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Problem of borderline hemoglobin A₂ levels in an Iranian population with a high prevalence of α - and β -thalassemia carriers

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Abstract

Background: It is difficult to classify a small fraction of α - and β -thalassemia (α - and β -thal) carriers based on their Hb A₂ levels. Here, we report the results of a molecular investigation in a cohort of thalassemia carriers with borderline Hb A₂ levels originated from western Iran.

Results: The documents of 5956 α - or β -thal carriers were reviewed. The frequency of individuals with borderline Hb A₂ levels in this cohort was 436 (7.32%). A total of 12 different α -thal and 27 different β -thal variants were identified in this study.

Conclusions: Our data showed that individuals with borderline Hb A₂ are not uncommon in our population. Moreover, preselection of α - and β -thal carriers with borderline Hb A₂ levels based on Hb A₂, mean corpuscular volume (MCV), and mean cell hemoglobin (MCH) is not advisable in our population. Therefore, it is necessary to investigate both α - and β -globin genes in cases with borderline Hb A₂ levels, especially if the partner is a carrier of β -thal or α^0 -thal.

Keywords: Thalassemia, Carrier screening, Hb A₂ borderline, Iran

Background

Hemoglobin F (Hb F; $\alpha_2\gamma_2$), hemoglobin A₂ (Hb A₂; $\alpha_2\delta_2$), and hemoglobin A (Hb A; $\alpha_2\beta_2$) are known as three main types of hemoglobin observed in normal adults [1]. Alpha and beta thalassemia (α - and β -thal) are due to mutations in the alpha (*HBA1*: hemoglobin subunit alpha1 and *HBA2*: hemoglobin subunit alpha2) and beta (*HBB*: hemoglobin subunit beta) genes, respectively [2]. Each normal individual has four α -globin genes [2]. Deletion of one or two α -globin genes leads to α^+ - or α^0 -trait in carriers, respectively. Although the carriers of α^+ -trait are asymptomatic with mildly microcytic and normal levels of Hb A₂ (2.4–3.2%), the carriers of α^0 -trait

are characterized by low-to-low normal range of Hb A₂ (1.5–2.5%) and slightly reduced in mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) [3]. On the other hand, the Hb A₂ level in β -thal carriers is increased to more than 3.5% [4].

Hb A₂ quantification is used as a valuable test to differentiate α - and β -thal carriers in clinical laboratories. However, it is difficult to classify a small fraction of thalassemia carriers because of their Hb A₂ borderline levels [3]. Regardless of the fact that different studies have considered different ranges of Hb A₂ to define the borderline level [3–11], subjects with borderline levels of Hb A₂ should be carefully screened to avoid missing at-risk couples.

Because thalassemia is a serious issue in the Iranian health system [12–14], the National Thalassemia Screening Program has been conducted in Iran for more than 20 years [15]. Implementation of this program in Iran has reduced the birth rate of β -thal by 90.13% [16]. Since

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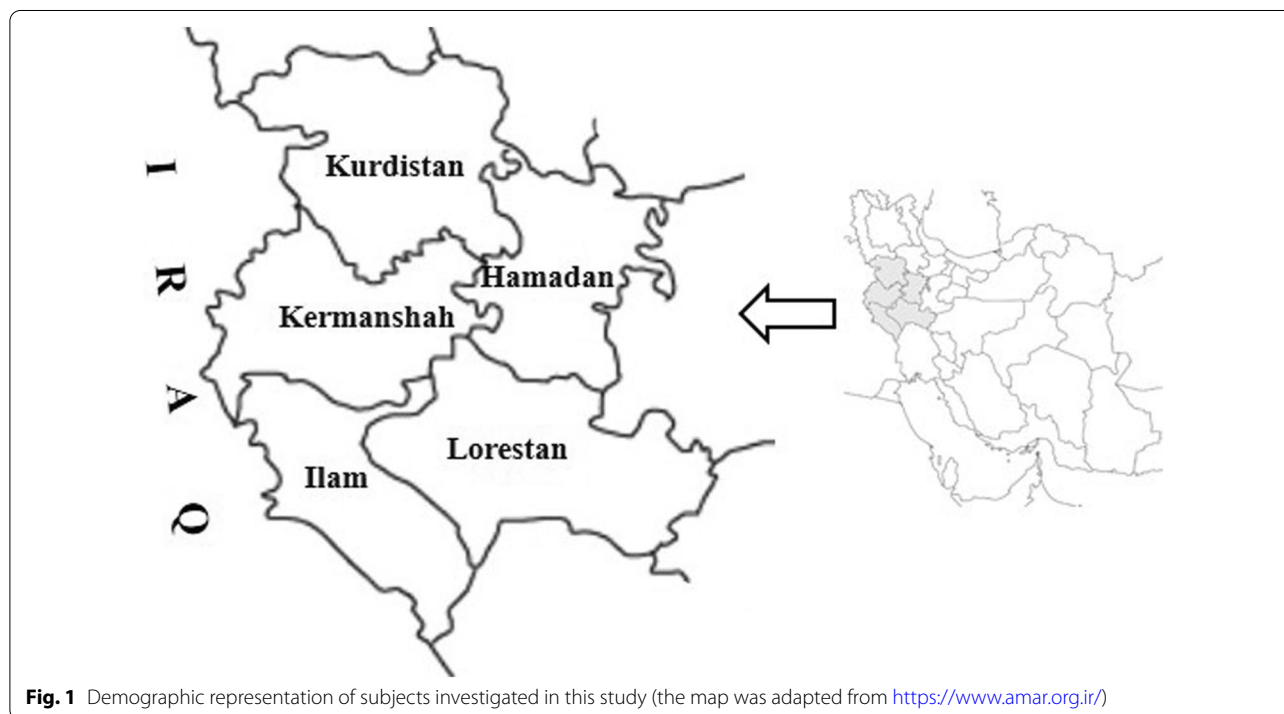
2009, thalassemia carriers from western provinces of Iran including Kermanshah, Kurdistan, Hamadan, Ilam, and Lorestan have been referred to our laboratory (Kermanshah reference Laboratory, Kermanshah, Iran), as one of the designated centers of genetic diagnosis of couples at risk for thalassemia. In this study, we aimed to evaluate the prevalence of subjects with borderline Hb A₂ levels among the total population of thalassemia carriers in western Iran and to determine the spectrum and frequency of thalassemia mutations in this group.

