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RESEARCH ARTICLE

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Alpha-globin gene mutation spectrum in patients with microcytic hypochromic anemia from Mazandaran Province, Iran

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Abstract

Background: It is estimated about 7% of the world population is carriers of hemoglobin diseases. Alpha-thalassemia is one of the most common hereditary hemoglobin disorders in the world. This study investigated alpha-globin mutations in potential carriers with hypochromic and microcytic anemia from Mazandaran, in northern Iran.

Methods: A total of 859 subjects were selected; genomic DNA was extracted and examined for the presence of mutations in the alpha-globin genes.

Results: Mutation analysis of alpha-globin genes revealed 27 different mutations. Seven variants were seen in 91.45% of all alpha-1 and alpha-2 mutations among patients in this study. The 3.7 kb deletion is the most frequent mutation with a frequency of 49.53%, followed by PolyA2 (15.19%), −4.2 deletion (8.76%), --MED (5.84%), IVSI-5nt deletion (5.49%), Hb constant spring (3.62%), and Cd 19 (−G; 3.04%), respectively. There are also seven new variants which were reported for the first time either in alpha-1 or alpha-2 genes, including codon 9 (C > A; α2), deletion of codon 60 (AAG deletion; α2), duplication of codon 94-100 plus 3 base pairs of intron 2 (IVSII + 3; α1), codon 99 (C > A; α2), codon 108 (A > G; α2), codon 128 (A > T; α2), and codon 129 (T > G; α2), respectively. The MLPA method also revealed three rare and novel deletions in alpha-cluster region with about 30 kilobases long.

Conclusion: This study showed an efficient identification of α-thalassemia can be achieved using standard hematological indices in our population. The details of these variations will help local genetic services for diagnostic and prenatal diagnosis services.

KEYWORDS

alpha-thalassemia, Iran, Mazandaran, mutation, thalassemia

1 | INTRODUCTION

Thalassemia is a global public health problem. It is estimated up to 7% of the world population are carriers of different hemoglobin disorders, including β-thalassemia, α-thalassemia, hemoglobin S, hemoglobin E, and other less common variants.[1-3] It has been also estimated 300 000-400 000 babies are born with severe forms of these diseases each year.[3,4] Alpha-thalassemia (α-thal) is one of the most common hereditary disorders in the world, primarily affects South-East Asia, Mediterranean and Middle Eastern populations, India, and Sub-Saharan Africa.[5-11] To date, about 812 different variants (361 in alpha-1 gene and 451 in alpha-2 gene) are reported to the

hemoglobin database "A Database of Human Hemoglobin Variants and Thalassemias" or Hb Var; (http://globin.bx.psu.edu/hbvar/menu.html) from different countries in alpha-thalassemia.[12] A variety of clinical phenotypes are dependent on the kind of variation as well as the number of mutated genes.

Each person has four copies of α-globin genes, two copies (α1 and α2) on each short arm of chromosome 16 (16p33). Different clinical conditions are expected for α-thalassemia. (a) Individuals with one α-globin gene defect that are known as silent carriers with a mild reduction in hematologic indexes, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). (b) Individuals with two genetic defects usually known as alpha-thalassemia minor with more reduction of MCV and MCH compared with individuals with one gene defects. (c) Individuals with three α-globin gene defects that cause hemoglobin H or HbH disease.[7] Different clinical manifestations from mild to severe have been observed in HbH disease, and there is no genotype-phenotype correlation, even in the presence of similar genotype.[7,13]

Both gene deletion and point mutation are common in alpha-thalassemia. Different kinds of deletion like alpha-1 (*HBA1*) or alpha-2 (*HBA2*) gene deletion as well as deletion of both alpha gene on the same chromosome (αα/- -) have been reported previously.[14] The most frequent mutation of alpha-globin gene is single-gene deletion (such as −3.7 or 3.7 kb deletion), usually makes little or no hematological disorders.[7] Also, many point mutations either in alpha-1 or alpha-2 genes are reported in different countries which lead to mild microcytic hypochromic anemia with normal HbA_2 levels (Hb Var database). Variations in three out of four alpha-globin genes (deletion or point mutation) cause HbH disease, which is characterized from mild to severe anemia and remarkably unbalanced globin chain synthesis ratios (β4 tetramers). Inheritance of four affected alpha-globin genes (- -/- -) is usually incompatible with life and leads to Hb Bart's hydrops fetalis (γ4 tetramers).[5-7,14,15]

