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ORIGINAL ARTICLE

RARE AND NEW MUTATIONS OF B-GLOBIN IN AZARI POPULATION OF IRAN, A CONSIDERABLE DIVERSITY

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ABSTRACT

Background

Thalassemia, as the most common single-gene genetic disorder, is related to a defect in the synthesis of one or more hemoglobin chains. More than 200 mutations have been identified in the β-globin gene. Globally, every susceptible racial group has its own specific spectrum of the common mutations that are well-known to a particular geographic region. On the other hand, varying numbers of diverse rare mutations may occur.

Materials and Methods

The subjects of the study included 2113 heterozygote or homozygote β-thalassemia cases selected among couples who participated in the Iranian national thalassemia screening program from January 2011 to November 2019. Molecular characterization of the β-thalassemia mutation was initially carried out by the amplification-refractory mutation system-polymerase chain reaction (ARMS–PCR) technique for common mutations, followed by sequencing, Gap PCR, and Multiple ligation-dependent probe amplification (MLPA) methods - in cases not detected by the ARMS-PCR.

Results

The existence of 39 rare and new point mutations and 4 large deletions were described in our cohort. Sicilian (-13,337bp) deletion, CD36/37 (-T), and CD15 TGG>TGA were encountered more often than the others in a decreasing order, in terms of frequency. The least frequent mutations/deletions were deletion from HBD exon 1 to HBB promoter, 619 bp deletion, Deletion from up HBBP1-Exon3 HBBP1 and up HBB-0.5Kb down HBB, CAP+8 C>A, CD37 (G>A), CD6 (-A), IVSI-2 (T>C), IVSII-705 T>G, and IVSII-772 (G>A). Each occurred once. Five mutations/variants were also determined which have not been reported previously in Iran.

Conclusion

According to the findings of the study, the Northwestern Iranian population displayed a wide variety of thalassemia allelic distributions. Identification of rare and new mutations in the β-thalassemia in the national population is beneficial for screening programs, genetic counseling, and prenatal diagnosis

Keywords: Azeri Turkish Population, β-globin, Rare Mutations, Iran

BACKGROUND & OBJECTIVES

The β-thalassemia, as the most common single-gene disorder is found worldwide. It is related to abnormal hemoglobin synthesis, and is more prevalent among the Indian, Mediterranean, and Middle Eastern populations, including Iranians (1, 2). The carrier rate for the β-globin gene mutations is approximately 4-8% in most regions and provinces among the nearly 80 million people living in Iran (3, 4).

Thalassemia is a heterogeneous hereditary anemia characterized by the reduced (β^{+}) or lack of (β^{0}) synthesis of the β-globin chains of the hemoglobin (Hb) tetramer composing of two α and two β-globin chains ($\alpha^{2}\beta^{2}$) (5, 6). According to a comprehensive review of the studies by

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Weatherall & Clegg, 2001 and Cao & Galanello, 2011, the disease is diverse at the molecular level (2, 5). More than 200 mutations have been identified worldwide (6-9) resulting in the β-thalassemia major or intermedia. Fortunately, only a subset of the prevalent common mutations is frequently encountered and has been well identified in the at-risk ethnic groups (7).

Globally, every high-frequency carrier population has a small number of common mutations that are specific to a particular region, in combination with varying numbers of rare ones (6). Molecular analysis of the patients with β-thalassemia in Iran has led to the identification of 50 various β-globin gene mutations responsible for the disease, thus reflecting the heterogeneity of the Iranian population (10, 11). The diversity of these mutations reflects a historical basis for the admixture of the genes in the country (7, 12). Therefore, it is necessary to determine the frequency and distribution of the β-thalassemia mutations in different regions and provinces.

These diverse mutations in the β-globin gene have different frequencies in different regions of Iran and within different ethnicities. In each province, some of these mutations are categorized as common, while others are known to be rare. Collectively, in the 30 provinces of Iran, 13 mutations encompass 70-90% of the patients with β-thalassemia these mutations are classified as common (4, 8, 13-16). The majority of the mutations are single nucleotide substitutions or point mutations. Unlike the α-globin gene, large deletions have been rarely reported in the β-globin gene (11). A geographic distribution with specific mutations could provide information about the place of origin of the genetic change that has generated it.

The identification of the particular mutations for prenatal diagnosis during the first trimester of pregnancy is the principal goal for molecular diagnosis of β-thalassemia (17). At the same time, molecular diagnosis of β-thalassemia is more intricate because of the diverse mutations causing defective synthesis of β-globin chain (4).Therefore, the current study was conducted to analyze the rare or lesser known β-globin mutations in the population of Northwestern Iran. The common β-thalassemia mutations in this region have been extensively studied (18).

Thus, the purpose of this study was to identify the uncommon and rare β-thalassemia mutations, in order to improve the quality and rate of molecular diagnosis in patients. A timely diagnosis of a mutation in a pregnant woman enrolled in the national prenatal diagnosis (PND) program in Iran is vital, but it is limited to 18 weeks post-conception. Awareness of the spectrum of the common and rare mutations would help to detect the responsible mutations in an accurate and timely manner.

