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RESEARCH ARTICLE

Molecular Detection of Some Mutation Which Causes β-thalassemia in AL-Muthanna Province-Iraq

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Abstract

This study was conducted to detect of mutations which cause β-thalassemia by ARMS- PCR assay for the first time in Al-Muthanna province – Iraq, during the period from October-2013 up to March–2014. The study diagnosed three types of mutation in β-thalassemic patients by ARMS-PCR assay (IVS- I-5, Codon 8\9 , Codon15), the highest percent of β-thalassemic patients mutation is IVS-I-5(53.8 %) followed by Codon 8\9 and Codon15 with percentage (27.6%) and (18.4%) respectively.

Introduction

Thalassemia (from Greek, thalassa: sea, eima: blood; British spelling, "thalassemia"). The thalassemias are hereditary anemia's caused by mutations that affect the synthesis of the globin, the protein component of the hemoglobin that thalassemias produce a massive public health problems in many parts of the world (Vichinsky,2005). They are two types of thalassemia are α and β-thalassemia, β-thalassemia is a common genetic disorder caused by mutations in one or more of the β-globin gene loci that result in reduced β-globin production (Mok *et al.*,2011) .

Most types of β -thalassemias are due to point mutations and large deletion mutations are found in rare cases, and β-thalassemia syndromes arise from mutations that affect every step in the pathway of β-globin gene expression: transcription, mRNA processing , mRNA translation, and post translational integrity of the β polypeptide chains (Galanello and Origa, 2010; Danjou *et al*., 2011). The beta-thalassemia as one of most common autosomal recessive disorders worldwide, high prevalence is present in populations in the Mediterranean, Middle-East, transcaucasus, central Asia, Indian subcontinent, and far East, it is also relatively common in populations of African descent, also, common in Northern Europe, north and south America, Caribbean, and Australia (Mok *et al.*, 2011).

Thalassemia in Iraq is a real problem mainly due to the deficiency in the equipments and drugs during different periods of lack of security and wars, out of 1064 couples recruited from the Public Health Laboratory in Basra, southern Iraq, about 5% had beta-thalassemia trait and the carriers of major beta-globin disorders comprised 11.48%. (Hassan *et al.*, 2003).

Jalal *et al.* (2010) carried out a study to determine the molecular basis of β-thalassemia in the Kurdish population of northeastern Iraq by using multiplex polymerase chain reaction and direct sequencing, a total of 11 different β-thalassemia mutations are identified in the studied samples : IVS- II-1 (G-to-A), IVS-I-110 (G-to-A), codon 8 (-AA), codon 8/9 (+G), IVS-I-5 (G-to-C), codon 5 (-CT), IVS-I-6 (T-to-C) and IVS-I-1 (G-to-A). Other mutations are less common or sporadic.

Al-Abboodi (2011) studied some of the mutations in thalassemic Iraqi population by developing a single-tube multiplex amplification refractory mutation system (multiplex ARMS), the multiplex ARMS has been developed using two sets of ARMS primers. Seven different β-thalassemia mutations are identified in the studied samples; these are (IVSI-110, codon 39, codon 37 IVS2 nt.1, codon 8/9, IVS1 nt.1 and IVS1 nt.6).

Also, Saud *et al.* (2013) recorded seven different β-thalassemia mutation in different Iraqi provinces which are :IVS-I-5 (G-C), codon 8/9(+G), codon 8 (-AA), codon 15(G-A), codon 41/42(-TCTT), codon 30(G-C).

The aims of study identify the causative mutation(IVS-1-5, codon8/9, codon15) of β -thalassemic Patients in Al- Muthanna Province -Iraq.

2-Materials and Method:-

2-1. Method

One hundred patients with thalassemia participated in the present study as well as fifty apparently healthy people were selected as the control group. All patients and control group ages ranged between 2-20 years old during the period from October -2013 up to March – 2014. These patients were registered as thalassemic patients in "Thalassemia Unit" at "Feminine and Children Hospital" in Al-Muthanna province- Iraq.

