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ORIGINAL ARTICLE

Molecular spectrum and distribution of hemoglobinopathies in southwest of Iran: a seven-year retrospective study

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Abstract

Thalassemia is one of the most common inherited autosomal recessive disorders around the world. Over 200 mutations in the beta-globin gene and 70 mutations in the alpha-globin gene have been identified. This study aimed to assess a comprehensive prevalence of most frequent thalassemia mutations in Khuzestan Province, where is a belt of thalassemia in Iran. A total of 6946 subjects were enrolled for evaluating alpha-beta thalassemia from 2012 to 2018. In order to determine the silent mutations, subjects with microcytic hypochromic without anemia with normal Hb, HbA2, and HbF were included too. Genomic DNA was extracted, and ARMS-PCR, Gap-PCR, and DNA sequencing were used to detect thalassemia mutations. Of 6946 individuals, just 880 (12.6%) were normal, and 6066 (87.3%) were the carrier for thalassemia. The most frequent phenotype was alpha thalassemia (3984; 57.4%), followed by beta (1429; 20.6%), and alpha-beta thalassemia (653; 9.4%), respectively. The most frequent alpha mutation was − 3.7 (68.6%), followed by − α (6.2%), Codon 19 (2.9%), Poly A2 (2.7%), and − 5 nt (2.4%), respectively. In beta thalassemia, the more often mutations have been HbS (20.2%), IVSI-I (11.3%), Codon 36/37 (11.2%), and IVSI-110 (7.4%), respectively. Additional analysis showed that the most frequent genotypes in alpha and beta thalassemia were heterozygous carriers with − 3.7α (52.2%) and HbS (21.3%) mutations, respectively. The lowest Hb was found in heterozygote beta thalassemia carriers with IVSII-1β mutation (11.6 g/dl). Our findings showed that the distribution of beta thalassemia mutations differs from other previous data reported from Khuzestan and other provinces. This study is useful for screening and preventing thalassemia.

Keywords Co-inheritance · Alpha thalassemia · Beta thalassemia · Mutations

Introduction

Thalassemia is one of the most common inherited autosomal recessive disorders around the world. Estimated nearly 30,000 children born each year with this disorder, and this has made thalassemia increasingly important [1]. The alpha-globin gene is localized in the telomeric region of chromosome 16 (16p 13.3) and beta-globin gene cluster is located in chromosome 11 [2, 3]. Mutation or deletion in alpha and beta genes, causing a decline or absence in the production of the globin chains. Depending on the type of mutations and their effects on globin gene expression, the severity of anemia varies from slight to severe. In the alpha-globin gene, non-deletional mutations (NDM) like − 3.7α, by involving the different stages of gene expression, mRNA translation, and alpha-globin stability, produce a slighter phenotype than deletional mutations (DM). Mutation in beta genes by decreasing beta-globin mRNA stability affects mRNA processing and reduces beta chain synthesis [4]. In beta thalassemia, due to the reduction of beta-

Highlights • Prevalence alpha, beta, and co-inheritance alpha-beta thalassemia in southwest of Iran
• Most thalassemia mutations and genotypes in southwest of Iran
• Comparing Hb level between different genotypes

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globin synthesis, the imbalance of the alpha-beta chains increases. Since free alpha-globins can not constitute tetramer, their accumulation in the cells causes damage cell surface, generating reactive oxygen species (ROS) and finally causing oxidative stress that promotes hemolysis. Macrophages in bone marrow, by phagocyting the precipitated erythroblasts, causes developing ineffective erythropoiesis. Extending ineffective erythropoiesis and hemolysis leads to splenomegaly, bone deformities, increase iron absorption, and metabolic disorder. Subjects with just one mutation in alpha or beta genes do not have a difference with healthy subjects and are identified just in the molecular examination [5, 6]. Nonrecognition of these carriers causes more prevalence of thalassemia.

The heterogeneity in thalassemia mutations has been the subject of intense debate within the scientific community. This has led to the proliferation of studies with contradictory findings of the thalassemia mutations and their prevalence around the world; these disagreements may be due to the different ethnicities and the low sample size. For example, the most reported mutations in alpha genes were DMs, whereas United Arab Emirates (UAE) reported the NDMs-like PollyA1 are more frequent [7–10]. These contradictory results are more seen in beta gene mutations; even in Iran, there has been a little agreement about beta thalassemia mutations spectrum. The most-reported mutations in beta genes in Iran are Codon 36/37 and I VS II-1 mutations; however, there are several differences reports [11–15]. Khuzestan Province in the southwest of Iran with a heterogeneous population is one of the most prevalent points of thalassemia in Iran and the Middle East. In order to evaluate the success of the thalassemia-preventing program and identifying common mutations in this region, several investigations were conducted, whose results indicated in addition to the above mutations, HbS has a high frequency in this province [16]. The aim of this survey was to investigate the most mutations in both alpha and beta genes and their association with Hb level.