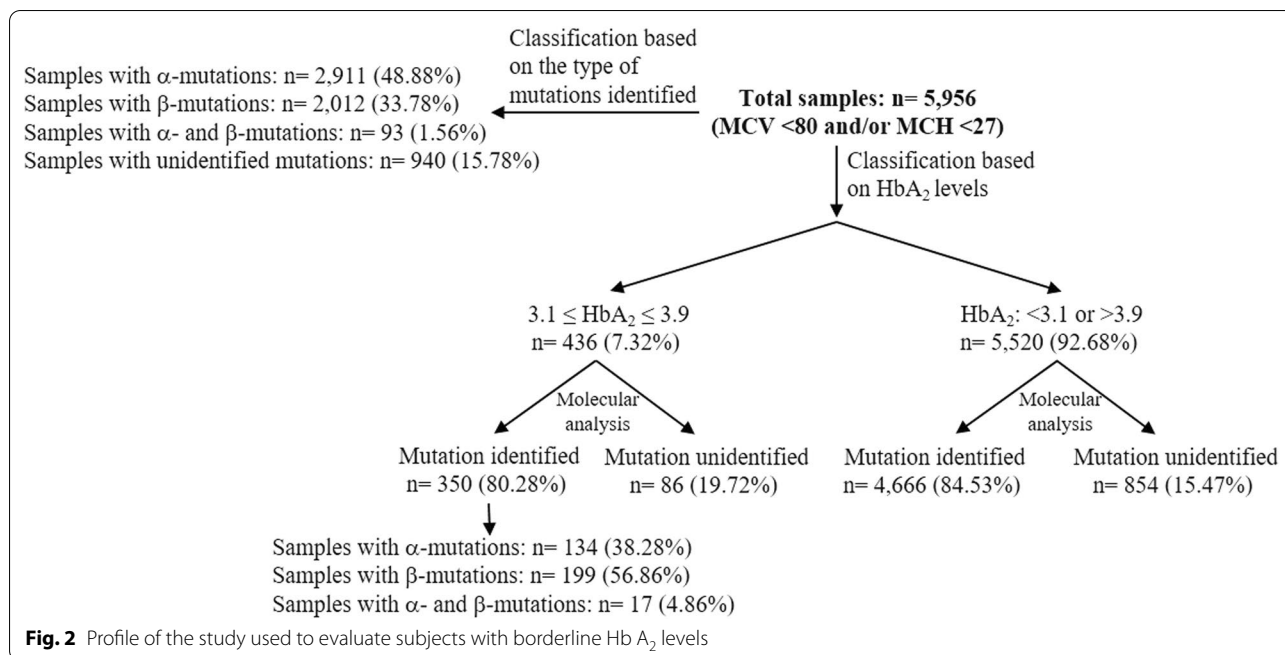
Methods

According to the National Thalassemia Screening Program in Iran, marriage registrars refer couples planning to get married to a designated local laboratory for premarital screening. The process of screening has already been discussed elsewhere [15, 17]. In the present study, the results of genetic analysis of 5,956 suspected carriers of thalassemia who were referred to Kermanshah Reference Laboratory, Kermanshah, Iran, between 2009 and 2019 were reviewed. The MCV and/or MCH of these subjects were <80fl and <27 pg, respectively. They were originated from five provinces located in the western part of Iran including Kermanshah, Kurdistan, Hamadan, Ilam, and Lorestan (Fig. 1).