2-2. Blood Samples:

Three ml of venous blood were collected from each person (patients and controls) involved in the present study samples, and the collected blood was put into EDTA polypropylene tube that preserved in refrigerator under -20^0C .The blood in the EDITA tubes was used to perform PCR test. About 400 ul of the EDTA blood was used for DNA extraction and purification in order to carry out molecular diagnosis to identify the causative mutation using PCR –based techniques.

2-3.ARMS-PCR(Amplification Refractory Mutation System):

ARMS-PCR assay was performed for detection of point mutations directly by the presence or absence of amplification using allele specific primers in β-Globin Gene of β-thalassaemia patients. For the diagnosis of specific point mutation a pair of allele specific primers one of which has its 3′ terminal nucleotide complementary to the point mutation (Mt ARMS primer) and other to the normal DNA sequence (N ARMS primer) the method was carried out according to method described by (Rahimi *et al.*,2010).

2-3-1.Genomic DNA Extraction:

Genomic DNA from blood samples were extracted by using Genomic DNA mini kit extraction kit (Frozen Blood) Geneaid. USA.

2-3-2.GenomicDNAProfile:

The extracted genomic DNA from blood samples was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

2-3-3. ARMS PCR master mix preparation

For each mutation, a number of samples are tested according to phenotypes of the disease for both patient and control groups. Two ARMSPCR reactions (two tubes) are performed for each sample: one for identifying the presence of the normal allele (using the normal primer) and the other for the presence of mutant allele (using the mutant primer). ARMS PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as explained following table:

ARMS PCR Master mix	Volume
DNA template	5µl
Internal Control Primer A (10pmol)	1µl
Internal Control Primer B (10pmol)	1µl
Common Primer C or D (10pmol)	1µl
Normal or Mutant Primer (10pmol)	1µl
PCR water	11µl
Total volume	20µl

After that, these ARMS PCR master mix component that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermo cycler (THECHNE.USA).

2-3-4. ARMS PCR Thermocycler Conditions :

ARMS-PCR program (1): To detect the mutations: IVS1nt.5, codon 8/9, was adopted **Table (2-1).**

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	25 cycle
Annealing	65C	30sec	
Extension	72C	1.5min	
Final extension	72C	7min	1
Hold	4C	Forever	-

ARMS-PCR program (2): To detect the codon 8 mutations was adopted **Table(2-2).**

PCR step	Temp.	Time	Repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	35 cycle
Annealing	57C	30sec	
Extension	72C	1.5min	
Final extension	72C	7min	1
Hold	4C	Forever	-

2-4. Statistical Analysis

Statistical analysis was conducted to determine the statistical differences among different groups using differences among different groups using ready – made statistical design statistical package for social science. Probabilities of ($P \leq 0.05$) were considered statistically significant.(SPSS version 13).

3-Results and Discussion:-

3-1. DNA Isolation

The band integrity and DNA concentration were found to be different according to the yielded amount of genomic DNA and its purity depend on the amount of white blood cells in the blood samples. In addition, the use of fresh blood samples was found to be better, therefore, the DNA isolation should be applied as early as possible and DNA concentration in the present study was 10-100 ng (figure 3-1).