Thalassemia carrier frequency in Iran is high as it is a part of the so-called thalassemia belt.[16] A national program of prevention and treatment has been implemented for thalassemia in 1997, and now

TABLE 1 The frequency of different mutations identified in both alpha-1 and alpha-2 genes in patients from northern Iran (n = 859)

Mutations	Gene	Mutation type	(n = 859)	Frequency (%)	Reported or Novel	Reference
-α 3.7	Mainly α1	del	424	49.36	Reported	12,14
PolyA2 (AATAAA > AATGAA)	α2	PM	130	15.13	Reported	12,14
-α 4.2	Mainly α2	del	75	8.73	Reported	12,14
--MED	α1 and α2	del	50	5.82	Reported	12,14
IVSI-5nt (TGAGG)	α2	PM	47	5.47	Reported	12,14
Cd 142 (TAA > CAA) Hbconst spring	α2	PM	31	3.61	Reported	12,14
Cd 19 (−G)	α2	PM	26	3.03	Reported	12,14
20.5	α1 and α2	del	15	1.75	Reported	12,14
PolyA1 (AATAAA > AATAAG)	α2	PM	15	1.75	Reported	12,14
Anti-3.7 kb/ ααα triplication	Duplication	dup	13	1.51	Reported	12,14
Cd 59 (GGC > GAC) Hb Adana	α1	PM	7	0.81	Reported	12,14
Cd 99 (AAG > TAG)	α1	PM	3	0.35	Reported	12,14
Cd 142 (TAA > AAA) Hb Icaria	α2	PM	2	0.23	Reported	12,14
Cd26 (GCG > ACG) Hb Caserta	α2	PM	1	0.12	Reported	12,14
Cd 64 (GAC > CAC) Hb-Q India	α1	PM	1	0.12	Reported	12,14
Cd 77 (CCC > CAC) Hb Toulon	α2	PM	1	0.12	Reported	12,14
Cd 90 (AAG > TAG)	α2	PM	1	0.12	Reported	12,14
Cd 109 T > G/Hb Suan Dok	α1	PM	1	0.12	Reported	12,14
−24 (C > G)	α2	PM	5	0.58	Reported	12,14
Deletion/non-characterized	α1 and α2	del	3	0.35	Novel	
−24 (C > G)	α1	PM	1	0.12	Reported	9,28
Cd 9 (AAC > AAA)	α2	PM	1	0.12	Novel	
Cd 60 deletion (AAG deletion)	α2	PM	1	0.12	Novel	
α dup (94-99)	α1	PM	1	0.12	Novel	
Cd 99 (AAG > AAT)	α2	PM	1	0.12	Novel	
Cd 108 (ACC > GCC)	α2	PM	1	0.12	Novel	
Cd 128 (AAG > ATG)	α2	PM	1	0.12	Novel	
Cd 129 (CTG > CGG)	α2	PM	1	0.12	Novel	

Abbreviations: del, Deletion; dup, Duplication; PM, Point Mutation.

it is known as one of the biggest such screening programs in the world.[17] Many reports are published about the frequency and mutation spectrum of beta-thalassemia in Iran to date.[18-20] Compared to beta-thalassemia, α-thal is usually milder with fewer consequences for the patients; however, our knowledge about the frequency and spectrum of variations as well as hematological indices is low in northern Iran. This study investigated the spectrum and frequencies of alpha-globin mutations in potential carriers with hypochromic and microcytic anemia from Mazandaran, a province in northern Iran. A more comprehensive study compared with past few reports on α-thalassemia will be useful to establish a prevention strategy for accurate thalassemia diagnosis in this region.

