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Molecular Study of Patients with Thalassemia Major in Ardabil Province

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ABSTRACT

Introduction: Thalassemia is a common disease caused by mutations in the beta globins gene. Today, this disease is high frequency due to some factors including lack of control, the increase of population growth, and lack of implementation of appropriate methods of family planning. In present study, the molecular β-thalassemia has been investigated in patients with thalassemia major in Ardabil, Iran.

Methods: Blood samples of patients were collected over the province and in the process of collecting; sampling of venous blood was performed under blood expert guidance and with the consent of 50 patients with β-thalassemia major. The samples were stored at -70 ºC in the freezer and DNA after extraction was amplified by amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) method.

Results: The results showed that the frequency of mutation was 16%, 14% and 4% for IVSI-110, IVSII-1 and IVSI-5 mutations, respectively.

Conclusion: It can be concluded that the incidence of β-thalassemia major in Ardabil IVSI-110 has the highest ratio of the disease, IVSII-1 is in the second place and IVSI-5 has a bit effect on the creation of thalassemia major patients. It is recommended to create a database of mutations in β-thalassemia patients to find appropriate therapeutic solutions.

Keywords: thalassemia, ARMS-PCR, mutation, ardabil, blood

INTRODUCTION

β-thalassemia is the most common autosomal single gene disorders in the world characterized by hypochromic anemia due to deficient synthesis of β polypeptide chains of human hemoglobin on chromosome11 and has been spread in more than 60 countries up to 150 million, especially in the Middle East and Asian countries (1, 2). This disease was named due to high frequency among people living around the Mediterranean Sea. In severe cases, no blood transferring leads to death of the child (2, 3 and 4). A limited number of haplotypes are found in each population so that 80% of the mutations are related to only 20% different haplotypes. So far, more than 200 different mutations of this gene have been identified with different prevalence in different populations (5, 6, 7 and 8). One of the reasons for high prevalence of thalassemia in some ethnic families in Iran is intra-group marriages about 25% (9). The β-thalassemia major is the most severe form including main public health problems in the endemic areas. Each province of Iran has its own characteristic spectrum of mutations, with a handful of frequent mutations and several rare ones. Searching for and identifying rare or new mutations is a constant priority in population screening, genetic counseling and prenatal diagnosis of thalassemia (10). About 13 beta globin mutations encompass 70 - 90% of mutation spectrum in Iran. These mutations are called common beta globin mutations (11). Therefore, in order to achieve the optimal situation in this field and to identify the carriers of the disease, identifying all the common mutations that cause the disease in each region is a basic requirement. The ARMS-PCR technique

Table 1. Primer sequences used for the detection of the β-thalassaemia mutations by ARMS-PCR and normal control.

Mutations	Size (bp)	Forward primers sequence 5'-3'	Reverse primers sequence 3'-5'
IVSI-110	419	ACCAGCACCTAGGGTGGGAAAATAGAT	ACCTCACCCTGTGGAGCCAC
IVSII-1	639	AAGAAAACATCAAGGGTCCCATAGACTGAT	ACCTCACCCTGTGGAGCCAC
IVSI-5	289	CTCCTTAAACCTGTCTTGTAACCTTGTTTAG	ACCTCACCCTGTGGAGCCAC
normal control	861	GAGTCAAGGCTGAGAGATGCAGGA	CAATGTATCATGCCTCTTTGCACC

is based on the principle of allele-specific priming of the PCR process, i.e. a specific primer will only permit amplification to take place when its 3' terminal nucleotide matches with its target sequence (12).This study was investigated using ARMS-PCR technique in the field of molecular biology to detect the common type of mutations including IVSI-110 (G →A), IVSII-1 (G →A) and IVSI-5 (G →C) related to β-thalassemia major in Ardabil, Iran.