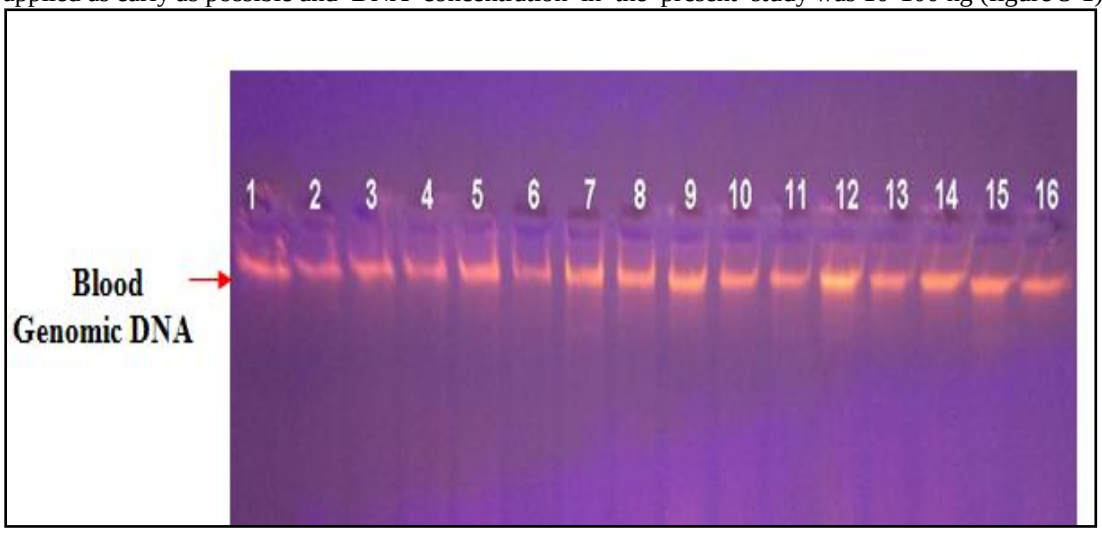


Figure (3-1): Genomic DNA bands extracted from human blood samples on 1 % agarose gel at 100Vand 80 Am for one hour.

3-2. ARMS-PCR Analysis

Each region and population had been shown to have their own profile of common mutations. Defining the precise spectrum of mutations in a given populations was important to implement strategies for genetic counseling (Chan *et al*., 2010).

In the present study, ARMS-PCR technique was used for molecular screening three studied types of β-thalassaemia mutations using a specific set of primers for each mutation and a set of internal control primers.

3-3. ARMS-PCR Screening

The internal control product of 861 bp molecular weight was showed in all ARMS-PCR reactions, which was considered as a mandatory sign of successful reaction upon gel electrophoresis, and its band was located between 800 bp and 900 bp bands . Also, the ARMS-PCR products for normal, heterozygous and /or homozygous cases for each type of the studied mutations were observed. For normal cases, the ARMS-PCR products were found within the normal primer reactions while in positive diagnosed patients, the ARMS-PCR products were found within both normal and mutant reactions in heterozygous cases and only within mutant primer reactions in homozygous cases.

This approach proved to be useful for mutation screening of β -thalassaemia alleles in Iraqi populations considering the following advantages (Tienthavorn *et al.*, 2006).

(1) High detection rate and sensitivity.

(2) Use simple methodology based on a non-radioactive detection method.

The pattern of results of the studied types of β-thalassaemia mutations was shown as the following:-

3-3-1. Intervening Sequences-I-5 (IVS-I-5) Mutation.

In figure (3-2) IVS-I-5 (G-to-C), 285 bp ARMS-PCR products were observed .ARMS-PCR products of IVS-I -5 mutation turn on a 1% agarose gel. Lane 4: DNA ladder marker . All samples contain an internal control band (861 bp).

Sample 1: contains an amplified product in both normal (N) and mutant (M) primers, assigning the individual is heterozygous genotype.

Sample 2: contains an amplified product in the mutant (M) primers indicating the homozygous genotype.

Sample 3: contains an amplified product in the normal (N) but lacks it in the mutant (M) primer; hence, implying a healthy individual.