2 | MATERIALS AND METHODS

2.1 | Study population

As part of the national thalassemia screening program in Iran, marriage registrars refer prospective couples to a designated local laboratory for premarital screening. People were first screened by CBC (complete blood count) and hemoglobin electrophoresis using cellulose acetate method at alkaline pH.[21] Those who had reduced MCV (MCV ≤ 80 fL) or MCH (MCH ≤ 27 pg/cell), and normal HbA_2 levels (1-3.5%) were subjected to the further study and referred to a local genetic laboratory as a routine national services.[17] A total of 859 subjects, originating from Mazandaran province, northern Iran, were selected from who have been referred to the Novin Medical Genetics Center (Mazandaran, Iran), between 2010 and 2014. These individuals all were hematological ascertained not to be carried of beta-gene mutations because of their genetic analysis results (beta-thalassemia or other beta-globin variant carrier). All individuals were informed about this research and written consent obtained from each patient.

2.2 | DNA extraction and amplification

A total of 5 mL of peripheral blood was collected in EDTA from each person, and genomic DNA was extracted according to established protocols.[22,23] The GAP-PCR protocol, described by Baysal and Huisman, was primarily applied for all samples to detect α3.7 (3.7 kb), α4.2 (4.2 kb), --MED, --20.5, and --SEA variations, as a primary screening test.[24] This was followed by ARMS-PCR described by Foglietta et al [25] with little modification to detect the following variations including codon 59 (GGC > GAC; Gly > Asp) or Hb Adana in the alpha-1, and 9 variations in the alpha-2 gene including codon 19 (GCG > GC-), IVSI-5 nucleotide deletion (del TGAGG), codon 26 (GCG > ACG; Ala > Thr) or Hb Caserta, codon 59 (GGC > GAC; Gly > Asp), codon 142 (TAA > CAA; stop codon > Gln) or Hb Constant Spring, codon 142 (TAA > AAA, stop codon > Lys) or Hb Icaria, polyadenylation1 or polyA1 (AATAAA > AATAAG), polyadenylation2 or polyA2 (AATAAA > AATGAA), and anti-3.7 or α-triplication. The ARMS-PCR also was applied for all 859 samples subsequently.

2.3 | DNA sequencing

Samples that showed no mutations using conventional Gap-PCR and ARMS-PCR methods were subjected to further investigations using automated DNA sequencing (Applied Biosystems) method. In brief, different specific primers were used to amplify entire coding and non-coding regions of the *HBA1* (α1) and *HBA2* (α2) genes separately, followed by DNA sequencing to find any variations or short deletion/insertions as described previously.[26] A total of 242 samples were sequenced in this study. Because of specific amplification of two similar genes, *HBA1* and *HBA2*, by specific primers, sequence analysis was performed using reference sequences from GenBank database. *Gene Runner* software (http://www.generunner.com) was used to align patient's gene sequence, and Ref. sequence along with manual check of chromatogram from patient's DNA sequence using *FinchTV* chromatogram viewer software (Geospiza) also was applied.

2.4 | Multiplex ligation-dependent probe amplification (MLPA)

Samples that showed no variations using different methods mentioned earlier were subjected to the MLPA method (MRC-Holland). In brief, entire coding and non-coding regions of the *HBA1* (α1) and *HBA2* (α2) genes were screened using "SALSA MLPA probemix P140 HBA" including 35 specific probes covering approximately 700 kb of human alpha-globin gene cluster in chromosome 16p13.3 as described previously.[27] Ten samples were subjected to MLPA analysis for *HBA1*/*HBA2* genes in this study.