Using a Capillarys 2 Flex Piercing instrument (Sebia, Lisses, France), the capillary electrophoresis had been performed for all of the subjects, accordingly, they were classified to have borderline (3.1–3.9%) or other Hb

A₂ levels (<3.1 or >3.9) (Fig. 2) [4]. After the collection of peripheral blood samples and genomic DNA extraction using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), the quality and quantity of extracted DNA were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). We used three different molecular methods to identify α -thal causing mutations: (1) multiplex gap-polymerase chain reaction (gap-PCR) for detection of $-\alpha^{3.7}$ (NG_000006.1: g.34164_37967del3804), $-\alpha^{4.2}$, and $-\alpha^{MED 1}$ (NG_000006.1: g.24664_41064del16401) deletions; (2) amplification refractory mutation system (ARMS)-PCR for detection of $\alpha^{-5 nt \alpha}$ (*HBA2*: c.95+2_95+6delTGAGG), polyadenylation signal A2 or polyA2 (α^{polyA2} α , *HBA2*: c.*92A>G), polyA1 (α^{polyA1} α , *HBA2*: c.*94A>G); $\alpha\alpha^{Hb Adana}$, *HBA1*: c.179G>A), and $\alpha^{codon 19}$ α (*HBA2*: c.56delG); and (3) direct DNA sequencing of *HBA1* and *HBA2* genes for the analysis of samples whose mutations were not in the list of mutations examined by the gap-PCR and ARMS-PCR methods. Moreover, ARMS-PCR technique was used for detection of *HBB* gene mutations. Samples negative on ARMS-PCR were analyzed by direct sequencing of the *HBB* gene. The protocols, primer sequences, and PCR cycling conditions have been described by us in our previous studies [18–26]. Some samples negative on the above-mentioned methods had been sent to Dr. Zeinali's Medical Genetics Laboratory, Kawsar Human Genetics Research Center, Tehran, Iran, for multiplex





ligation-dependent probe amplification (MLPA) analysis of the α - and β -globin gene clusters.

Statistical data analysis was carried out using the Statistical Package for the Social Sciences software, version 25.0 (<https://ibm.com/SPSS-Statistics/>). The independent samples t test, the analysis of variance (ANOVA), and Scheffe post hoc analysis were performed to determine the significance of differences between hematological factors in thalassemia genotypes identified in our study. The p values less than 0.05 were considered to be significant for all described tests.

Results

In this study, the documents of 5,956 α - or β -thal carriers were reviewed. The frequency of subjects with borderline Hb A₂ levels in this group was 436 (7.32%), in which α - and/or β -variants were identified in 350 subjects (mutation detection rate: 80.28%) (Fig. 2). These subjects classified as carriers with α -thal mutations ($n=134$: 38.28%), β -thal mutations ($n=199$: 56.86%) and coexistence of α - and β -thal mutations ($n=17$: 4.86%), respectively (Fig. 2).

A total of 12 different α -thal and 27 different β -thal mutations were identified in the present study. The most frequent mutations are shown in Table 1 (data not shown in detail). The α -globin mutations including $-\alpha^{3.7}$ deletion, α^{PolyA2} , $\alpha^{\text{Hb Adana}}$, $-\alpha^{4.2}$ deletion, and $\alpha^{-5\text{nt}}$ were detected with the frequencies of 62.57, 8.19, 7.02, 5.85 and 5.85%, respectively. On the other

hand, Hb D-Punjab (*HBB*: c.364G >C) was the most frequent β -globin variant (21.92%), followed by IVS-II-1 (G>A) (*HBB*: c.315+1G>A), IVS-I-6 (T>C) (*HBB*: c.92+6T>C), codons 8/9 (+G) (*HBB*: c.27_28insG), IVS-I-110 (G>A) (*HBB*: c.93-21G>A), -101 (C>T) (*HBB*: c.-151C>T), Hb Erz (HBB: c.371C>A), and IVS-I-128 (T>G) (*HBB*: c.93-3T>G) with the frequencies of 16.89, 13.70, 7.76, 7.31, 4.57, 4.11 and 3.65%, respectively, among our samples.

Out of 59 couples who were at risk for having a child with thalassemia, eight and 51 couples had α -thal and β -thal mutations, respectively. Fifty-two couples were referred for premarital genetic diagnosis, whereas seven couples were referred to us as they already had a child with thalassemia (Table 2). In addition, 37 out of 59 couples were from consanguineous families.