MATERIALS/PATIENTS AND METHODS

Study Subjects

EDTA tubes containing venous blood samples were collected from 5190 presumptive hemoglobinopathy carriers who were at the premarital or PND stages from January 2011 to November 2019. The carriers had been referred for confirmation by the molecular analysis under a national prenatal screening program (19). The selection criteria in this program were based on the hematological indices (MCV<80 fl, MCH<27 pg). If both in the couple are microcytic, hemoglobin A2 concentrations are then checked by the hematology laboratories.

As a part of the 7-year nationwide screening program for thalassemia at our medical genetic center, molecular analysis was performed for 5190 potentially at-risk individuals, mostly among the Azeri community. In this population, 4749 cases belonged to the couples who were in the pre-marital stage, and the remaining 441 cases were related to fetal samples (samples obtained from the amniotic fluid or chorionic villus).

This nationwide program has been implemented by Iran's Ministry of Health and Medical Education to reduce and control the incidence of thalassemia. Ethical approval was obtained to report the results from our institutional review board.

Molecular Analysis

Screening and full genotyping of thalassemia were performed on all individuals. Based on the hematologic and hemoglobin electrophoresis results, they were tested for the β-thalassemia (1389 cases) or both β and α-thalassemia (3801 cases) by molecular analysis methods. Those diagnosed with a heterozygote or homozygote β-thalassemia mutation, 2113 cases, were included in this study.

First, the ARMS-PCR technique was used to screen the ten well-known common thalassemia mutations in this region including the IVSII-I(G>A), IVSI-110(G>A), codon-8 frameshift FSC8(-AA), FSC8/9(+G), IVSI-I(G>A), IVSI-6(T>C), IVSI-5(G>C), CD39(C>T), CD44(-C), and (-30) (T>A).

In individuals who were negative in terms of these mutations, Sanger sequencing analysis (Sanger, 1997) was performed using an ABI automated sequencer analyzer (ABI-3730XL Capillary, Applied Biosystem, USA), according to the manufacturer's instructions. Gap-PCR and multiplex ligation-dependent probe amplification (MLPA) (SALSA MLPA P102 HBB kit) techniques were also used for detection of the large possible deletions. Gap-PCR method was used for evaluation of 619 bp-deletion, HPFH1, HPFH2, HPFH3, *Sicilian* (-13,337bp) deletion,

and Hb Lepore, as well as Spanish, Asian-Indian, and Turkish δβ-thalassemia deletion. Mutations with frequencies equal or less than 2% were considered as rare.

RESULTS

In total, 2113 cases (40.7 %) were found to be affected, either as homozygote/compound heterozygote patients (122 offspring) or β-thalassemia carriers (1991 cases).

Among 2113 detected cases in this family-based study, 572 individuals were excluded for statistical analysis, because they were relatives [consanguineous couples or heterozygote offspring (450 cases) and homozygote offspring (122 cases)]. Hence, the analysis was conducted on **1541** independent individuals considering the consanguinity.

Of the 1236 subjects, the β-globin gene showed the common mutations mentioned above, and 305 subjects (19.79 %) were found to have rare mutations. Thirty-nine rare mutations were identified in this cohort study. (Table 1)

All the mutations were identified by Sanger sequencing techniques with the exception of five mutations: *Sicilian* (-13,337bp) deletion*, Lepore* deletion from HBD exon 1 to HBB promoter, deletion from up HBBP1-Exon3 HBBP1 & up HBB-0.5Kb down HBB and 619 bp deletion. *Sicilian* (-13,337bp) deletion*, Lepore* and 619 bp deletion mutations were detected by the Gap PCR. *(Figure 1)* The large deletions identified by the MLPA techniques were the "deletion from HBD exon 1 to HBB promoter" and "*Deletion from up HBBP1-Exon3 HBBP1 and up HBB-0.5Kb down HBB"(Figure 2)*.

The Sicilian (-13,337bp) deletion, CD36/37(-T), CD15 TGG>TGA, and CD22/23/24-7 bp(-AAGTTGG) were encountered more frequently than the other rare mutations (frequency of above 1%). Eight mutations (IVS-II-745 (C>G), IVS-I-128(T>G), CAP+20 (G>A), 25bp del, (-28) TATA Box, CAP+22 (G>A), IVS-I (-1), or codon 30 (G>A), (-88) C>A) were observed with a frequency of approximately 0.5-0.1%. The mutations with a frequency between 0.5 – 0.1% were as follows: IVS-I-130(G>C), CD82/83 (-G), CD35(C>A), IVS-II-848(C>A), -87(C>T), CD29(C>T), CD25/26(+T), (-87)C>G), (-101) C>T, CD41/42(-CTTT), CAP+1548 (A>G), (-86) C>G, Hb Lepore, CAP+1 (A>C), CD126 (GTG>GGG), CD69 (GGT>AGT), Hb City of Hope, CAP + 1570 T>C, CD16(-C), and deletion from HBD exon 1 to HBB promoter. The least frequent mutations/deletions (less than 0.1%) were the 619 bp deletion, CAP+8 C>A, CD37 (G>A), CD6 (-A), IVSI-2 (T>C), IVSII-705 T>, IVSII-772 (G>A) (Figure 3) and deletion from up HBBP1- Exon3 HBBP1 & up HBB-0.5Kb down HBB (Figure 4) each with one mutation/deletion