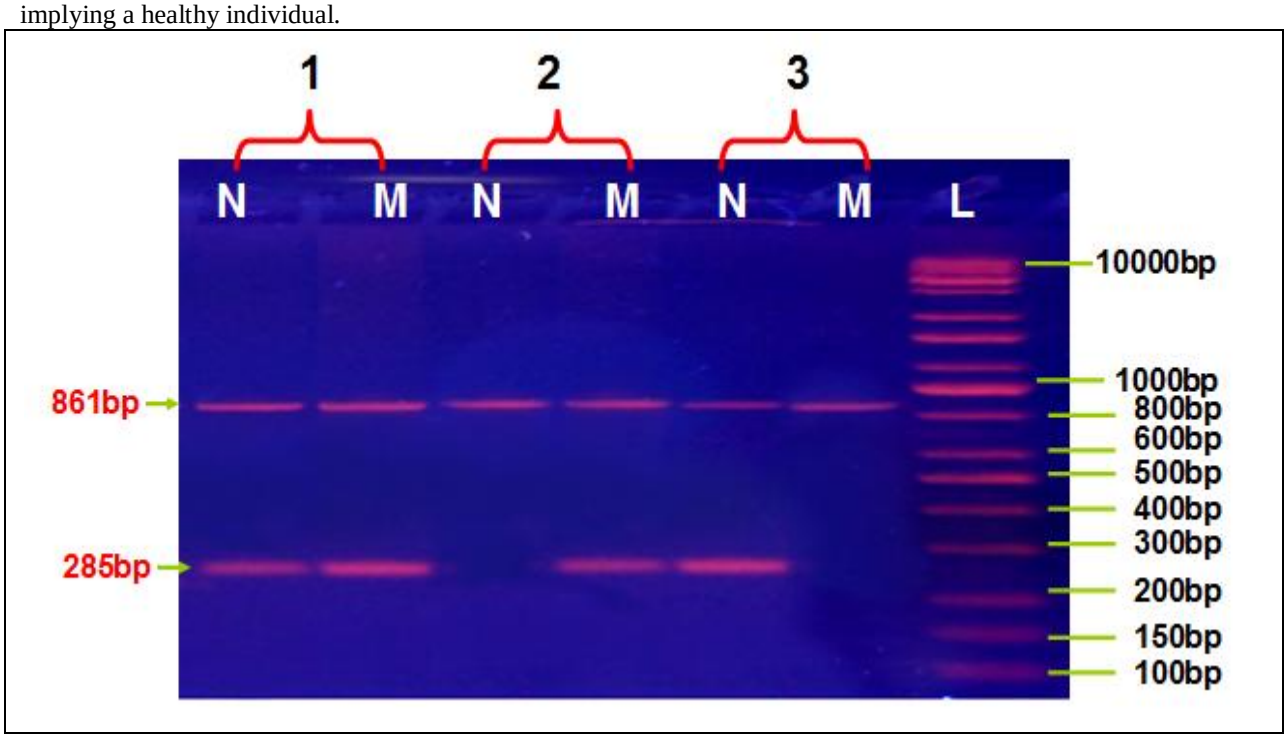


Figure (3-2): ARMS-PCR products of IVS-I-5 β-thalassaemia mutation on 1% agarose gel at 100 voltages for one hour. Sample 1: Heterozygous genotype, Sample 2: Homozygous genotype, Sample 3: Normal genotype. l: ladder DNA. **N**: Normal; **M**: Mutant.

3-3-2.Codon 8 \ 9

In figure (3-3) 225bp ARMS-PCR products were observed . ARMS-PCR products of cd 8/9 mutation turn on a 1% agarose gel. All samples contain an internal control band (861 bp). Lane 1 : DNA Ladder.

Sample 1: contains an amplified product in both normal (N) and mutant (M) primers; assigning the individual to heterozygous genotype.

Sample 2: contains an amplified product in the mutant (M) primers indicating to the homozygous genotype.

Sample 3: contains an amplified product in the normal (N) but lacks it in the mutant (M) primer; hence, implying a healthy individual.