MATERIALS AND METHODS

The present study was conducted during 2015. Fifty venous blood samples including 24 females (48%) and 26 males (52%), under the supervision of specialists in blood and after filling the consent form out by patients, were randomly taken from patients with β-thalassemia major referring to hospitals in Ardabil. The samples were stored at -70 ºC in freezer. DNA was extracted from leukocytes of whole blood by phenol-chloroform method (13). After DNA extraction, spectrophotometer was applied to quantify of DNA. The amplification was done by ARMS-PCR method to detect mutations of the DNA samples obtained from β-thalassemic patients. A typical ARMS test for a single mutation consists of two amplifications in the same reaction mixture using the same genomic DNA as substrate. One amplification product results from the specific ARMS primer and its primer pair (when the mutation is present in the genomic DNA) and the

Table 2. PCR programme in three mutations IVSI-110, IVSI-5, IVSII-1 and normal

No. of cycle	Steps	Mutations & control	Temperature (ºC)	Time (min)
1	**Initial denaturation**	IVSI-110	95	2
		IVSI-5	95	2
		IVSII-1	95	2
		control	95	2
34	**Denaturation**	IVSI-110	94	1
		IVSI-5	90	0.50
		IVSII-1	94	1
		control	95	0.25
	Annealing	IVSI-110	63	0.25
		IVSI-5	60	0.50
		IVSII-1	62	1
		control	61.5	0.25
	Extension	IVSI-110	72	1.5
		IVSI-5	72	0.50
		IVSII-1	72	1.5
		control	72	0.50
1	**Final extension**	IVSI-110	72	1
		IVSI-5	72	1
		IVSII-1	72	1
		control	72	5

Table 3. Distribution of age and number of patients with β-thalassemia

Sex	Number	Frequency (%)	Age (Mean ± S.D.)
Male	26	52	20.35±9.95
Female	24	48	21.75±9.05
Total	50	100	-

Note: S.D.: Standard deviation.

Table 4. Distribution of number and frequency of IVSI-110, IVSII-1and IVSI-5 mutations in patients with β-thalassemia

Mutation	Number	Frequency (%)
IVSI-110	8	16
IVSII-1	7	14
IVSI-5	2	4

other amplification results from two primers that generate a control fragment in all cases. The generation of control product indicates the reaction mixture and thermal cycler is working optimally.

One hundred nanogram extracted DNA from each sample was amplified by ARMS- PCR using primers as described in table 1(14). The 25µL reaction mixture contained 2.5µL PCR buffer, 0.75 µL $MgCl_2$ (1.5 mM), 0.5 µL of all four deoxynucleoside triphosphates (each at 0.2 mM), 0.5 µL of each forward and reverse primers (each at 25 pmol/µL), and 2.5 U of Taq DNA polymerase. The reactions were carried out to detect the mutations IVSI-110, IVSI-5 and IVSII-1 the programme as shown in Table 2 (15). As negative control, a PCR reaction without genome was achieved to control for cross-contamination.

The frequency of mutations IVSI-110 (G→A), IVSI-5 (G→A) and IVSII-1 (G→C) was determined by Agarose Gel Electrophoresis through 1.5% agarose gel (1.5g agarose/100 ml 0.5X TBE buffer) and run at 70 voltages for an hour in two rows. The gel was stained with ethidium bromide solution (0.5µg/ml) for 15-30 min, bands of primers mutations were visualized under UV trans- illuminator and then photographed using photo documentation system (2, 16 and 15). DNA ladder (100 bp) was applied for estimating the molecular size of the bands.

RESULTS

Table 3 summarizes the demographic data for age and number of patients with β-thalassemia. The number of males and females are 26(52%) and 24(48%), respectively. The average age in female group with β-thalassemia major was 21.75±9.05 and in male patients was 20.35±9.95.

The samples of extracted DNA were analyzed using a spectrophotometer; in most cases, the samples had desirable quality and the ratio 280A/260A was appropriate among 1.8 to 2.

In this study, three of the most frequent mutations IVSI-110, IVSII-1and IVSI-5 was investigated in the provinces of Ardabil, which conducted by ARMS-PCR method. Among these mutations, IVSI-110 with a frequency of 16% was identified as the most common mutations. In second place IVSII-1 was observed with a ratio of 14% mutations. Mutation IVSI-5 with 4% frequency was the lowest among patients (Table 4).

