacement of a base at the DNA level, causes the displacement of one amino acid with another amino acid. Normal typeG6PD is known type B. About 20 percent of black African have G6PDtype (A +) which is normal in terms of activity but is different with type B in terms of one amino acid and can be identified by electrophoresis (Luzzatto, 1973). Some African descents with G6PDtype (A-) have unstable G6PD because of mutations in the DNA.

Type Mediterranean G6PDis the most common type of G6PD and its symptoms are more severe than of type (A-). This variant can be found in the Mediterranean regions (Italy, Greece, Sardinia), the Jewish Kurds and Arabs, India and in Southeast of Asia (Luzzatto, 1973; Oppenheim *et al.,* 1993).

According to previous studies, the most prevalent mutations in Iran are Mediterranean, Chatham and Cosenza. The purpose of this study is to investigate the frequency rate of the Mediterranean mutation in north-west of Iran.

Materials and Methods

In this cross-sectional study, 77 Peripheral blood samples (2-5 ml in 300 µ EDTA 0.5 M) from unrelated patients with G6PD deficiency were collected from the hospitals of the north-west of Iran (including Ardebil, Tabriz and Urmia provinces). The blood samples collection process

was performed with the ethics committee approval and the patients' informed consent through telephone correspondences. and until the DNA extraction, the falcons were held in the temperature of -20^{o}C.Qualitative measurement of the enzyme activity was performed using Fluorescent Spot Test and Saba laboratory kit. The basis of this method is the catalytic activity of G6PDenzyme in conversion of G6PD Glucose 6-phosphate to 6-Phosphogloconate and simultaneous revival of NADP to NADPH2. The produced NADPH2 has fluorescent specifications under UV (365nm). The fluorescence intensity in the blood of healthy individuals is positive (strong) and in the blood of patients with G6PD deficiency is low or negative.Extraction and purification of the samples' DNA Genomic, from Peripheral blood leukocytes, was performed by Rapid Genomic DNA Extraction (RGDE) method.

RGDE method was performed as follow (Saremi *et al.,* 2010):

Pouring 500 µl or 0.5 gr of blood sample into a 1.5 ml microfuge tube and 1000 µl of Cell Lysis buffer.

Shaking microfuge tube gently, and then centrifuging it for 2 minutes at 6000 rpm.

Removing and discarding supernatant and repeating steps 1-3 two or three.

Adding 300 µl of Nuclei Lysis buffer to the microfuge tube and keeping the tube in room temperature for 2 minutes to prevent clot formation.

Adding 100 µl of saturated NaCl and 600 µl of Chloroform to the microfuge tube, shaking it gently then centrifuging it for 2 minutes at 6000 rpm.

Transferring 300-400 µl of supernatant to a new 1.5ml microfuge tube.

Adding 600µlof cold Isopropanol to it; shaking it gently then quickly.

Centrifuging the microfuge tube for one minute at 13000 rpm to precipitate, and then removing some of supernatant and letting completely dried in room temperature and keeping it for later uses.

Amplification of exon 6 from the G6PD gene was performed by primers F-Med [5'CCC CGA AGA GGA ATT CAA GGG GGT 3'] and R-Med [5'GAA GAG TAG CCC TCG AGG GTG ACT 3'] 92 in thermal cycler (Senso Quest-Germany) .For each subject patient, 3µl of the mentioned primers, DFS-Taqmaster mix 12/5 µl (Bioron-Germany) was added to the PCR tube. Then 2µl of the DNA was added to the tube and until the tube's final volume 25µl distilled water (dH2O) was added to it.PCR conditions were as follows: Initial denaturation of DNA at 95 ˚C for 5 min. 35 cycles each consisting of three steps of denaturation at 94 ˚C for 1 min, annealing at 59 ˚C for 1 min, extension at 72 ˚C for 1 minute and final extension at 72˚C for 5 minutes. PCR Products were electrophoresed on 1.5% agarose gel at presence of DNA Ladder 50-700 bp (Bioron-Germany) and negative control samples. (fig. 1)Then583bp fragment, the amplified product, was digested by enzymes *MboII*.