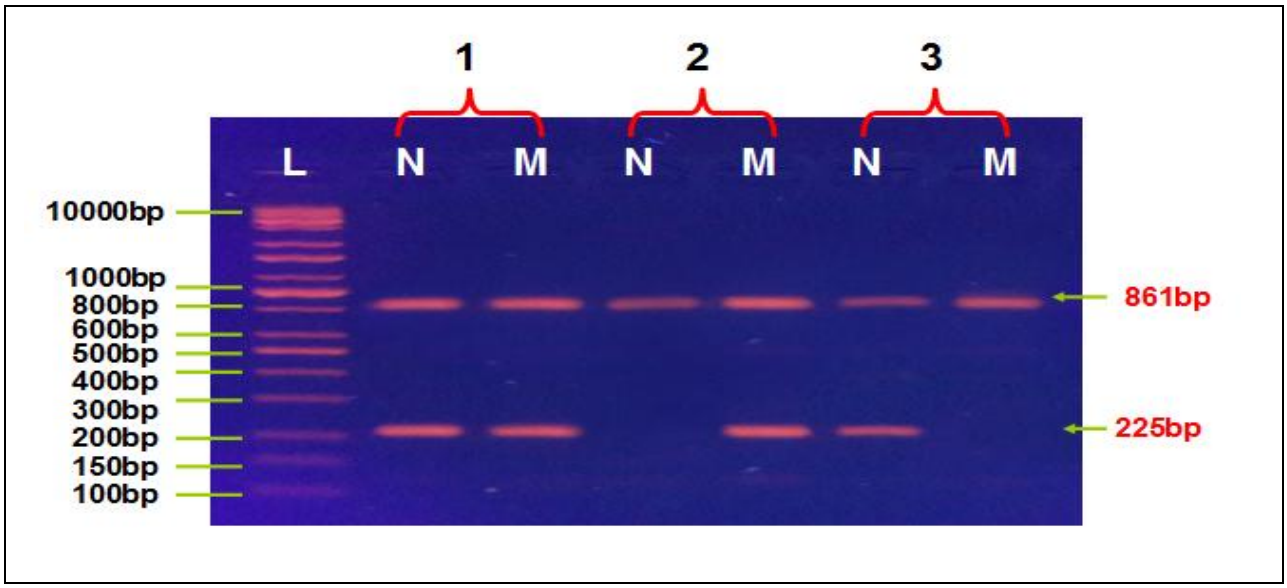


Figure (3-3): ARMS-PCR products of codon 8/9 β-thalassaemia mutation on 1% agarose gel at 100 voltages for one hour. Sample 1: Heterozygous genotype, Sample 2: Homozygous genotype, Sample 3: Normal genotype. l: ladder DNA. **N**: Normal; **M**: Mutant.

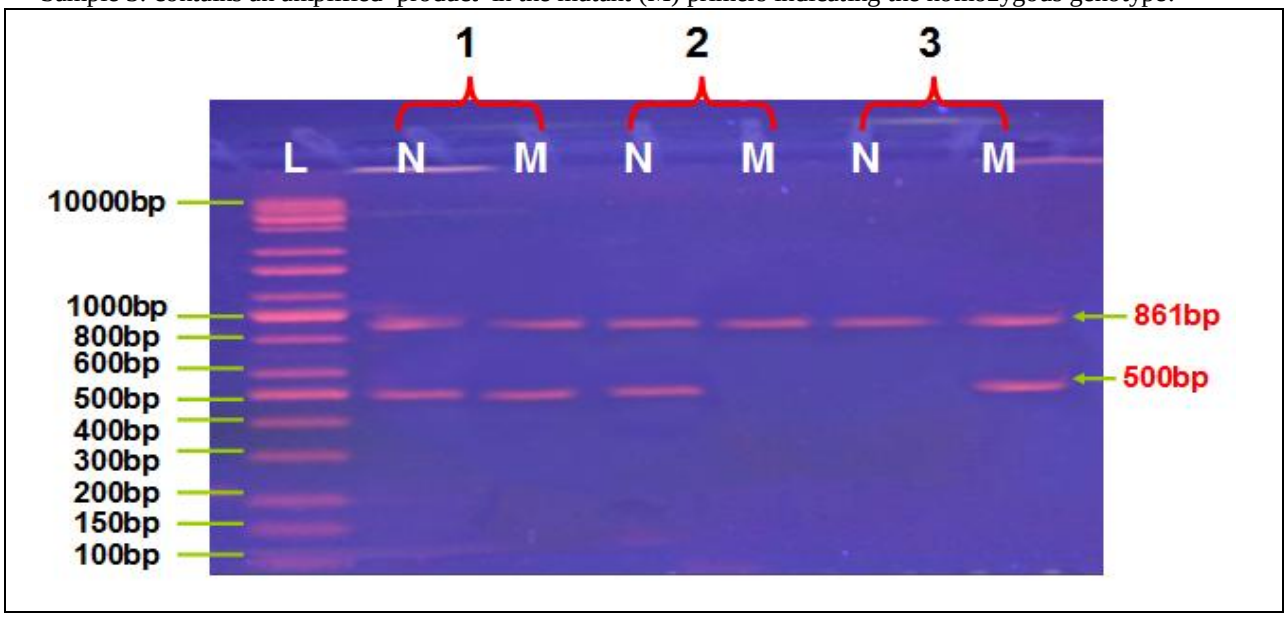
3-4-3-3. Codon 15

Figure (3-4) showed 500bp products which observed in ARMS-PCR products of codon 15 mutation turn on a 1% a garose gel. All samples contain an internal control band (861 bp). Lane1 for DNA ladder

Sample 1: contains an amplified product in the normal (N) but lacks it in the mutant (M) primer; hence, implying a healthy individual.