As shown in Table 3, Hb A₂, MCV, and MCH were significantly different between α -thal and β -thal carriers ($p < 0.0001$). Post hoc analysis showed that except for -101 (C>T) and IVS-I-128 (T>G) mutations, the other most prevalent β -thal mutations in this study caused a significant reduction in MCV and MCH levels compared with α -thal mutations ($p < 0.05$) (Table 4). On the other hand, in contrast to α or $\alpha/\alpha^{\text{non-deletional}}$ ($\alpha/\alpha^{\text{ND}}$) or $\alpha/\alpha^{\text{ND}}$ genotypes, there was no statistically significant difference in the mean levels of MCV and MCH factors between β -thal and $-\alpha$ -/ α or α -/ α genotypes ($p > 0.05$) (Table 5).

Table 1 List of the common α - and β -globin gene mutations identified in samples with borderline Hb A₂ levels

Mutation name	HGVS name	Type	Allele frequency (%)
α -Globin gene mutations			
$-\alpha^{3,7}$	NG_000006.1: g.34164_73967del13804	α -thal del	107 (62.57)
PolyA2 (AATAAA>AATGAA)	HBA2: c.*92A>G	α -thal	14 (8.19)
Codon 59 (GGC>GAC) (Adana)	HBA1: c.179G>A	Variant/ α 1-thal	12 (7.02)
$-\alpha^{4,2}$	NA	α -thal del	10 (5.85)
IVS-I-1 (-5 nt) ^a	HBA2: c.95+2_95+6delTGAGG	α -thal-2	10 (5.85)
$\alpha\alpha/\alpha\alpha^{\text{Anti-3.7}}$	NA	α -thal	5 (2.92)
PolyA1 (AATAA > AATAAG) ($\alpha^{\text{T-Saudi}}$)	HBA2: c.*94A > G	α -thal-2	4 (2.34)
_MED1	NG_000006.1: g.24664_41064del	α -thal del	4 (2.34)
Other			5 (2.92)
Total			171 (100)
β -Globin gene mutations			
Hb D-Punjab	HBB: c.364G>C	Hb variant	48 (21.92)
IVS-II-1 (G>A)	HBB: c.315+1G>A	β^0	37 (16.89)
IVS-I-6 (T>C)	HBB: c.92+6T>C	β^+	30 (13.70)
Codons 8/9 (+G)	HBB: c.27_28insG	β^0	17 (7.76)
IVS-I-110 (G>A)	HBB: c.93-21G>A	β^+	16 (7.31)
-101 (C>T)	HBB: c.-151C>T	β^+	10 (4.57)
Hb ErnZ	HBB: c.371C>A	Hb variant	9 (4.11)
IVS-I-128 (T>G)	HBB: c.93-3T>G	β^+	8 (3.65)
Codons 36/37 (-T)	HBB: c.112delT	β^0	7 (3.20)
IVS-I-1 (G>A)	HBB: c.92+1G>A	β^0	5 (2.28)
Codons 82/83 (-G)	HBB: c.250delG	β^0	4 (1.83)
Other			32 (14.61)
Total			219 (100)

^a IVS-I-1 (-5 nt): GAGGTGAGG>GAGG-----

Discussion

Iran is a vast country with high ethnic diversity. These factors have not only led to a high prevalence of a number of genetic diseases, but have also led to a wide range of mutations associated with each of these diseases [27–32]. The above issues are more evident in the western region of Iran [19–26, 33–38]. In this study, we report the hematological and molecular findings in a group of α - and β -thal carriers with borderline Hb A₂ levels (3.1–3.9%). They were originated from western provinces of Iran with Kurd, Lur, and Lak ethnicities.

Various mutations were detected on α - and β -globin genes. As shown in Table 1, the borderline Hb A₂ level is not specific to just one or a few α -thal mutations in our population. In addition, all main types of α -thal genotypes including $\alpha\alpha/-\alpha$, $-\alpha/-\alpha$, $\alpha\alpha/-$, $\alpha\alpha/\alpha^{\text{ND}}$ $\alpha\alpha$ or $\alpha\alpha/\alpha^{\text{ND}}$ were associated with borderline Hb A₂ levels. This finding is in line with previous studies in which different α -thal mutations were associated with borderline Hb A₂ levels [4, 5, 8–10, 39, 40]. Previous studies have shown that variations in the erythroid Krüppel-like factor (*KLF1*) gene lead to a borderline Hb A₂ in normal

individuals as well as α^+ - or α^0 -thal carriers [40, 41]. As shown in Fig. 2, our population has a high frequency of α -thalassemia carriers with borderline Hb A₂ levels. Therefore, the *KLF1* gene mutations may be a possible cause of increased Hb A₂ levels in our population.

