1. INTRODUCTION

Thalassemia is a genetic abnormality involving mutations of the genes responsible for the production of hemoglobin in the blood. There are two broad types of thalassemia, α- and β-thalassemia; each of which has a different prevalence among certain ethnicities or population groups. Approximately 4.5 of every 10,000 live births throughout the world are affected by thalassemia (http://www.ironhealthalliance.com/disease-states/thalassemia/epidemiology-and-pathophysiology.jsp). α-Thalassemia is more frequent in Southeast Asia than in other areas of the world, and up to 40% of genetic traits have been found in thalassemia traits (TTS) (1–30%). People living in the Mediterranean, African, and South Asian areas are more likely to be affected by β-thalassemia. Genetic prevalence of β-thalassemia throughout the world is 2–18% (affected by a gene mutation) in the Eastern Mediterranean and 0–11% in Southeast Asia [1]. Approximately 5% of the global population have a variation in the α or β part of the hemoglobin molecule, although some of these are asymptomatic and known as silent traits. In fact, only 1.7% of the global population have signs as a result of the gene mutations, known as α-TT. However, tribal or ethnic groups are more likely to be affected and 5–30% of the population may be symptomatic among these groups [1].

Iran has an area of 1,648,000 km², and like many other countries in the region, has a large number of patients with major thalassemia. α-Thalassemia is infrequent in Iran. Gene frequency of β-thalassemia is high, and it varies considerably from area to area. Its highest rate (>10%) is found around the Caspian Sea and Persian Gulf. Prevalence of β-thalassemia in other areas is between 4% and 8% [2]. In Southern Iran, the IVS-II-1 (G → A) mutation is the most frequent (31%) mutation for β-thalassemia. In Khuzestan Province, in Southern Iran, the frequency of β-thalassemia minor is also high and reaches 10% [2]. According to a study conducted by Zandian et al. [3] in Khuzestan, from 152 volunteer couples (342 individuals) from Arab ethnic groups in Dashte–Azadegan and Khorramshahr cities, 3.63% and 10.57%, respectively, had sickle cell trait; Of these, 84.21% had normal hematological indices [mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH)]. A few studies on genetic diversity of β-thalassemia mutations and sickle cell anemia (SCA) have been conducted in Iran, particularly in Khuzestan Province. Therefore we decided to carry out this cross-sectional and prospective study to assess the genetic diversity and prevalence of α- and β-thalassemia and their relationship with each other, detection of genetic diversity of α- and β-thalassemia minor, and detection of genetic diversity of SCA among 17,581 volunteer couples living in Khuzestan Province, Southwest Iran. Eventually data for the analysis of mutated genes, mutational pattern, as well as their prevalence were submitted to the Research Center of Thalassemia and Hemoglobinopathies, Jundishapur University of Medical Sciences, Ahvaz, Iran. Data were then submitted to Khuzestan
Healthcare and Treatment Institute, Khuzestan, Iran to develop a control program in order to reduce the prevalence of thalassemia.

2. MATERIALS AND METHODS

2.1. Study Design

This was a prospective study that was carried out in the Department of Pathology and Thalassemia Control Cell, Shafa Hospital in Ahvaz, the capital city of Khuzestan Province, Southern Iran (Latitude: 31.436015, Longitude: 49.041312) between February 2014 and December 2017. A total of 17,581 prospective couples from Ahvaz (Latitude: 31.3183272, Longitude: 48.67061869999998) and Abadan (Latitude: 30.347296, Longitude: 48.2934) cities of Khuzestan Province were screened for the presence of thalassemia or any structural variant. All participants and their family members with hematological indices were questioned about their medical history. A 5-mL intravenous blood sample was collected in ethylenediamine tetracetic acid (EDTA) anticoagulant. Red cell indices were measured on an automated hematology analyzer (Sysmex KX 21; Sysmex Corporation, Kobe, Japan). Hemoglobin (Hb)A2 and HbF were studied by high-performance liquid chromatography (HPLC) used for chromatographic separation of human Hb [4,5].

2.2. Sample Collection and Preparation

Five milliliters of intravenous blood was collected in a vacuum collection tube containing EDTA, which was stored at 3–8°C for a maximum 7 days if processing were delayed. HbA2 calibrators and normal and abnormal controls were analyzed at the beginning of each run.