2.5 | Variation nomenclature

Nomenclature to report variations in this study was according to HB Var database with traditional numbering.[12]

3 | RESULTS

Mutation analysis of alpha-1 and alpha-2 genes in 859 subjects revealed 27 different variations as well as three large deletions as listed in Table 1 and Figure 2. Some deletion variations like −3.7, −4.2, --MED, and --20.5 and other point mutations such as Hb constant Spring, α2IVSI-5nt deletion, α2PolyA2, α2PolyA1, and α2Codon 19 were more frequent. According to our results, the 3.7 kb deletion is the most frequent variation with a frequency of 49.53%, followed by PolyA2 (15.19%), −4.2 deletion (8.76%), --MED (5.84%), α2IVSI-5nt deletion (5.49%), Cd142 (TAA > CAA) or Hb constant spring (3.62%), and α2Cd 19 (−G; 3.04%), respectively (Table 1). These seven variations were seen in 91.45% of all variations in alpha-1 or alpha-2 or both genes in this population. Some other known point mutations such as α1Cd59 (GGC > GAC) or Hb Adana, α2Cd142 (TAA > AAA) or Hb Icaria, Cd26 (GCG > ACG) or Hb Caserta, Cd 64 (GAC > CAC) or Hb-Q India, Cd 77 (CCC > CAC) Hb Toulon, and Cd 109 (T > G)/Hb Suan Dok were also found with low frequency (Table 1).

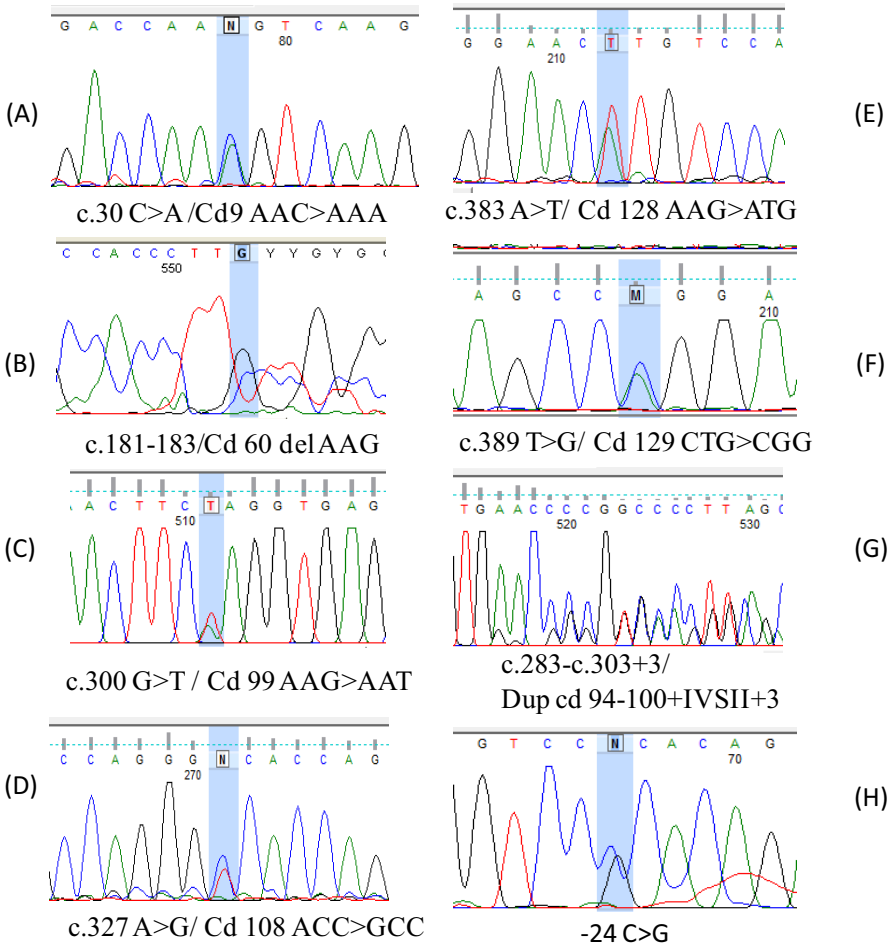

FIGURE 1 Seven novel variations identified in either alpha-1 (*HBA1*) or alpha-2 (*HBA2*) gene using Sanger DNA sequencing method. Chromatogram A-F shows six candidate variations in alpha-2 gene (*HBA2*), and chromatogram G shows a change in alpha-1 gene (*HBA1*). Chromatogram H demonstrates a promoter change, −24 C > G in alpha-1 gene (*HBA1*) identified in this study