Materials and methods

Ethical statement of the study

The study is base on the approval of the Medical Ethics Committee of Ahvaz Jundishapur University (reference number: Th-9808). The screening and identifying thalassemia were done according to the last updated National Thalassemia Prevention Guidelines in Iran provided in 2012.

Study design and population

The Khuzestan Province is a region with different ethnicities and one of the most centers of thalassemia in Iran. To evaluate the most prevalent thalassemia mutations in alpha and beta genes, and their effect on the phenotype of thalassemia, 6946 subjects were enrolled across 2012–2018. All participants were recruited to a private clinic for premarital screening.

Inclusion criteria

Accordance with the National Thalassemia Prevention Guidelines, subjects with mean cell volume (MCV) below 80 (Fl), mean cell hemoglobin (MCH) below 27 (pg), or both have to be considered for evaluating their HbA2 level by column chromatography. The HbA2 level between 3.5 and 7% was considered as beta thalassemia carrier, and > 7% was suspected as hemoglobinopathies. In the heterozygote (minor), alpha thalassemia, MCV, and MCH have just reduced. In the current investigation to determine the silent mutations, subjects with microcytic hypochromic without anemia with normal Hb, HbA2, and HbF were included too.

Hematological analysis

Blood samples were collected from among all subjects into ethylenediaminetetraacetate acid (EDTA) tubes. The cell blood counter (CBC) was conducted using the SYSMEX XN1000 (JAPAN) hematology analyzer. HbA, HbA2, and HbF were measured by Hb electrophoresis (Helena Laboratories).

Genotyping analysis

DNA was extracted from peripheral blood cells using MagCore-automated nucleic acid extraction (Switzerland). The purity and concentration of the extracted DNA were measured using the NanoDrop spectrophotometer (Thermo). Gap-polymerase chain reaction (Gap-PCR) technique was used to detect common DMs in alpha genes. Point mutations in alpha and beta genes were detected by the amplification refractory mutation system (ARMS). The detecting mutation panel for both alpha and beta genes was shown in ESM Appendix 1. In cases that PCR could not detect common mutations, DNA sequencing by ABI-3130XL (USA) was used.

Statistical analysis

All analyses were carried out using SPSS version 24. The normalization of data has been checked by Kolmogorov-Smirnov. Kruskal-Wallis test was used to determine the correlation between genotype and Hb level with a 95% confidence interval (CI). Mann-Whitney test was used to compare means. The P value of less than 0.05 was considered as significant statistically.

Results

In this study, 6946 individuals, including 3473 (50.0%) females, 3455 (49.7%) males, and 18 (0.3%) children, were investigated for thalassemia, of which just 880 (12.6%) and the remainder, i.e., 6066 (87.3%), respectively, were normal, and the carriers of thalassemia gene were defects. As shown in Table 1, the most frequent type of thalassemia was alpha thalassemia (3984; 57.4%), followed by beta (1429; 20.6%), and alpha-beta thalassemia (653; 9.4%), respectively. Further analysis showed a significant difference in Hb and MCV when they were adjusted for gender ($P < 0.001$).

Post hoc analysis showed that the means of the hematological parameters, including Hb, MCV, and MCH, were significantly lower in beta and alpha-beta thalassemia (Table 2). As expected, HbA2 and HbF were higher in beta and alpha-beta thalassemia carriers ($P < 0.001$) (Table 2). No significant differences were found between alpha thalassemia carriers compared with healthy subjects for HbA2 ($P = 0.815$) and HbF ($P = 0.491$) (Table 2). After comparing different genotypes for Hb level with normal subjects, it was found that nine mutations in heterozygous caused a significant reduction in Hb level; of these, the lowest Hb was observed in subjects with a harboring IVSII-I mutation ($P < 0.001$) (Table 3).