Mutations in the two cases remained unknown, despite all types of molecular analysis. These two cases were classified as a β-thalassemia carrier as supported by the hematological indices and the result of Hb electrophoresis (mean MCV=65 and MCH= 20 and mean HbA2=% 4.7).

Overall, 44.7 % of all rare mutations had the β^0 phenotype, while 31.6% of them showed β^+phenotype. Remaining rare mutations were silent β^{++}(15.8%) and β^0 (2.6%).

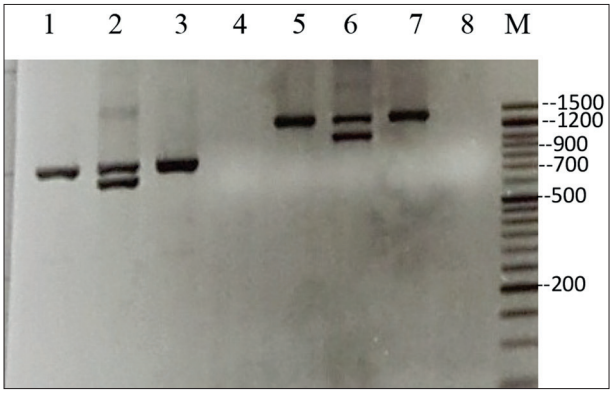

Figure 1. Representative samples of Lepore and sicillian deletions detected by GAP-PCR. Lane 2 corresponds to heterozygous case of Lepore deletion (upper band normal and lower band mutant) and Lane 6 corresponds to heterozygous case of sicillian deletion (upper band normal and lower band mutant). Cases 1, 3, 5, and 7 show norma- related PCR fragments. Lane 4 and 8 corresponds to negative PCR control. M corresponds to molecular marker 5obp.

DISCUSSION

Northwest Iran consists of three provinces, East - and West -Azerbaijan, and Ardebil. This area is located on the border with Iraq, Turkey, Armenia, the Republic of Azerbaijan, and the Nakhichevan enclave. Azeri Turkish people account for most of the population, but Kurds and other minorities are also scattered throughout the region. Regarding the variation in the mutation frequencies in different parts of Iran, it can be stated that the existence of different ethnicities, such as Fars, Azeri, Kurd, Baluch, and Lur could be the most possible reason. On the other hand, historically, a variety of the mutations have been introduced to Iran as a consequence of years of wars, invasions, and massive migrations.

In Iran, the prevalence rate of β-thalassemia carriers is about 4%, while in northwest of Iran, it is half the country's average rate (less than 2%) (20).

With the development of molecular techniques, the discovery rate of common mutations has increased remarkably. Over the past two decades, ARMS-PCR has been used as the main technique for diagnosing known mutations (21), however, this method cannot detect all the unknown mutations. Inconclusive results obtained in approximately 20%