Seven novel mutations which are not reported before in HB Var, ClinVar, HGMD, and other related databases are also reported in this study. One variation is in alpha-1 gene, a duplication from IVSII + 118 to IVSII + 148 followed by duplication from codon 101 to codon 142 in alpha-1 gene (MF970421). Another variation in alpha-1 gene, a C > G in −24 position, in the promoter region (MF970420) also was found in this study. Although this variation is not reported in HB Var or other databases as a mutation, it was mentioned earlier in previous studies as a variant.[9,28]

There are also six new variations in alpha-2 gene (Table 1 and Figure 1), including codon 9 (AAC > AAA; MF970422), deletion of codon 60 (AAG deletion; MF970423), duplication of codon 94 to 100 plus 3 base pairs of intron 2 (IVSII + 3) in alpha-2 gene (MF970424), codon 99 (AAC > AAT; MF970424), codon 108 (ACC > GCC; MF970425), codon 128 (AAG > ATG; MF970426), and codon 129 (CTG > CGG; MF970427) in alpha-2 gene, respectively (Table 1 and Figure 1).

Also, three samples that showed no variations using DNA sequencing were analyzed by MLPA method and results showed three

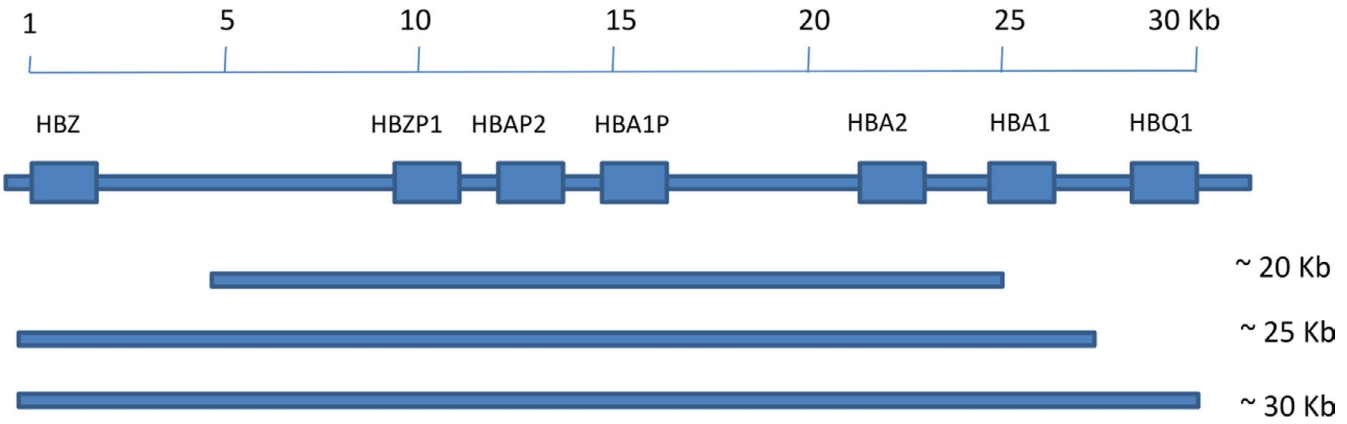

FIGURE 2 Three different alpha-cluster gene deletions achieved from three different patients using MLPA method. The length and exact area of these deletions are not reported before. In these three patients, different length from 20 kb to about 30 kb was deleted

TABLE 2 Alpha-globin gene mutation list and related hematological indexes in patients with α-thalassemia from northern Iran (n = 859)