For digestion of 10µl PCR products, 2µl of 10X Enzyme buffer (B^{+}) and 1µl *MboII* Enzyme (Fermentas Co.) were mixed in a tube and until the tube's final volume, 31µl dH2O was added to it. The Mixture was transferred to the Thermal Cycler device and for each of the cycle, the temperature of 65˚C for 2 hours and the temperature of37˚C for 25 minutes were adjusted and digested products were electrophoresed on 1.5-2% agarose gel at presence of DNA Ladder and negative control samples. In case of the absence of Mediterranean mutation and the enzymatic digestion, 379 bp, 120 bp, 60 bp, 24 bp bands are visible. If there is a mutation, 379bp band is cut and turns to 276bp and 103 bp. (fig. 2). To study the G6PD genetic changes (exon 6) accurately, DNA Sequencing was performed in a randomly selected sample of Mediterranean Mutant. To do so the PCR product was sent to Fazapazhouh Company (USA) and the response was analyzed by means of Chromas Lite version 2.33 software (fig. 3).

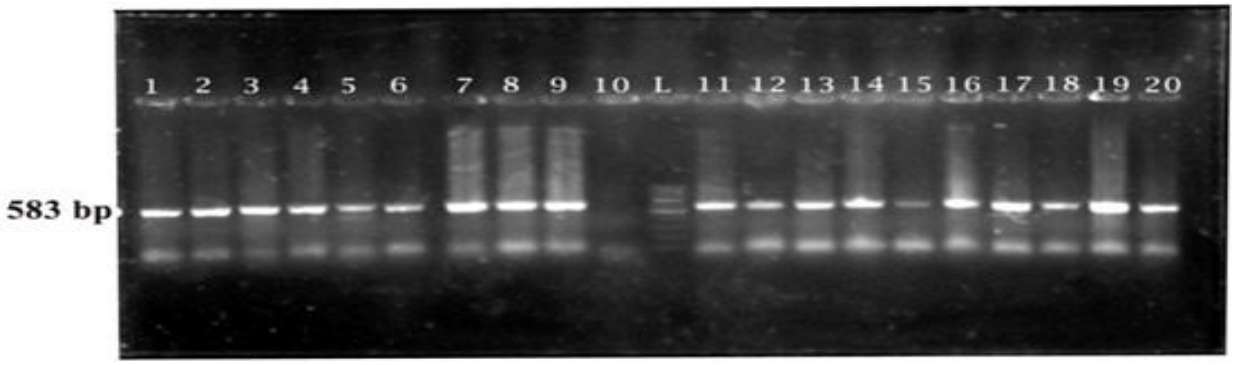


Fig. 1: The results of amplification of nucleotide sequence containing Mediterranean mutation. Lanes1-20: PCR product. Lane 10: negative control. L:50 bp plus DNA Ladder.