The results of electrophoresis of PCR products in three mutations IVSI-110, IVSI-5, IVSII-1 and normal control have been illustrated in Figures 1, 2, 3 and 4.

DISCUSSION

β-thalassemia is a heterogeneous disease at the molecular level, which in most cases is caused by mutations in the beta globin locus. Majority of these mutations are including single nucleotide substitutions, deletion and addition of the oligonucleotide that alter the genes frame (17).

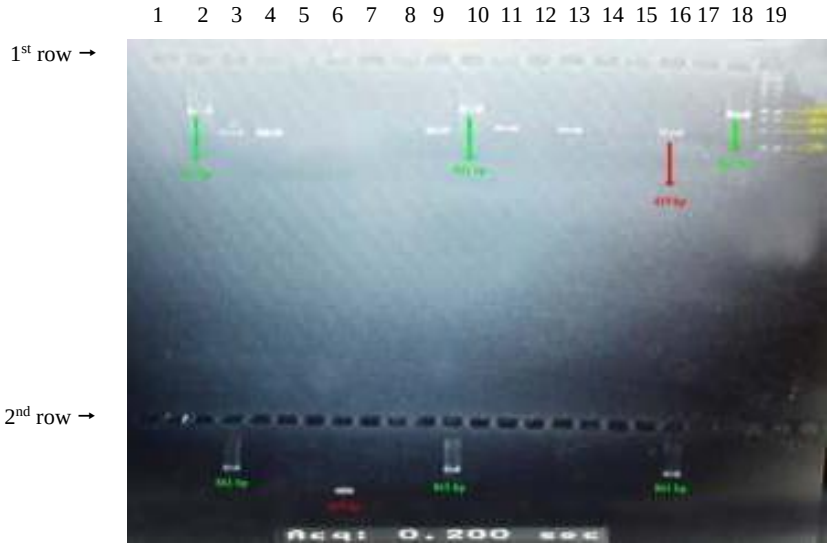

Figure 1. PCR products of IVS1-110. 1st row: Lane 1: Negative control. Lanes 3,4,9,11,13and 16; 2nd row: Lane 16 PCR Products of IVS1-110 β-thalassemia mutation detected at mol, Size of 419bp; 1st row: Lanes 2,10 and 18; 2nd row: Lane 3,10 and16 normal control; Lane 19: DNA marker.

According to the annual birth rate of thalassemia, prenatal diagnoses are priority of healthcare to prevent this type of disease. In the past two decades, numerous studies have been used for prenatal diagnosis; therefore, the numbers of abortion, health care costs, and psychological problems have been reduced in families with thalassemia (18).

For this purpose, in this research of 50 patients with β-thalassemia major three mutations IVSI-110, IVSI-5 and IVSII-1 were studied, which mutation IVSI-110 with a frequency of 16% was the most common reported already in the west and southwest of the Mediterranean. The frequency of this mutation has increased from east to west in Iran (19). The Arab regions have high frequency 12-38%, while the countries around the Persian