		MCV			MCH		
Mutations	**Number (n)**	**Range (fL)**	**Mean**	***P* Value**	**Range (pg/cell)**	**Mean**	***P* Value**
-α 3.7	287	72.1-86.6	77.6	ns	21.3-27.2	24.9	ns
PolyA2 (AATAAA > AATGAA)	109	61.2-85.8	76.5	ns	19.7-26.6	24.6	ns
-α 4.2	59	72.3-86.4	77.5	ns	21.4-27.1	24.9	<.01
--MED	47	60.6-74.2	67.4	ns	18.6-25	20.6	.0019
IVSI-5nt (TGAGG)	32	71-82.4	75.6	<.05	21.7-26.4	24.1	ns
Cd 142(TAA > CAA) Hb constant spring	25	71.2-81.7	76.6	ns	22.5-27.5	24.6	ns
Cd 19 (−G)	20	71-80.5	76.2	ns	22.7-25.6	24.5	ns
--20.5	13	59.4-78.4	69.7	ns	19.4-26	22.3	.0019
PolyA1 (AATAAA > AATAAG)	12	71-81.9	75	ns	22.08-26.9	24	<.01
Anti-3.7 kb/ααα triplication	11	72.3-79.2	75.2	ns	23.6-26.6	25	ns
Cd 59 (GGC > GAC) Hb Adana	5	72-80	75	ns	23.9-25.8	24.7	ns
−24 (C > G; α2)	5	67.9-82.1	76.42	ns	24.5-27.2	25.66	ns
Cd 99 (AAG > TAG)	3	77.7-69.8	72.13		21.7-25.4	23.33	
Deletion/non-characterized	3	62.6-70	67		20-20.7	20.6	
Cd 142 (TAA > AAA) Hb Icaria	2	74.7-76.1	75.4		24.5-25.3	24.9	
−24 (C > G; α1)	1		77.7			26.5	
Cd 9 (AAC > AAA)	1		71.5			23.6	
Cd26 (GCG > ACG) Hb Caserta	1		77.1			24.7	
Cd 60 deletion (AAG deletion)	1		77.9			26.4	
Cd 64 (GAC > CAC) Hb-Q India	1		85.7			30	
Cd 77 (CCC > CAC) Hb Toulon	1		69.2			22.5	
Cd 90 (AAG > TAG)	1		70			22.7	
α duplication (Cd 94-99)	1		74.7			24.2	
Cd 99 (AAG > AAT)	1		82.1			25	
Cd 108 (ACC > GCC)	1		77.5			25.1	
Cd 109 T > G/Hb Suan Dok	1		68.7			21.8	
Cd 128 (AAG > ATG)	1		82.2			26.7	
Cd 129 (CTG > CGG)	1		71.1			22.5	
Normal range			80-100			27-31	

Abbreviations: Cd, Codon; fL, femtoliter; Hb, Hemoglobin, ns, not significant; pg, pictograms.

different deletions that are not similar to the known common deletions like those mentioned earlier in the paper (Figure 2).

Hematological data of patients with corresponding variations are also shown in Table 2. The highest average of MCV and MCH values (85 and 30) was also observed in variation of codon 64 (GAC > CAC) in alpha-1 gene. Statistical analysis using Dunn's multiple comparison test was applied to compare MCV and MCH volume between people with different variations. Result of the comparison between MCV and MCH in people with one gene defect (either single-gene deletion or point mutation) is shown in a column with header *P* value in Table 2. There was no significant difference between MCV/MCH reduction in people with one of these mutations (−3.7, −4.2, α2Poly A2, Cd 19, Hb constant spring, as well as alpha triplication or anti-3.7). Difference between MCV in people with 3.7 mutation and people with polyA1 mutation in alpha-2 gene was significant ($P < .05$). For the MCH, also difference between people with 3.7 variant and α2IVSI-5nt deletion or 3.7 variant with α2PolyA1 was significant ($P < .01$; Table 2). Comparison between two gene deletions, --MED and 20.5, showed no significant difference in MCV, but difference in MCV volume was significant ($P = .0019$; Table 2).

4 | DISCUSSION

Iran with a population of approximately 80 million represents a highly heterogeneous gene pool and diverse mutation spectrum due to geographical, cultural, and ethnical diversity. There are at least eight different ethnics in Iran, and Mazandarani is one of them who live in Northern province, Mazandaran, with about 3.2 million individuals in the south coast of Caspian Sea.[18] Few previously

reports are available about the alpha-globin gene variations in Mazandaran, these papers analyze fairly large samples to evaluate the spectrum of alpha-globin gene variations in α-thal patients from this province.