In an analysis of beta mutations, fifty mutations were detected, of which Hb-S (20.2%), IVSI-1 (11.3%), Codon36/37 (11.2%), and IVSI-110 (7.4%) were the more often mutations (Table 4). There are several rare detected mutations with less than 1% frequency, including − 87, Codon 30, Codon 17, IVSI-726, 30, +20, +22, IVSII-850, Codon 30, Codon 41/42, Codon 27, IVSI-13, − 71(c > T), IVSI-108, Codon 26, IVSII-848, IVSII-849, IVSI-128, IVSII-II, + 1479, − 56, and unknown (ESM Appendix 2). The most detected genotypes in beta and alpha-beta thalassemia carriers were β/HbS (21.3%) and − 3.7/S (54.0%), respectively.

In alpha mutation analysis, forty-four mutations were identified, from these, − 3.7 mutation with a frequency of 68.6% was the most prevalent, followed by -α (6.2%), Codon 19 (2.9%), Polly A2 (2.7%) and − 5 nt (2.4%), respectively (Table 5). There were the rarest mutations with a frequency of less than 1% in alpha genes like PollyA4, Codon 142, and PollyA6 (ESM Appendix 2). The most frequent genotype in alpha thalassemia patients was − 3.7/αα (52.2%).

Table 1 Genotypes frequency of patients

Genotype	Frequency (%)
Alpha-thalassemia	3984 (57.4%)
Beta-thalassemia	1429 (20.6%)
Alpha-beta thalassemia	653 (9.4%)
Normal	880 (12.6%)
Total	6946 (100.0%)

Table 2 Subgroup analysis of hematological parameters between different thalassemia phenotypes

Patient genotypes	Hb	Sig	95% CI for difference	
Normal	13.4		Lower bound	Higher bound
Alpha-thalassemia	13.3	0.84	−1.03	1.26
Beta-thalassemia	12.6	0.22	−0.5	2.13
Alpha-beta thalassemia	14.8	0.083	−3.05	0.18
Normal	MCV	Sig	95% CI for difference	
			Lower bound	Higher bound
	80.9			
Alpha-thalassemia	77.4	$P < 001$	2.4	4.5
Beta-thalassemia	68.4	$P < 001$	11.2	13.7
Alpha-beta thalassemia	71.2	$P < 001$	8.2	11.2
Normal	MCH	Sig	95% CI for difference	
			Lower bound	Higher bound
	26.5			
Alpha-thalassemia	25.0	$P < 001$	0.9	2.1
Beta-thalassemia	22.6	$P < 001$	3.1	4.6
Alpha-beta thalassemia	23.2	$P < 001$	2.4	4.2
Normal	HbA2	Sig	95% CI for difference	
			Lower bound	Higher bound
	2.8			
Alpha-thalassemia	2.7	0.815	− 0.458	0.582
Beta-thalassemia	5.2	$P < 001$	− 2.97	− 1.78
Alpha-beta thalassemia	4.3	$P < 001$	− 2.23	− 0.779
Normal	HbF	Sig	95% CI for difference	
			Lower bound	Higher bound
	0.7			
Alpha-thalassemia	0.6	0.491	− 0.245	0.511
Beta-thalassemia	1.7	$P < 001$	− 1.438	− 0.599
Alpha-beta thalassemia	1.5	0.003	− 1.271	− 0.265

Discussion

The present study was designed to determine the most prevalent type of thalassemia and mutations in Khuzestan Province, southwest of Iran. Based on the conducted studies, beta thalassemia is more prevalent in Iran and is more severe than alpha-thalassemia [17]. Prenatal diagnostic tests in Iran were established from 1997 to prevent beta thalassemia. For this, evaluating the rate of thalassemia and most mutations has led to a proliferation of studies in this field. Previously published studies are limited to investigating just one type of thalassemia or evaluating a small sample size in Khuzestan [18, 19]. It has been demonstrated that in Khuzestan province, beta thalassemia is higher than the other provinces. However, it is in contrast with our finding that alpha-thalassemia is more prevalent [20] (Table 1).