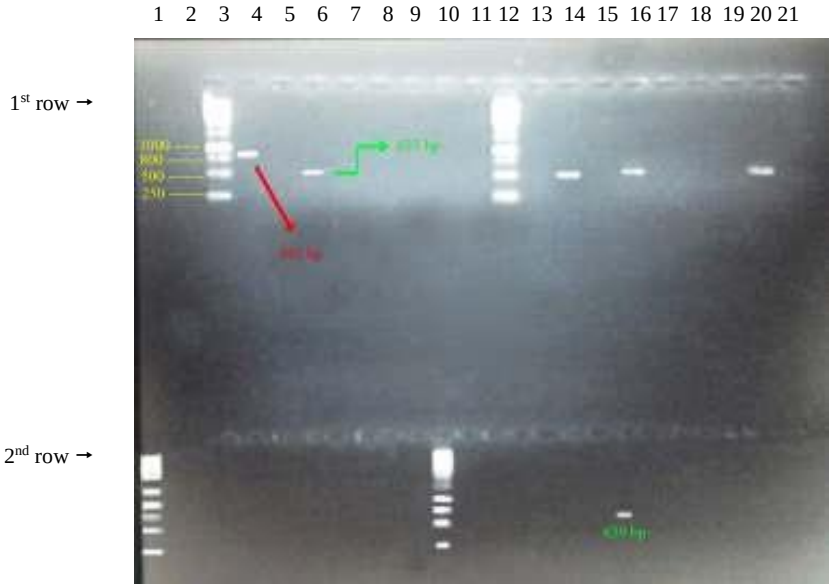

Figure 2. PCR products of IVSII-1. 1st row: Lane 3, 12; 2nd row: Lanes 1 and 10 DNA markers. Lanes 6,14,16 and 20; 2nd row: Lane 15 PCR Products of IVSII-1 β-thalassemia mutation detected at mol, Size of 639 bp; 1st row: Lane 4: PCR Products of IVSII-1 β-thalassemia mutation detected at mol, Size of 419 bp.

Gulf show less frequency 2%. Recently, there are reports that the six mutations: IVSI-110 (G > A), IVSII-1 (G > A), IVSI-1 (G > A), IVSI-5 (G > C), CD 36 - 37 (-T), and IVSI (-del 25 nt) with 50% are the common mutations in southwest of Iran (20). Lowest rates have been described in South with 0.3% frequency for FSC8/9+G and Southeast with 1% occurrence for IVSI-110 (C>T) (21). This mutation likely has been arisen from Turkey or Greece and probably expanded by migrating to eastern countries (2, 17). IVSII-1 with frequency of 14% in Ardabil is a Mediterranean type and its prevalence has been reported in the provinces of north and northwest of Iran (18). With a mean overall frequency of 23%, this mutation is the most frequent β-thalassemia mutation observed in Iran. However, its prevalence is not similar in all regions, with the highest frequency is observed in Northern regions (66%) (22). IVSII-1 (G>A) has also been reported at relatively high ratios in West, Central, Northwest, and Southwest of Iran (21). Some of mutations that are frequent in different parts of Iran (Qazvin, Khuzestan, Mazandaran, Zanjan) are IVSII-1 (G > A), IVSI-1 (G > A), IVSI-6 (T > C), IVSI- 110 (G > A), Fr 8/9 (+G), IVS1- 5 (G > C), CD 44 (-C) etc… that of which the IVSII-1 (G > A) is the most common mutation in Iran (10, 18). Results of β-thalassemia mutational gene analysis showed the frequency of CD36-37(-T) and IVSII-1 (G> A) mutations to be 23.4%. Our data showed that the frequency of CD 36 - 37 (-T) and IVSII-1 (G > A) mutations are first in the Bakhtiary background (23).

In this study, the frequency of mutation IVSI-5 was 4%. According to previous studies, the frequency of this mutation has increased from north to south and reduced from east to west as well as has been introduced the most common mutation in the central, southern and southeastern of Iran. In prior studies, this mutation has been reported the most common mutation in the Middle East, India, South and South-East Asia with a frequency of 47% (15, 18). IVS-I-5 (G> C) is also the most detected mutation in South of the country (66%) (24, 25). Northeast comprises third common area of IVSI-5(G>C) detection. Beside these three main geographical locations, IVSI-5(G>C) mutation is also among three most common observed defects in patients of North and Central districts. However, this mutation is observed in lower ratios in Western lands (21).

CONCLUSION

Given to the results of present study the frequency of mutation was 16% for IVSI-110, 14% for IVSII-1 and 4% for IVSI-5 in Ardabil province, Iran. It can be concluded that the incidence of β-thalassemia major in Ardabil IVSI-110 has the highest ratio of the disease, IVSII-1 is in the second place and IVSI-5 has a bit effect on the creation of thalassemia major patients. It is recommended to create a database of mutations in patients to find appropriate therapeutic solutions.

DECLARATIONS

Authors have no conflict of interest to declare.

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