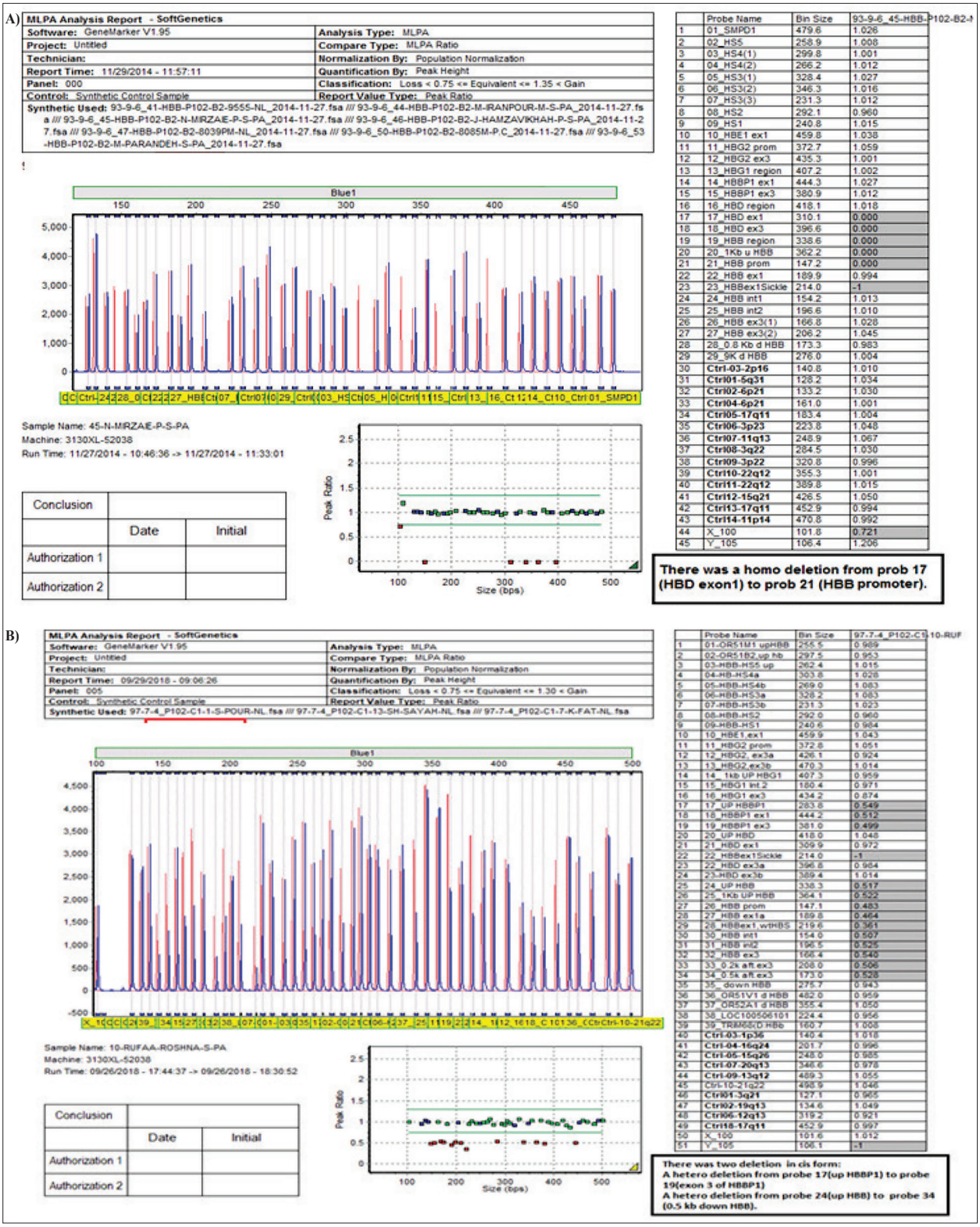

Figure 2. MLPA analysis of β-globin locus using the SALSA MLPA Probemix P102 HBB- panel A and B illustrates deletion from HBD exon 1 to HBB promoter "and "Deletion from up HBBP1-Exon3 HBBP1 and up HBB-0.5Kb down HBB, respectively.

of our β-thalassemia cases tested by the standard ARMS-PCR necessitated the use of additional methods, such as sequencing, Gap–PCR, and MLPA techniques.

More than 200 mutations of the β-globin gene have been recognized globally, primarily categorized as point mutations (9). The prevalence of mutations differs according to race and ethnicity, and in each geographical area, several mutations are more prevalent than the others.

Codon 39 C>T mutation has been found in 95.7% of Sardinian patients and 25% of the patients with β-thalassemia major in Saudi Arabia (22, 23). However, this mutation is found only in 2.5% of patients with -thalassemia in Iran (18). Similarly, CD41-42 (-CTTT) mutation is rare in Iran, however, it is most common (45.81%) in China (Guangxi) (24) . Some rare or unknown mutations have been found among the β-thalassemia cases, and their identification can improve the quality of screening protocols for precise detection of the carriers and rapid detection of the fetuses affected with β-thalassemia major.

Table 1 and Figure 5 summarize the findings of the current study and compare them with the data obtained from the reports published from the Northern, Central, and Southwestern regions of the country, as well as two larger general studies. Furthermore, it shows the frequencies reported by the neighboring countries including Turkey (25), Iraq (26), the Republic of Azerbaijan (27, 28), and Pakistan (29).

Derakhshandeh-Peykar et al. surveyed the presence of β-globin gene mutations among 394 heterozygote

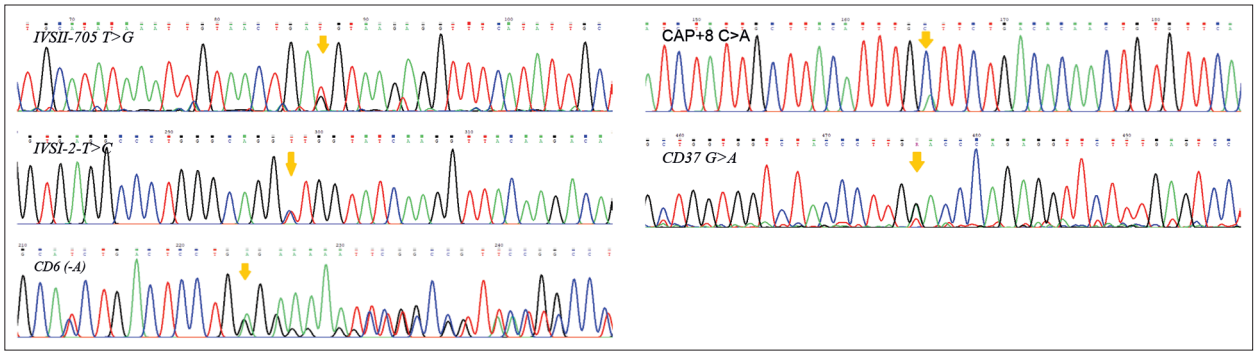

Figure 3. Sequencing results of rare point mutations: The name of mutation is denoted on the chromatogram. Arrows indicate the mutation positions.