After mutation analysis, our data revealed that five mutations, including HbS, Codon 36/37, IVSI-1, IVSI-110, and

Table 3 The mean Hb level in different genotypes comparing with normal genotype

						95% confidence interval	
	Frequency	Percentage (%)	Hemoglobin (g/dl)	SD	Sig.	Lower bound	Upper bound
Normal	863		13.1 (7.7–18.4)	1.6			
Alpha-thalassemia							
− 3.7/− 3.7	473	11.9%	12.7 (7.8–18.8)	1.5	$P < 0.001$	0.251	0.628
-med/αα	60	1.5%	12.3 (7.9–15.2)	1.5	$P < 0.001$	0.375	1.253
Beta-thalassemia							
N/Cd6 (Hb S)	208	20.3%	13.8 (7.5–18.6)	1.8	$P < 0.001$	− 0.928	− 0.404
N/IVSI-I	176	17.2%	11.8 (7.2–16.1)	1.4	$P < 0.001$	1.058	1.605
N/Cd36–37	177	17.3%	11.7 (9–15.1)	1.3	$P < 0.001$	1.152	1.696
N/IVSI-110	109	10.6%	12.2 (7.6–15.2)	1.5	$P < 0.001$	0.645	1.316
N/Fr8–9	74	7.2%	11.8 (9.3–19.6)	1.3	$P < 0.001$	0.940	1.757
N/IVSII-I	106	10.3%	11.6 (8.3–14.9)	1.4	$P < 0.001$	1.171	1.863
N/Cd5	47	4.6%	11.7 (9.4–15)	1.4	$P < 0.001$	0.920	1.905
N/− 25b	51	5%	12.2 (8.9–15.2)	1.5	$P < 0.001$	0.469	1.425

IVSII-1, account for over than 50% beta thalassemia mutations in our population (Table 4); this is in agreement with finding of Tosun et al.'s study in Turkey [21] while inconsistent with previous Iranian studies' results in a geographical difference, where IVSII-1 was most prevalent [11, 22, 23]. A survey conducted by Kiani et al. showed that Codon 36/37 is more frequent and different from other parts of Iran [14].

Table 4 An overview of the most frequent mutations in the beta gene and their phenotypes

Beta-thalassemia mutations	Frequency	Percentage (%)	Phenotype
HbS	419	20.2	β^0
IVSI-1	234	11.3	β^0
Cd36-37	233	11.2	β^0
IVSI-110	154	7.4	β+
IVSII-1	143	6.9	β^0
Fr8-9	104	5.0	β^0
HbD	81	3.9	β+
-25 del	79	3.8	β+
− 101	72	3.5	β+
Cd5	67	3.2	β^0
IVSI-6	53	2.6	β+
− 88	48	2.3	β+
Cd39	44	2.1	β+
IVSI-5	43	2.1	β^0
IVSII-745	36	1.7	β+
Cd82-83	35	1.7	β+
Cd44	33	1.6	β^0
− 28	28	1.4	β+
Initiation Cd	20	1.0	β^0

Cd, Codon

An explanation for this might be due to the difference in ethnic and smaller sample sizes. Compared with the other studies around the world, Chinese researchers reported that Codon 41/42 mutation is more prevalent [24, 25].

Except for IVSI-110, other mutations develop β^0 phenotype, which in the heterozygous genotype significantly reduces Hb compared with normal subjects. It can be seen in Table 3, that the subjects with IVSII-1 heterozygote genotype had significantly lower Hb compared with the other genotypes. This finding supports the previous research that in coexisting alpha thalassemia with beta thalassemia or hemoglobinopathies, due to the reduced accumulation of free alpha-chains, the toxic effect decreases and reduces the severity of thalassemia [26–28].

The Hb H disease is an unstable form of Hb, which is the result of the homozygote for NDMs or heterozygous for both DMs and NDMs [29]. Our findings demonstrated that more

Table 5 The most frequent alpha gene mutations

Alpha-thalassemia mutations	Frequency	Percentage (%)	Phenotype
− 3.7	3169	68.6	α+
− a	288	6.2	α+
Cd19	135	2.9	α+
Polly A2	123	2.7	α^0
5 nt	109	2.4	α+
-med-	73	1.6	α^0
− 4.2	56	1.2	α+
Anti 3.7	49	1.1	α+
Initiation codon	45	1.0	α+
Not found	350	7.6	–