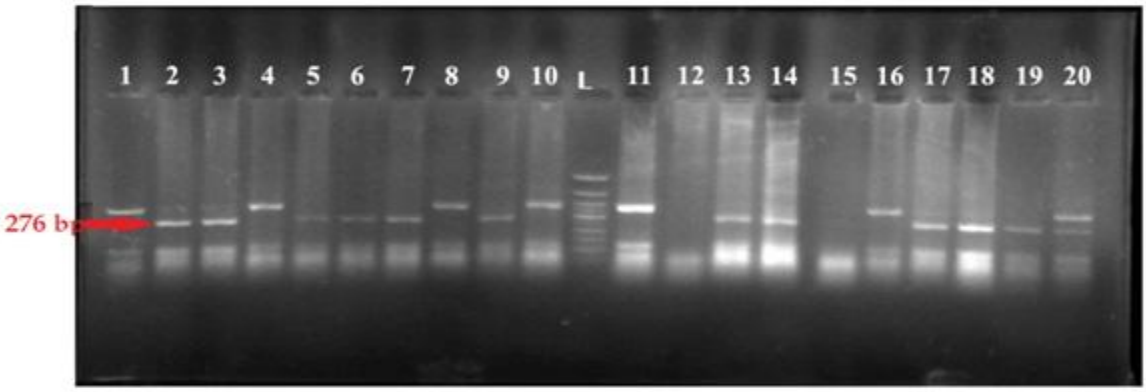
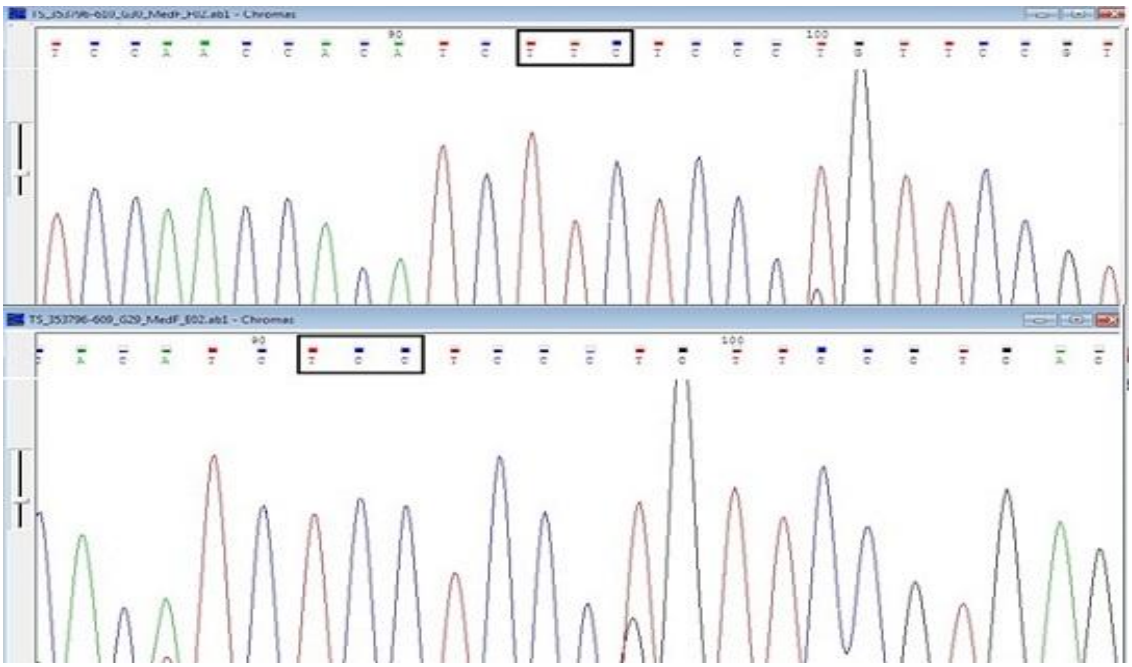


Fig. 2: Restriction digestion analysis of PCR products related to G6PD Mediterranean mutation withMboII.Lanes:1,4,8,10,11,16:normalsamples.Lane 2: G6PD-Med Positive controlLanes:3,5,6,7,9,13,14,17,18,19:G6PD Mediterranean mutation.Lanes:12,15:negative control. Lane 20: A heterozygote sample.



The results of DNA Sequencing for a sample of patient with 563 mutation (C-T) (top) and healthy individual (bottom).

As it was shown in the rectangular, in the patient sample C base has been replaced with T base.

(TCC 188 TTC) ⟶ (Ser 188 Phe)

Results

This study was conducted on 77 individuals with deficient G6PD enzyme activity. Individuals' ages ranged from one month to 21 years.G6PD Mediterranean mutation (563 C-T) was observed in 48 (37 males and 9 females) cases, which represents the prevalence rate of 62/3%. 29 patients (37/7%) weren't affected by the Mediterranean-type mutation. Mediterranean defect prevalence rate was 77/1% in men and 22/9% in womenand it shows the high incidence of this disease in males and X-Linked recessive pattern of inheritance. Among women, 9 cases (81/8%) were heterozygote females and 3 cases (18/2%) were homozygote.