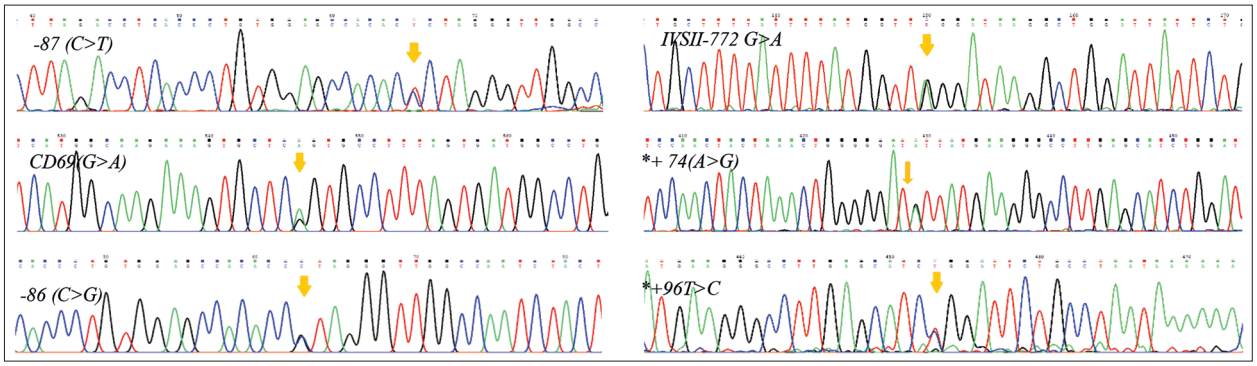

Figure 4. Sequencing results of point mutations which reported for the first time in Iran: The name of mutation is denoted on the chromatogram. Arrows indicate the mutation positions.

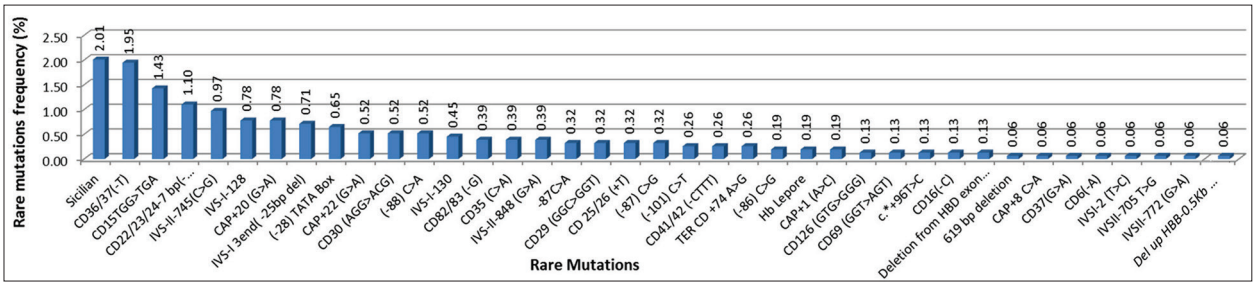

Figure 5. Frequency of the rare mutations in the Azeri population of Northwestern Iran.

Table1. Distribution of thirty nine rare mutations in Iran and neighboring countries