Table 6 Distribution type of thalassemia in various regions of Iran

Area	Hemoglobinopathies	alpha-thalassemia	beta-thalassemia	Ref.
North of Iran		− 3.7α (44.9%)	IVSII-I (56.1%)	[13, 34]
Northwest of Iran		− 3.7α (51.7%)	IVSII-1 (23.6%) IVSI-110 (29.5%)	[32, 41, 42]
Northeast of Iran			IVS-I-5 (42.03%)	[43]
Central regions of Iran	Hb-D (75.67%)			[44]
West of Iran			CD36/37 (33.8%) IVSII-1 (33%)	[14, 45]
Southwest of Iran	HbS (21.0%)	− 3.7α (20.0%)	IVSII-1 (11.7%) CD36/37 (13.9%)	[15, 16, 30]
South of Iran			IVSI-5 (69%)	[46]

than 90% of mutations were α^+ and just 6.3% were α^0phenotypes. The more often α^+ mutations were − 3.7, − α, Polly A2, and -med accounts for more than 95% α0 mutations (Table 5). The most frequent genotype in alpha-thalassemia carriers was − 3.7/αα (52.2%); this is in accordance with findings of Keikhaei et al.'s study [19]. Our results are in agreement with data obtained from similar studies conducted by some researchers in Iran, Turkey, and Israel, where − 3.7 mutation is the most frequent defected gene [30–35], whereas the conducted studies in Malaysia and China revealed that the -SEA mutation has the most prevalent [36, 37].

Further analysis showed that the severity of alpha-thalassemia is less than beta and alpha-beta thalassemia (Table 2), and the significant reduction was not found in HbA2 and HbF in alpha thalassemia carriers compared with normal subjects. This result may be explained by the fact that there are four copy numbers of the alpha-globin gene in normal individuals, encoded by two adjacent *homologous* genes, alpha 1 and alpha 2; these units were divided into X, Y, and Z *homologous* units [4]. The recombination of Z units causes developing the rightward deletion (− 3.7α), which is accountable for the most frequent NDMs [3]. Additionally, there is no compensatory increase in the remaining functional gene, which causes producing unstable Hb and reduction of Hb [3]. The severity of alpha thalassemia is somewhat proportional to the number of affected alpha alleles. Sometimes thalassemic erythrocytes produce more alpha chains than predicted by the number of affected alleles. There are two possible explanations for this. First, the alpha 2-gene produces two or three times the amount of mRNA than the alpha1-gene [38, 39]; therefore, the mutation in the alpha 2-globin gene would reduce two up to three times in alpha-chain production [3, 39]. Second, erythrocytes have an internal mechanism to compensate for deleted genes that stimulate the more production of alpha chains from the unaffected genes. Depending on the amount of the involved alpha gene, exceeds beta-chains accumulate and combine as a tetramer (β4).

Geographical distribution of thalassemia and hemoglobinopathies in Iran

Despite its great clinical success in the premarital diagnosis of thalassemia, 3 to 100 patients per 100,000 are the carriers for thalassemia in Iran [40]. A considerable amount of literature has been published about the type of thalassemia, mutations, and their influence on the phenotype of thalassemia in Iran, but these contradictory findings show the distribution of thalassemia and hemoglobinopathies entirely dependent on the geographical distribution. Table 6, shows the molecular spectrum and hemoglobinopathies in different provinces according to the comprehensive investigations. As shown in Table 6, the most reported mutation in beta gene was IVSII-I [13, 15, 16, 30, 41, 42]. After hemoglobinopathies frequency analysis, more prevalence of HbS and HbD was reported in southwestern and central regions of Iran, respectively [16, 44]. As discussed earlier, the results obtained from previous studies about alpha thalassemia mutations showed that most of them are in agreement that − 3.7α is the most frequent alpha gene defect (Table 6).

Limitations

The current study is limited by the lack of positive samples for δ hemoglobinopathies, so further investigation and experimentation for δ hemoglobinopathies are strongly recommended. The data recorded in this study belonged only to those who met the inclusion criteria, and lack of access to the initial number of subjects is another limitation of the study.

Conclusion

This study set out to determine the most frequent type of thalassemia, mutations, and their association with hematological parameters in the Khuzestan Province, southwest of Iran. Previously published studies are limited to investigating just

one type of thalassemia or evaluating the low sample size in Khuzestan [18, 19]. The result of this study showed that alpha thalassemia is more frequent and is accompanied by a slight to mild phenotype. According to our findings, the normal level of HbA2 and HbF cannot rule out alpha thalassemia. This study, by providing comprehensive data about all types of thalassemia frequency and indicating the prevalence of thalassemia carriers with normal electrophoresis, can be more helpful in preventing thalassemia.

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Author contributions F.R conceived the manuscript and revised it. M. A and J.M.A wrote the manuscript and prepared tables and figures.

Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

Ethical approval This article was approved by the sponsor and the applicant with respect to scientific content and compliance with applicable research and human subjects' ethical regulations.

Informed consent For this type of study, informed consent was not required.

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