Alpha-thalassemia like sickle cell anemia and beta-thalassemia occurs at high frequencies throughout all tropical and subtropical regions of the world, and in some areas, the carrier frequency may be as high as 80%-90% of the population.[29] It is believed that all globin gene disorders such as α-thal have been selected because of protective potential of the carrier against malaria.[4,30] Among different globin gene disorders, α-thal are clinically very mild and most of the time may not be noticed during life and it may be one of the reasons that α-thal is more widely distributed than other type of globin gene disorders.[14] This study investigated 859 individuals and revealed more than 28 different variations, including 22 different point mutations and four different deletions. The α3.7 deletion was the most frequent variation (49.36%), similar to frequencies obtained from Tamaddoni et al study in Mazandaran (44.9%), Hadavi et al study in Gilan (42.5%), another neighbor province, and Zandian et al study in a southern province, Khuzestan (41.4%).[31-33] In addition, polyadenylation2 or PolyA2 (AATAAA > AATGAA; Turkish type), a point mutation in the alpha-2 gene, was the second most common variation with a frequency of 15.13% which followed by −4.2 deletion with 8.73% frequency. PolyA2 and −4.2 variations were also reported as second and third most common variations in Tamaddoni et al study with a frequency of 18.2% and 9.1% in Mazandaran, respectively.[31] There were also 9 new variations found in this study that were not reported previously (Table 1). Among point mutations, variations in alpha-2 gene were more frequent than in the alpha-1 gene, and 16 different variations found in this study in alpha-2 compared with six different variations in alpha-1 gene, respectively. The most common α_0-thal deletion or double gene deletion found in this study was --MED (5.82%), which was in agreement with previous results from Mazandaran (4.3%) and was reported from other provinces in Iran as well.[31-33] Another double gene deletion was --20.5, represented 1.75% of the identified variations. MLPA method also revealed rare or novel deletion variations from three patients with a α_0-thal which their mutations were not characterized previously (Figure 2). MLPA result indicated three different lengths of deletion that are not similar to those are common in the region like --MED, --20.5, or --SEA. Although the actual size of the deletions need to be identify properly in a separate study later, which was not possible in this study.

Duo to the national thalassemia screening program in Iran, reduced hematological indices (MCV ≤ 80 fL and MCH ≤ 27 pg/cell) are used as cutoff for microcytic and hypochromic anemia.[19] Also, HbA_2 level more than 3.5% is used to detect beta-thalassemia carriers. In this study, microcytic and hypochromic individuals with normal HbA_2 level were investigated. Different previous studies showed reduction in MCV/MCH in alpha-thalassemia carriers.[6,15,34-38] In this study, level of MCV/MCH in carriers with single-gene deletion/variation and double gene defect was compared. Among single-gene defect carriers, except significant difference between MCV in people with 3.7 and polyA1 variations, MCV values in other individuals were not significant (Table 2). Also for MCH value, difference between people with 3.7 and α2IVSI-5nt deletion or PolyA1 was significant ($P < .01$; Table 2). Number of individuals with polyA1 or α2IVSI-5nt variation in this study was relatively low compared with individuals with 3.7 variations, and it may affect the statistic results (Table 2). Comparison between individuals with two gene deletions (--20.5 and --MED) also revealed statistical difference between MCH level in these two group. The difference between the MCH level in individuals, who had --MED variation (47 individual with mean of 20.6) compared with --20.5 carriers (13 individual with mean of 22.3) was 1.7 (Table 2). It may the difference in the number of people in two group affect the statistical results.

Many common variations have been reported from different provinces of Iran as well as Mazandaran previously,[14,39] and this study revealed some novel and rare along with the previously known variations in this population (Figures 1 and 2, Table 1). These data revealed that the mutations causing α-thal in Iran are highly heterogeneous. This study along with the previous report confirmed that an efficient identification of α-thal can be achieved using standard hematological criteria in various populations. The spectrum of alpha-globin variations achieved from the present study will provide valuable support for the Iranian national thalassemia screening program and improving it.

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