	Mutations	β^{0}/β^{+}	HGVS	Current Study No (%)	North of Iran (%) (30)	South-West Iran No (%) (31)	Central Iran (%) (32)	General in Iran – I (%)(33)	General in Iran – II No (%) (34)	Pakistan (%) (29)	Turkey (%) (25)	Iraq (%) (26)	Azerbaijan (%) (27, 28)
1	Sicilian(-13,337bp)	$\delta\beta^{0}$	NG_000007.3:g.64336_77738del13403	31(2.01)				(1.26)					
2	CD36/37(-T)	β^{0}	HBB:c.112delT	30 (1.94)	(0.8)	(14)	(19.7)	(5.52)			(0.23)	(0.19)	(1.18)
3	CD15(TGG>TGA)	β^{0}	HBB:c.47G>A	22 (1.42)		(2.1)			3 (3.99)	(4.6)			
4	CD22/23/24-7 bp(-AAGTTGG)	β^{0}	HBB:c.68_74delAAGTTGG	17 (1.1)	(3)	(0.64)	(1.4)	(0.95)			(0.29)	(0.19)	(0.29)
5	IVS-II-745 (C>G)	β^{+}	HBB:c.316-106C>G	15 (0.97)	(2.3)	(1.3)	(4.8)	(1)			(3.87)	(1.34)	(0.88)
6	IVS-I-128(T>G)	β^{+}	HBB:c.93-3T>G	12 (0.78)		(1)			4 (5.32)				
7	CAP+20 (G>A)	β^{+}	HBB:c.-31C>T	12 (0.78)		(1)		-					
8	25bp del	β^{0}	HBB:c.93-22_95del	11(0.71)	(1.20)	(5.6)	(4.8)	(2.23)				(0.96)	
9	(-28) TATA Box	β^{+}	HBB:c.-78A>G	10 (0.65)	(0.5)	(0.36)			1 (1.33)				
10	CAP+22 (G>A)	β^{+}	HBB:c.-29G>A	8 (0.52)		(0.8)			1 (1.33)				
11	IVS-I (-1) or CD 30 (G>A)	β^{0}	HBB:c.92G>A	8 (0.52)	(7.7)	(0.68)		(1.92)		(2.8)		(0.38)	(0.59)
12	(-88) C>A	β^{+}	HBB:c.-138C>A	8 (0.52)	(0.3)			(0.9)					
13	IVS-I-130(G>C)	β^{0}	HBB:c.93-1G>C	7 (0.45)	(1.6)	(0.8)			11(14.6)				
14	CD82/83 (-G)	β^{0}	HBB:c.251delG	6 (0.38)	-	(2.1)		-	9(11.97)				
15	CD35(C>A)	β^{0}	HBB:c.108C>A	6 (0.39)	-	-							
16	IVS-II-848(C>A)	β^{+}	HBB:c.316-3C>A	6 (0.39)	-	(0.82)		-					
17	-87(C>T)	β^{+}	HBB:c.-137C>T	5 (0.32)					1(1.33)				
18	CD29(C>T)	β^{+}	HBB:c.90C>T	5 (0.32)	-	-							
19	CD25/26(+T)	β^{0}	HBB:c.78_79insT	5 (0.32)	-	-		(0.3)	2(2.66)				
20	(-87)C>G	β^{+}	HBB:c.-137C>G	5 (0.32)	-	-		-					

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21	(-101)C>T	β^{++}(silent)	HBB:c.-151C>T	4 (0.26)	-	(0.64)		-	2(2.66)			
22	CD41/42(-CTTT)	β^{0}	HBB:c.126_129delCTTT	4 (0.26)	-	(0.36)		(0.3)	4(5.32)			
23	CAP+1548 (A>G)	unknown	HBB:c.*74 (A>G)	4 (0.26)	-	-		-				
24	(-86) C>G	β^{+}	HBB:c.-136C>G	3 (0.19)	-	-		(2.5)				
25	Hb Lepore	β^{0}	NG_000007.3:g.63564_70978del	3 (0.19)	-	-		(9.38)				
26	CAP+1 (A>C)	β^{++}(silent)	HBB:c.-50A>C	3 (0.19)				-		(3)		
27	CD126 (GTG>GGG)	β^{++}(silent)	Hb Neapolis	2 (0.13)				-				
28	CD69 (GGT>AGT) Hb City of Hope	silent Hb variant	HBB: c.208G>A	2 (0.13)								
29	CAP + 1570 T>C	β^{++}(silent)	HBB:c.*96T>C	2 (0.13)	(0.3)							
30	CD16(-C)	β^{0}	HBB:c.51delC	2 (0.13)	(0.3)		(0.9)	(2.5)	7(9.31)	(0.7)		
31	Deletion from HBD exon 1 to HBB promoter	β^{0}		2 (0.13)								
32	619 bp deletion	β^{0}	NG_000007.3: g.71609_72227del619	1 (0.06)						5.3		
33	Deletion from up HBBP1-Exon3 HBBP1 and up HBB-0.5Kb down HBB	β^{0}		1(0.06)								
34	CAP+8 C>A	β^{++}(silent)	HBB:c.-43C>T	1 (0.06)								
35	CD37(G>A)	β^{0}	HBB:c.114G>A	1 (0.06)	0.5							
36	CD6(-A)	β^{0}	HBB:c.20delA	1 (0.06)								
37	IVS-I-2 (T>C)	β^{0}	HBB:c.92+2T>C	1 (0.06)	2				1(1.33)			
38	IVS-II-705 T>G	β^{+}	HBB:c.316-146T>G	1 (0.06)								
39	IVSII-772 (G>A)	unknown	HBB:c.316-78A>G	1 (0.06)								
40	Unknown			2 (0.13)	40 (10.3)			10 (13.3)				
	Total			305 (19.79)								

β-thalassemia cases using the ARMS-PCR and DNA sequencing methods, in the northern Caspian Sea provinces of Gilan, Mazandaran, and Golestan. They identified 19 mutations, with IVS-II-1 (G>A) being the most frequent (51.6%). Table 1 shows 14 rare mutations found in their study.(30)

Galehdari et al. (2010) studied 1241 cases in the southwest of Iran and reported 14 rare mutations partially compatible with the current findings (Table 1) (31). CD36/37(-T) and IVS-I 3end (-25bp del) mutations were considered to be rare in our study and accounted for 14 and 5.6% of β-globin gene mutations in this geographic region, respectively.