Based on the identification of 27 different β -thal mutations associated with borderline Hb A₂ in this study (Table 1), it can be concluded that the borderline Hb A₂ level is not specific to just one or a few β -thal mutations in our population. Regardless of Hb D-Punjab and Hb ErnZ which are known as Hb variants, the IVS-II-1 (G>A), IVS-I-6 (T>C), codons 8/9 (+G), IVS-I-110 (G>A), -101 (C>T) and IVS-I-128 (T>G) were the most frequent *HBB* gene mutations among our samples. Previous studies have reported that some mild β -thal mutations are recurrently associated with normal/borderline Hb A₂ levels. However, their types and frequencies were not similar in different populations. Examples of these mutations are as follows: IVS-I-6 (T>C), -101 (C>T) and IVS-I-110 (G>A) in Italy [8, 10, 42]; cap+1 (A>C) (*HBB*: c.-50A>C) and IVS-I-5 (G>C) (*HBB*: c.92+5G>C) in India [6, 43]; IVS-I-6 (T>C), IVS-I-110 (G>A) and

Table 2 Couples with α - or β -thal genotypes at risk for having a child with thalassemia

Gender	Hb A ₂	Genotype	Gender	Hb A ₂	Genotype	Gender	Hb A ₂	Genotype
α-Thalassemia genotypes								
Male	3.5	$\alpha\alpha/_{-MED1}$	Male	2.3	$\alpha\alpha/\alpha^{PolyA1}\alpha$	Male ^a	3.1	$\alpha\alpha/\alpha^{Hb Adana}$
Female	2.7	$\alpha\alpha/_{-MED1}$	Female	3.2	$\alpha\alpha/_{-}\alpha^{3.7}$	Female ^a	2.8	$\alpha\alpha/\alpha^{Hb Adana}$
Male ^a	2.4	$\alpha\alpha/\alpha^{Hb Adana}$	Male ^a	3.0	$\alpha\alpha/\alpha^{Hb Adana}$	Male	3.1	$\alpha\alpha/\alpha^{PolyA1}\alpha$
Female ^a	3.2	$\alpha\alpha/\alpha^{Hb Adana}$	Female ^a	3.5	$\alpha\alpha/\alpha^{Hb Adana}$	Female	2.2	$_{-}\alpha^{3.7}/_{-}\alpha^{3.7}$
Male ^a	3.3	$\alpha\alpha/\alpha^{PolyA2}\alpha$	Male	2.1	$\alpha\alpha/_{-MED1}$			
Female ^a	2.8	$\alpha\alpha/\alpha^{PolyA2}\alpha$	Female	3.2	$\alpha\alpha/\alpha^{-5nt}\alpha$			
β-Thalassemia genotypes								
Male ^a	4.8	Codons 8/9 (+G)/W	Male ^a	3.6	IVS-II-1 (G>A)/ W	Male ^a	3.9	IVS-II-1 (G>A)/ W
Female ^a	3.4	Codons 8/9 (+G)/ W	Female ^a	5.0	IVS-II-1 (G>A)/ W	Female ^a	4.1	IVS-II-1 (G>A)/ W
Male ^a	2.8	IVS-I-6 (T>C)/ W	Male ^a	3.7	Codons 8/9 (+G)/ W	Male	3.7	IVS-I-110 (G>A)/ W
Female ^a	3.2	IVS-I-6 (T>C)/ W	Female ^a	4.4	Codons 8/9 (+G)/ W	Female	4.1	IVS-I-110 (G>A)/ W
Male ^a	3.7	IVS-I-6 (T>C)/W	Male ^a	4.5	IVS-II-1 (G>A)/ W	Male ^a	2.7	IVS-I-6 (T>C)/ W
Female ^a	5.9	Codon 39 (C>T)/ W	Female ^a	3.7	IVS-II-1 (G>A)/ W	Female ^a	3.9	IVS-I-6 (T>C)/ W
Male ^a	3.9	IVS-I-6 (T>C)/ W	Male	3.7	IVS-II-1 (G>A)/ W	Male ^a	3.9	IVS-II-1 (G>A)/ W