Discussion

Using RFLP-PCR, this study showed that62/3% of individuals with Mediterranean type enzyme deficiency has mutations in nucleotide 563 (C-T) of G6PD gene. In these individuals, at position 188, phenylalanine amino acid is replaced by serine. G6PD is one of the most common genetic diseases that many people in the world suffer from it. This disease has caused many damages on child development and it has taken into consideration by the World Health Organization.On the basis of different studies, Mediterranean mutation prevalence is different around the world and Iran.G6PD Mediterranean mutation prevalence is 79% in turkey (Keskin *et al.,* 2002), 80% in Saudi Arabia (Niazi *et al.,* 1996), 74% in Oman (Darr *et al.,* 1996), 55% in UAE (Bayoumi *et al.,* 1996), and 76% in Pakistan (Saha *et al.,* 1994). Noori-Daloii *et al.,* studies conducted in northern, central and southern provinces of Iran (table 1) (Noori-Daloii *et al.,* 207, 2006b), showed that the lowest and the highest prevalence of Mediterranean mutation in patients withG6PD deficiency belongs to Kerman 63% and Gilan 86.5% provinces, respectively.

Table 1: Prevalence of Mediterranean mutation in north,center and south of Iran.

	Area's group	
Northern provinces	**Central provinces**	**Southern provinces**
Gilan 86.4 %	Yazd 64 %	Sistan and Baluchestan 80%
Mazandaran 66.2 %	Kerman 63%	Hormozgan 79%
Golestan 69 %		
Khorasan 66%		

The Studies of Karimi *et al.,* in Fars Province (Karimni t al., 2003), Miri Moghaddam *et al.,* in Sistan and Baluchistan province (Mirimoghaddam *et al.,* 2002) and Mortazavi *et al.,* in Zanjan province (Mortazavi *et al.,* 2006) have reported 83.8%, 85.1% and 72.7%, respectively, prevalence in these provinces.the present study, prevalence rate of the mutation is 62.3% which, compared to the reported mutations, represents the lowest prevalence rate. This study and the previous studies, conducted in the provinces, reveal thatMediterraneanmutation type is the dominant mutation in these areas it means that inIran, G6PD dominant mutation is Mediterranean type. That this mutation has happened thousands of years ago and the mutant genes have been transferred to other territories through migration or population displacement due to war and etc.

Several studies have shown that patients with G6PD deficiencyareresistant against catching malaria (Guido *et al.,* 2007). Because the prevalence rate of Malaria in the northwest of the country is lower than the South and Southeast regions, so the prevalence of deficiency for this region is justifiable.

Although, due to the inheritance of X-linked recessive, it is expected that this deficiency should be observed only in males but based on the Lyon's hypothesis (random inactivation of sex chromosomes), although less prevalent, it is likely

to occur in females. In the present study, like other studies, a significant percentage of females (23%) had this deficiency.

29 patients (37.7%) of the subjects had no mutations. since more than 60 different mutations have been identified at the molecular level (Mortazavi *et al.*, 2006). So identification of mutations in these patients needs further molecular studies and it can be done through using SSCP and ARMS-PCR methods andsequencingthe G6PD gene.

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References

Arese P, Gallo V, Pantaleo A, Turrini F (2012). Life and Death of G6PD Deficient Erythrocytes-Role of Redox Stress and Band 3 Modification.Transfus Med. Hemother., 39: 328-334.

Arese P, De Flora A (1990). Pathophysiology of hemolysis in glucose-6-phosphate dehydrogenase deficiency. Semin Hematol., 27: 1-40.

Bayoumi RA, Nur-E-Kamal MSA, Tadayyon M, Mohamed KKA, Mahboob BH, Qureshi MM (1996). Molecular characterization of G6PD deficiency in Al-aindistrict,UAE. Hum Hered., 46: 136-141.

Beutler E (1994). G6PD deficiency. Blood., 84: 3613-17.

Beutler E (2001). Energy metabolism and maintenance of erythrocytes; in Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U (eds): Williams Hematology, 6th ed. New York, McGraw Hill, 319-332.