Najmabadi et al. (4) and Nejat Mahdieh et al. (34) conducted relatively comprehensive studies on the rare mutations in Iran as a whole. In contrast to the current findings, Najmabadi et al. showed that CD36/37(-T) and Hb Lepore had frequencies of more than 2% (5.52 and 9.8%, respectively) and were considered as the common mutations.

Nejat Mahdieh et al. (34) reviewed 32 published studies conducted in Iran on the 31734 β-thalassemia cases and reported lower frequencies of IVS-I-130(14.63%), CD16 (-C) (9.31%), CD82/8 (-G) (11.97%), CD41/42 (-CTTT) (5.32%), and IVS-I-128 (5.32%) mutations compared to our findings.

Sicilian (-13,337bp) deletion was the most frequent rare mutation in our study, accounting for 30 out of 1541 alleles (2.01%). Esteghamat et al. reviewed the deletional mutations in β-thalassemia, and reported a frequency of about 1.26% for the Sicilian (-13,337bp) deletion in 1500 independent cases in Iran (33). As reflected by its name, this deletion is originated from the Mediterranean region, especially Italy and Greece.

Codon 36/37 (−T) is the most frequent mutation (31-34%) in Lur and Bakhtiari, two Iranian minority ethnic groups living in the central parts of Iran (7). Galehdari et al. (35) and Derakhshandeh-Peykar et al. (32), in their studies in the southwestern and central parts of Iran, reported a frequency of 22.70 and 19.7% for this mutation, respectively. In the present study, this mutation was found in 1.94% of the independent individuals. This mutation has been rarely reported in the neighboring countries of Turkey (25), Iraq, and the Republic of Azerbaijan (34). It has been reported with a frequency of 0.5- 1.5% in Saudi Arabia (23). While it has been found to come from the Kurdish population of Iran (36), it is most commonly found in Khuzestan and Lorestan provinces. Increase in its frequency in these subpopulations could be due to gene flow and genetic drift.

CD15(G>A) mutation with Asian-Indian origin has been previously reported in Iran with low frequency, ranging from 2.1-3.9% (34). Yet, in our study, its frequency was equal to 1.4%. This mutation has been reported in Pakistan with a frequency higher than in Iran. In Pakistan, this mutation has a decreasing frequency from east to west; therefore, a lower frequency of this mutation is expected in northwest of Iran (1.4%) compared to Iran's general statistics (3.99%).

The CAP+22 (G>A) mutation of Mediterranean/Bulgarian origin was found in this study with a frequency of 0.53 % in eight out of 1541 analyzed subjects. Akhavan-Niaki et al. (10) screened for β-globin gene mutations among 1635 Iranian carriers in the north of Iran, and found that the CAP+22 (G>A) was the least frequent mutation, identified in 0.10% of the cases. This mutation has been reported very rarely (0.04%) in Iran and has been mainly detected in Azeri populations in other surveys.(25, 37)

CD25/26, which originally is a Tunisian mutation, is among the rarest mutations in Iran. It was introduced by Haghi et al. in 2009 in Tabriz, East Azerbaijan province, Iran (13). In this study, this mutation was identified in five out of 1541 subjects (0.33%).

The CD16 (-C) β^0 mutation of Asian-Indian origin is a rare mutation with a frequency of 0.9% in Isfahan province (Central Iran) (32) and had a frequency of 0.13 % in our study.

Roudkanar et al. (38), Najmabadi et al. (37), and Rahim et al. (39) described the IVSI-130 (G>C) mutation of Middle Eastern origin (36) as the most common rare mutation in Iran with 4, 11, and 1 alleles, respectively. Yavarian reported a frequency of 0.41% for this mutation in Southern Iran (40). Similarly, in the present study, this mutation was observed in 0.35% of the cases (7 alleles). Ayçiçek et al. reported this mutation with a frequency of 3.5% in Turkey (41). This mutation has been reported with a frequency of 4.3% in the eastern provinces of Saudi Arabia (23).

In central Iran, IVSI 3'end 25 del mutation accounts for about 5% of mutations in the β-globin gene. It is also frequent in Bahrain and Saudi Arabia with frequencies of 36 and 14%, respectively (42) . Although this mutation has been proposed to have an Asian-Indian origin, southern Iran, particularly the Persian Gulf area, has also been proposed as the actual place of origin for this mutation (7, 42). The frequency of this mutation is equal to 1.2% in Northern Iran (30), and has a descending trend of frequency from the south to the north in Iran. In our research, this mutation was found in 11 subjects (0.71%).

A study on patients with thalassemia in Northeastern Iran in 2018 (43) showed that the frequency of CD 29 (GGC>GGT) was seven in 100 cases (7%), but in the current study, its frequency was reported to be five (0.32%).