Female ^a	3.9	IVS-I-6 (T>C)/ W	Female	4.4	Codons 82/83 (-G)/ W	Female ^a	4.6	IVS-II-1 (G>A)/ W
Male	3.8	IVS-II-1 (G>A)/W	Male ^a	3.7	IVS-I-110 (G>A)/ W	Male ^a	4.6	IVS-II-1 (G>A)/ W
Female	3.9	IVS-II-1 (G>A)/W	Female ^a	4.5	IVS-II-1 (G>A)/ W	Female ^a	3.9	IVS-II-1 (G>A)/ W
Male ^a	3.7	IVS-I-6 (T>C)/ W	Male ^a	3.8	Codons 8/9 (+G)/ W	Male ^a	4.3	IVS-I-1 (G>A)/ W
Female ^a	4.2	IVS-I-6 (T>C)/ W	Female ^a	3.7	Codons 8/9 (+G)/ W	Female ^a	3.9	IVS-I-1 (G>A)/ W
Male ^a	4.2	Codon 44 (-C)/ W	Male ^a	3.6	IVS-I-128 (T>G)/ W	Male	3.8	IVS-I-6 (T>C)/ W
Female ^a	3.9	Codon 44 (-C)/ W	Female ^a	3.6	IVS-I-128 (T>G)/ W	Female	6.2	Codon 39 (C>T)/ W
Male ^a	3.9	Codons 25/26 (+T)/ W	Male	5.0	IVS-II-1 (G>A)/ W	Male	3.8	IVS-I-6 (T>C)/ W
Female ^a	4.6	Codons 25/26 (+T)/ W	Female	3.7	IVS-I-6 (T>C)/ W	Female	5.2	IVS-II-1 (G>A)/ W
Male ^a	4.3	IVS-II-1 (G>A)/ W	Male ^a	3.5	IVS-I-6 (T>C)/ W	Male ^a	3.9	IVS-I-6 (T>C)/ W
Female ^a	3.6	IVS-II-1 (G>A)/ W	Female ^a	3.9	IVS-II-1 (G>A)/ W	Female ^a	3.9	IVS-I-6 (T>C)/ W
Male	3.8	IVS-I-6 (T>C)/ W	Male	4.4	IVS-II-1 (G>A)/ W	Male ^a	3.9	IVS-I-6 (T>C)/ W
Female	4.6	Codons 8/9 (+G)/ W	Female	3.8	Codons 36/37 (-T)/ W	Female ^a	3.9	IVS-I-6 (T>C)/ W
Male	4.1	IVS-I-110 (G>A)/ W	Male ^a	3.8	IVS-I-6 (T>C)/ W	Male ^a	4.5	IVS-II-1 (G>A)/ W
Female	3.6	IVS-I-110 (G>A)/ W	Female ^a	2.7	IVS-I-6 (T>C)/ W	Female ^a	3.8	IVS-II-1 (G>A)/ W
Male	3.8	Codons 82/83 (-G)/ W	Male ^a	5.3	IVS-II-1 (G>A)/ W	Male ^a	3.9	IVS-II-1 (G>A)/ W
Female	4.4	Codons 82/83 (-G)/ W	Female ^a	3.5	-101 (C>T)/ W	Female ^a	4.0	IVS-II-1 (G>A)/ W
Male	6.0	Codons 8/9 (+G)/ W	Male ^a	4.5	Codon 15 (G>A)/ W	Male ^a	4.7	Codon 22 (G>T)/ W
Female	3.8	Codons 8/9 (+G)/ W	Female ^a	3.7	Codon 15 (G>A)/ W	Female ^a	3.9	Codon 22 (G>T)/ W
Male	4.0	IVS-I (25bp del)/ W	Male ^a	5.0	IVS-I-110 (G>A)/ W	Male	3.9	-101 (C>T)/ W
Female	3.9	IVS-I-1 (G>A)/ W	Female ^a	3.5	IVS-I-128 (T>G)/ W	Female	5.5	-101 (C>T)/ W
Male	3.7	-101 (C>T)/ W	Male	3.9	-101 (C>T)/ W	Male ^b	4.5	Codons 82/83 (-G)/ W
Female	5.6	IVS-I-6 (T>C)/ W	Female	5.0	Codon 44 (-C)/ W	Female ^b	3.9	Codons 8/9 (+G)/ W
Male ^{a,b}	3.9	IVS-I-6 (T>C)/ W	Male ^{a,b}	3.9	IVS-II-1 (G>A)/ W	Male ^b	3.9	-101 (C>T)/ W
Female ^{a,b}	4.5	IVS-I-6 (T>C)/ W	Female ^{a,b}	6.2	IVS-II-1 (G>A)/ W	Female ^b	5.0	Codon 44 (-C)/ W
Male ^{a,b}	3.8	Cap+22 (G>A)/ W	Male ^{a,b}	4.9	Codon 5 (-CT)/ W	Male ^b	3.7	IVS-I-6 (T>C)/ W
Female ^{a,b}	3.7	Cap+22 (G>A)/ W	Female ^{a,b}	3.7	IVS-I-6 (T>C)/ W	Female ^b	5.7	Codons 8/9 (+G)/ W