Sample 2: contains an amplified product in both normal (N) and mutant (M) primers; assigning the individual to heterozygous genotype.

Sample 3: contains an amplified product in the mutant (M) primers indicating the homozygous genotype.



Figure(3-4) : ARMS-PCR products of codon 15 β-thalassaemia mutation on 1%agarosegel at 100 voltages for one hour. 1- Sample 1: Heterozygous genotype, , 2- Sample 2: Normal genotype. 3-Sample 3: Homozygous genotype. L: ladder DNA .

3-4-3-4.Types of some β-thalassemic patients Mutations

The present study showed three types of mutation in β-thalassemic patients (IVS- I-5, Codon 8\9, Codon15), the highest percent of β-thalassemic patients mutation is IVS-I-5 (53.8 %) followed by Codon 8\9 and Codon15 with percentage (27.6%) and (18.4 %) respectively, table (3-1).

Table(3-1): Types of some mutations in thalassemic patients.

Mutation type	Positive patients	Percentage %
IVS- I-5	35	53.8%
Codon 8\9	18	27.6%
Codon15	12	18.4%

The above results indicated that the types of some β-thalassaemia mutations (IVS-I-5, Codon 8\9, Codon15) in iraqi population were geographically distributed around the Iraqi regions and centralized within southern parts (Al-Muthanna Province), this results is in agreement with (Saud, 2013) in the sense that they found the most common β-thalassaemia mutations were (IVS- I-5, Codon 8\9, Codon15). Also the our results in figures (4-8 , 4-9, 4-10) are in agreement with (Saud, 2013) in the sense that she recorded that IVS-I-5 and codon 15 were more occurred in the middle and south of Iraq.

Al-Assadi (2007) showed that the frequency of IVS-l-5 was (12.5%) in thalassemic samples in Iraq while in this study the frequency of this mutation in thalassemic patient's samples was (35%) . The IVSI-5, a mutation of Asian- Indian origin, was the most common mutation in the United Arab Emirates (UAE) (55%) and Oman (62%), and was quite frequent in neighboring Kuwait and Saudi Arabia (17% –19%) (Al-Sultan *et al.,* 2011).

Also the most frequent and widespread Asian-Indian mutation in Arab countries is IVS-I-5 (G-to-C). As expected, it was frequency was highest in countries of the Arabian Peninsula, while it was lower frequency in Western Asian Arab countries might be explained by migration. The frequency of mutations in the study disagreed with the results of both (Haghi *et al.,* 2009; Rahimi *et al.,* 2010) who studied the prevalence and spectrum of β-thalassemia mutations in the population of Sunni Muslim Kurds and Kermanshah Province in Iran respectively, the common mutations were codon 8/9 (+G) (15.7%).

The results of our patients are in agreement with (Saleh-Gohariand Bazrafshani, 2010), in this study the most prevalent mutation in overall samples analyzed in Kerman Province in Iran was IVSI-5 (G-to- C), with the frequency of (66.2%), the second prevalent mutation was codon 8/9 (+G) (4.9%), the less frequent mutations include: codon 15 (G-A) (2.6%).

Al-Abboodi (2011) carried out a study to identify common ethnic specific β-thalassemia mutations which showed that the frequency of codon 8/9 has been (4.1%) in Baghdad and (18%) in Al-Muthanna province. This difference in percentage may be due to each province has experienced admixtures from various populations throughout history. Furthermore, migration between Arab countries has been common until the present times.

The heterogeneity of the Arab people was reflected in the 52 β- thalassemia mutations detected. These mutations were mainly of Mediterranean and Asian origin , and although some countries have unique mutations, no specific mutation seems to be confined to the Arabs (Akhavan-Niaki *et al.,* 2011).

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