The CD30 (AGG>AGC) mutation has a nearly 2% frequency in Iran (7, 34). In the current study, its frequency was equal to 0.52 %.

In this study, the Deletion from up HBBP1-Exon3 HBBP1 and up HBB-0.5Kb down HBB CD69 G>A (HBB: c.208G>A) (2 cases), c.*96T>C (2 cases), *HBB:c.*74(A>G)* (4cases), (-86) C>G (3cases), (-87) C>T (5cases), and *IVS-II-772 (G>A)* mutations/variants were reported for the first time in Iran (Figure 4).

In accordance with our findings, it has been previously reported that the CD69G>A(HBB: c.208G>A), Hb City of Hope is a rare and silent Hb variant. Hemoglobin electrophoresis cannot separate it from Hb A (44). CD69G>A appears to have no obvious functional effects on the β-globin chain properties in the heterozygotes, as do two other β variants at codon 69, Hb Kenitra and Hb J Cambridge (45, 46). But it has been reported that the compound heterozygote for this variant and β-globin mutations result in the development of β-thalassemia .(47)

In our study, the phenotype of the heterozygote cases for CAP + 1570 T > C (HBB:c.*96T > C) mutation was compatible with the silent carrier of β-thalassemia. This variant was reported previously by Vinciguerra et al. in 2015 (48) showing variable phenotype ranging from β-thalassemia carrier to mild form of β-thalassemia intermedia in the compound heterozygotes for this mutation and severe β-globin mutations. These findings allow us to better understand the clinical implications of this variant that can be categorized as a silent β-thalassemia defect.

Regarding phenotype of the heterozygote cases for CAP+1548 A>G (HBB:c.*74 A>G), it can be said that this variant might have decreased the MCV by 76 on average and also the MCH to 23.3 without elevation in the HbA2 level. As the iron deficiency in the population under study had been primarily excluded by the appropriate means and α-globin genes mutations were ruled out by the Gap-PCR and sequencing, lower hematological indices could be attributed to this variant. Therefore, according to these findings, it cannot be strongly concluded that this variant can be categorized as a β^{++} mutation.

The phenotype of the heterozygote cases for the IVS-II-772 mutation was found to be β^{++}. As these cases had the $\alpha^{3.7}$deletion in α-globin locus, in addition to this variant, the effect on the phenotype could not be concluded precisely. Consequently, the clinical significance of this variant remains unknown.

Although, CD126 GTG>GGG mutation (Hb Neapolis) was detected in only 0.13% of the studied population, reporting of this variant is important due to the fact that the heterozygote cases for this variant and β^0 mutations have previously shown typical characteristics of the thalassemia intermedia (49).

In the current study, some common mutations which were missed by the routine ARMS-PCR technique, were rediscovered by the sequencing technique demonstrating the inferior sensitivity of ARMS- PCR in comparison with more modern methods. This limitation should be considered for any molecular laboratory test involved in the β-thalassemia PND program.

Two cases remained unidentified, despite all efforts. There are various types of mutation in the β-globin locus influencing the gene action at any level of transcription, through translation, for example, mutation in the locus control region (LCR) or other regulatory regions can lead to β- thalassemia or β-hemoglobinopathy (50). Modern methods for molecular analysis, such as next generation sequencing (NGS), may be fruitful in these conditions.

CONCLUSION

Over the course of the last decade, multiple studies have been conducted about the frequency and distribution of the mutations in the beta-globin gene among many geographic areas of Iran. These data have been used to organize a country-wide network for the detection of the molecular patterns of beta-thalassemia carriers and patients in the population of a country like Iran, one that has very diverse ethnicities and races living together. Currently, there are many molecular diagnostic centers in Iran performing the prenatal molecular diagnosis of the beta-thalassemia aimed at reducing the population of the patients with transfusion-dependent beta-thalassemia.

Genetic counseling and identification of new mutations which cause clinically significant disease, together with PND are the best methods for effectively controlling the disease and prevention from the birth of new cases in the community. In Iran all of these services are routinely available. Finding new mutations showed that there are several rare mutations that can be easily missed during the screening of beta-thalassemia carriers, thus it is recommended to consider these mutations in the screening protocols for detection of the beta-thalassemia carriers, not only in Northwest Iran but also in other regions and provinces, as well as in nearby countries, due to the increased immigrations.

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Authors' Contribution

HN, MK, SD, and HMD collected the samples from the patients, analyzed the hematologic parameters, and

performed the molecular tests. MSK was involved in the data collection and resources management. AHPF was involved in the review of the literature and writing the manuscript. SMD supervised all the performed molecular tests and data analyses and was involved in the review of the literature and providing the manuscript draft. All the authors read and approved the final manuscript.

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Compliance with ethical standards

Research involving human participants or animals

This study was conducted in accordance with the Helsinki II declaration of good clinical practice.

Conflict of interest

The authors declare that they have no conflicts of interests.

Informed consent

Each subject signed an informed consent before participating in this study.

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