Codon 39 (C>T) (*HBB*: c.118C>T); Codon 44 (-C) (*HBB*: c.135delC); Codons 25/26 (+T) (*HBB*: c.78_79insT); Codon 15 (G>A) (*HBB*: c.48G>A); Codon 22 (G>T) (*HBB*: c.67G>T); IVS-I (25bp del) (*HBB*: c.93-21_del); Cap+22 (G>A) (*HBB*: c.-29G>A); Codon 5 (-CT) (*HBB*: c.17_18delCT)

^a Consanguineous couples

^b Couples who had a child with thalassemia

Table 3 Comparison of mean values of Hb A₂, MCV and MCH in α- and β-thalassemia carriers investigated in this study

Genotype	n	Hb A ₂			MCV			MCH		
		Min; max	Mean	p value	Min; max	Mean	p value	Min; max	Mean	p value
α-Thalassemia genotypes	127	3.1; 3.9	3.23 ± 0.18	<0.0001	59.9; 102.5	75.87 ± 5.69	<0.0001	18.3; 32.5	24.22 ± 2.31	<0.0001
β-Thalassemia genotypes	150	3.1; 3.9	3.71 ± 0.20		51.0; 88.0	66.66 ± 5.82		13.0; 27.7	20.54 ± 2.42	

Table 4 Comparison of mean values of Hb A₂, MCV and MCH in α-thalassemia genotypes (as a single group) with those in common β-thalassemia genotypes identified in this study

Genotype	n	Hb A ₂			MCV			MCH		
		Min; max	Mean	p value	Min; max	Mean	p value	Min; max	Mean	p value
α-Thalassemia genotypes	127	3.1; 3.9	3.23 ± 0.18		59.9; 102.5	75.87 ± 5.69		18.3; 32.5	24.22 ± 2.31	
IVS-II-1 (G>A)/W	36	3.1; 3.9	3.70 ± 0.23	<0.0001	58.6; 76.4	64.73 ± 4.40	<0.0001	17.0; 24.7	19.86 ± 1.70	<0.0001
IVS-I-6 (T>C)/W	30	3.1; 3.9	3.71 ± 0.22	<0.0001	63.0; 78.2	69.28 ± 3.83	<0.0001	19.3; 25.7	21.50 ± 1.45	<0.0001
Codons 8/9 (+G)/W	16	3.4; 3.9	3.73 ± 0.12	<0.0001	59.6; 67.6	63.20 ± 2.34	<0.0001	17.9; 21.1	19.04 ± 0.90	<0.0001
IVS-I-110 (G>A)/W	16	3.1; 3.9	3.72 ± 0.21	<0.0001	57.7; 69.5	64.53 ± 3.49	<0.0001	13.8; 21.8	19.46 ± 2.06	<0.0001
-101 (C>T)/W	8	3.4; 3.9	3.68 ± 0.20	<0.0001	72.8; 88.0	79.68 ± 4.78	0.935	23.0; 27.7	26.11 ± 1.77	0.677
IVS-I-128 (T>G)/W	8	3.5; 3.9	3.67 ± 0.15	<0.0001	68.0; 77.9	71.63 ± 3.52	0.870	20.4; 25.4	22.51 ± 1.58	0.806
Codons 36/37 (-T)/W	7	3.2; 3.8	3.62 ± 0.20	0.001	62.0; 70.4	66.24 ± 3.42	0.005	19.1; 22.0	20.67 ± 1.05	0.007
IVS-I-1 (G>A)/W	4	3.7; 3.9	3.82 ± 0.09	<0.0001	61.3; 62.9	62.12 ± 0.71	0.001	18.0; 19.8	19.02 ± 0.75	0.001
Codons 82/83 (-G)/W	4	3.2; 3.8	3.62 ± 0.28	0.078	53.1; 64.5	59.15 ± 4.77	<0.0001	16.2; 19.8	18.35 ± 1.60	<0.0001
Coexistence of α- and β-thalassemia genotypes	5	3.2; 3.7	3.40 ± 0.21	0.980	68.1; 79.0	72.9 ± 4.18	1.000	20.1; 25.4	22.62 ± 2.22	0.985

Table 5 Comparison of mean values of Hb A₂, MCV and MCH in β-thalassemia genotypes (as a single group) with those in α-thalassemia genotypes identified in this study