Cappellini MD, Fiorelli G (2008). Glucose-6-phosphate dehydrogenase deficiency. Lancet., 371: 64-74.

Daar S, Vulliamy T (1996). Molecular characterization of G6PD in Oman. Hum Hered., 49: 172-174.

Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN (1996). Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood., 87: 1595-1599.

Vulliamy T, Mason P, Luzzatto L (1992). The molecular basis of G6PD deficiency. Hematol., 8: 138-140.

Grimes AJ (1980). Human Red Cell Metabolism. Oxford, Blackwell.

Guindo A, Fairhurst RM, Doumbo OK (2007). X-linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria. Plo S Med., 4: 66.

Keskin N, Ozdes I, Keskin A, Acikbas I, Bagci H (2002). Incidence and molecular analysis of glucose-6-phosphate dehydrogenase deficiency in the province of Denizli, Turkey. Med. Sci. Monit., 8: 453-6.

Karimi M, Martinez di Montemuros F, Danielli MG, Farjadian S, Afrasiabi A, Fiorelli G (2003). Molecular characterization of glucose-6-phosphate dehydrogenase deficiency in the Fars province of Iran. Haematol., 88: 346-47.

Luzzatto L (1973). Studies of polymorphic traits for the characterization of populations. African populations south of the Sahara. Isr. J. Med. Sci., 9: 1181-94.

Mason PJ, Bautista JM, Gilsanz F (2007). G6PD deficiency: the genotype-phenotype association. Blood Rev., 21: 267-283.

Malluh AA, Inseeh G, Abu-osba YK, Hamdan JA (1992). Screening for G6PD Deficiency can prevent severe neonatal jaundice. Ann. Trop. Pediat., 12: 391-395.

Mirimoghaddam E, Pourfathollah AA, Moatabar M, Shahrakipour M (2002). The prevalence of glucose-6-phosphate dehydrogenase deficiency in sistani and baluchestani tribe leaving in sistan and baluchestan province. Modarres J. Med. Sci., 5: 129-133.

Mortazavi Y, Esmaeilzadeh A, Kalantari S (2006). Evaluation of molecular genetics of glucose-6-phosphate dehydrogenase deficiency and male individuals in zanjan city during (2001-2003). KAUMS J., 9(4): 1-6.

Noori-Daloii MR (2007). A comprehensive study on the major mutations in G6PD deficient polymorphic variants identified in the coastal provinces of Caspian Sea in the north of Iran. Clin Biochem., 40: 699-704.

Noori-Daloii MR, Hejazi SH, Yousefi A, Mohammad Ganji S, Soltani S, Javadi KR, Sanati MH (2006b). Identification of mutations in G6PD gene in patients in Hormozgan province of Iran. J. Sci. IR., 17: 313-316.

Niazi G, Adey O, Kunnu A (1996). Neonatal jaundic in saudi newborns with G6PD Aures. Am. Trop. Pediatr., 16: 33-37.

Oppenheim A, Jury CL, Rund D, Vulliamy TJ and Luzzatto L (1993). G6PD Mediterranean accounts for the high prevalence of G6PD deficiency in Kurdish Jews. Human Genet., 91: 293-4.

Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L (1995). Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J., 14: 5209-5215.

Ruwende C, Khoo SC, Snow RW (1995). Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. Nat., 76: 246-249.

Scott MD, Wagner TC, Chiu DT (1993). Decreased catalase activity is the underlying mechanism of oxidant susceptibility in glucose-6-phosphate dehydrogenase-

deficient erythrocytes. Biochim Biophys Acta., 1181: 163-168.

Mohammad S, Ali *et al.*, (2010). Rapid genomic DNA extraction (RGDE).Protocol Online PID: 4791, http://www.protocol-online.org.

Saha N, Ramzan M, Tay JS, Low PS, Basair JB, Khan FM (1994). Molecular characterisation of red cell glucose-6-phosphate dehydrogenase deficiency in north-west Pak. Hum. Hered., 44: 85-9.