Genotype	n	Hb A ₂			MCV			MCH		
		Min; max	Mean	p value	Min; max	Mean	p value	Min; max	Mean	p value
β-Thalassemia genotypes	150	3.1; 3.9	3.71 ± 0.20		51.0; 88.0	66.66 ± 5.82		13.0; 27.7	20.54 ± 2.42	
αα/-α	69	3.1; 3.8	3.21 ± 0.16	<0.0001	67.0; 102.5	78.27 ± 4.67	<0.0001	20.8; 32.5	25.32 ± 1.64	<0.0001
-α/-α	18	3.1; 3.9	3.28 ± 0.25	<0.0001	63.0; 74.0	69.22 ± 3.10	0.645	19.1; 23.9	21.25 ± 1.37	0.896
αα/-	4	3.1; 3.5	3.25 ± 0.19	<0.0001	66.9; 72.4	68.97 ± 2.60	0.991	20.6; 21.9	21.37 ± 0.55	0.993
αα/α ND α or αα/αα ND	36	3.1; 3.9	3.23 ± 0.19	<0.0001	59.9; 87.1	75.35 ± 5.39	<0.0001	18.3; 27.6	23.91 ± 2.25	<0.0001
Coexistence of α- and β-thalassemia genotypes	5	3.2; 3.7	3.40 ± 0.21	0.047	68.1; 79.0	72.9 ± 4.18	0.372	20.1; 25.4	22.62 ± 2.22	0.566

ND, non-deletional

IVS-I-1 (G>A) (*HBB*: c.92+1G>A) in Egypt [44]; and IVS-I-1 (G>A) in Malaysia [7]. All of these mutations were also observed in the present study (Table 2). Therefore, our findings are consistent with the results of the studies mentioned above.

In this study, we found eight couples with α-thal mutations in which one partner had a borderline Hb A₂ level (Table 2). According to previous studies [27, 45], there is a probability of 25% of having a child/fetus with Hb H disease or hydrops fetalis in these couples. On the other hand, out of 51 couples with β-thal mutations who were

at risk for having a child with thalassemia, we found 40 couples with borderline Hb A₂ in one partner and increased Hb A₂ level (>3.9) in the other partner; nine couples with borderline Hb A₂ levels in both partners; and two couples with borderline Hb A₂ in one partner and normal Hb A₂ level in the other partner. In addition, seven couples were referred to us as they already had a child with thalassemia (Table 2).

Our analysis showed that the mean values of Hb A₂, MCV, and MCH were significantly different between α-thal and β-thal carriers with borderline Hb A₂ levels

($p < 0.0001$) (Table 3). Two other statistical analyzes were performed. In the first analysis, the mean values of MCV and MCH in β -thal genotypes (as a single group) were compared with those in common β -thal genotypes (Table 4). The results showed that the mean values of these factors were not statistically significant in at least two β -thal genotypes, $-101 (C>T)/W$ and $IVS-I-128 (T>G)/W$, compared to α -thal genotypes. In the second analysis, the mean values of MCV and MCH were evaluated in β -thal genotypes (as a single group) compared to those in $\alpha\alpha/-\alpha$, $-\alpha/-\alpha$, $\alpha\alpha/-$, $\alpha\alpha/\alpha^{ND}\alpha$ or $\alpha\alpha/\alpha^{ND}$ genotypes (Table 5). According to the results of this analysis, there was no statistically significant difference between the mean values of these factors in $-\alpha/-\alpha$ and $\alpha\alpha/-$ genotypes compared to the β -thal genotypes. In addition, the mean values of MCV and MCH were not significantly different when we compared the combined α -thal and β -thal genotypes with α -thal genotypes (as a single group) or β -thal genotypes (as a single group) (Tables 4, 5, respectively). Therefore, preselection of α - and β -thal carriers based on MCV and MCH factors is not advisable in our population.

Conclusions

In conclusion, our data showed that thalassemia carriers with borderline Hb A₂ are not uncommon among all thalassemia carriers in our population. Out of 59 couples who were at risk for having a child with thalassemia, 37 couples were from consanguineous families. In addition, preselection of α - and β -thal carriers with borderline Hb A₂ levels based on Hb A₂, MCV, or MCH factors, alone or in combination, is not advisable in our population. Therefore, it is necessary to investigate both α - and β -globin genes in cases with borderline Hb A₂ levels, especially if couples are from consanguineous families or the partner is a carrier of β -thal or α^0 -thal. This strategy is cost-effective because it prevents the birth of children with thalassemia and, consequently, the high costs associated with caring of these patients.

Abbreviations

Hb A: Hemoglobin A; Hb A₂: Hemoglobin A₂; Hb F: Hemoglobin F; α -thal: Alpha thalassemia; β -thal: Beta thalassemia; HBA1: Hemoglobin subunit alpha 1; HBA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; MCV: Mean corpuscular volume; MCH: Mean cell hemoglobin; ARMS-PCR: Amplification refractory mutation system-polymerase chain reaction; MLPA: Multiplex ligation-dependent probe amplification.

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Authors' contributions

RA and KM had the idea of the topic. KM, SS, and AA collected the data in the documents. KM reviewed the data and drafted the manuscript. RA supervised

the study process and critically reviewed the manuscript. All authors have read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Ethics code: IR.KUMS.REC.1398.731, project number: 980659).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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