2.3. Thalassemia Screening

At the first, the men's red cell indices were checked by complete blood count. If they had microcytosis (MCH < 27 pg or MCV < 80, the women's red cell indices were tested too. When both were microcytic, their HbA2 concentrations were measured by HPLC (Model D10; Bio-Rad, France, using an ELITech Kit; ELITech Group, Puteaux, France). Tris-glycine buffer was used in order to measure HbA2 by HPLC. Electrophoresis was performed on cellogel at pH 8.5 in Tris-glycine buffer for 90 minutes, as described previously [4,5]. A concentration >3.5% was indicative of β-TT. Microcytic individuals with HbA2 concentration in the normal range (1.5–3.5%) were treated with iron and their indices rechecked. Patients who had TT (MCV < 80 fl, MCH < 27 pg/L, and HbA2 ≥ 3.5%) were examined using multiplex amplification refractory mutation system (M-ARMS) to detect α- and β-thalassemia mutations, direct DNA sequencing of α- and β-globin genes, and gap-polymerase chain reaction (PCR) for globin gene deletions, respectively. Inclusion criteria for the iron deficiency anemia (IDA) group were Hb <13 g/dL for men and <12 g/dL for women, MCV < 80 fl and MCH < 27 pg for both sexes, and ferritin < 28 ng/mL for men and < 6 ng/mL for women [6,7]. Exclusion criteria for the IDA group included the presence of mutations associated with α-TTs and/or β-TTs. For inclusion in the β-TT group, individuals had MCV < 80 fl, MCH < 27 pg, and HbA2 > 3.5% [4,5]. α-TT was confirmed by the presence of mutations (Fig. 1).

Silent α/β-thalassemia carriers have no signs or symptoms of the disease, but are able to pass it on to their children. In our study, silent thalassemia carriers showed a normal hematological picture or slight alterations in some hematological parameters (mild changes in erythrocyte morphology, or MCV below the normal mean), and normal Hb status or slight alterations in HbA2 level, while α/β-globin synthesis ratio was abnormal (>1 or <1). Genotype and some hematological parameters in some individuals with silent or α- or β-thalassemia are shown (see Table 1).

2.4. Thalassemia Mutation Analysis

Genomic DNA was extracted from peripheral blood lymphocytes using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA).

Figure 1  Inclusion and exclusion criteria. Hb, hemoglobin; IDA, iron deficiency anemia; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; TT, thalassemia trait.
Table 1 Distribution of some hematological parameters in subjects with silent α- or β-thalassemia

| Silent thalassemia                                      | Hb (g/dL) | MCV     | HbA2   | α/β ratio | Genotype         |
|--------------------------------------------------------|-----------|---------|--------|-----------|------------------|
| Silent β-thalassemia (−101 C → T mutation)             | 14.2 ± 1.6| 85 ± 1.2| 3.3 ± 0.3| 1.1 ± 0.14 | −101/b<sub>αα</sub> |
| Silent β<sup>+</sup>-thalassemia (IVS II 844 C → G mutation) | 14.4 ± 1.8| 86 ± 1.5| 3.4 ± 0.23| 1.10 ± 0.03 | AA<sup>♂</sup> ♂ |
| Silent α<sup>+</sup>-thalassemia (−α<sup>2NcoI</sup> mutation) | 13.6 ± 0.2 | 75 ± 0.2 | 2.3 ± 0.23 | 0.88 ± 0.08 | b<sup>NcoI</sup>α/aα |
| Silent α<sup>+</sup>-thalassemia (a<sup>2HphI</sup> mutation) | 13.5 ± 1.1 | 78 ± 1.2 | 2.4 ± 0.04 | 0.85 ± 0.12 | b<sup>HphI</sup>α/aα |

Data are presented as mean ± SD; Hb, hemoglobin; MCV, mean corpuscular volume.

Table 2 Multiplex gap-PCR protocol for the diagnosis of 3.7- and 4.2-kb α<sup>+</sup>-thalassemia deletions

| Primer sequences [1]                                      | Primer      | Description                        | Sequece                        | Annealing T(°C) |
|--------------------------------------------------------|-------------|-----------------------------------|--------------------------------|-----------------|
| Primer mix                                              | Component   | µL                                 |                                |                 |
| α2/3.7-F (10 µM)                                        | 1           | 1.0                               |                                |                 |
| α2-R (10 µM)                                            | 2           | 0.25                              |                                |                 |
| α2/20.5-R (10 µM)                                       | 3           | 1.0                               |                                |                 |
| 4.2-F (10 µM)                                           | 4           | 1.0                               |                                |                 |
| 4.2-R (10 µM)                                           | 5           | 1.5                               |                                |                 |
| 10x buffer (750 mM Tris-HCl pH 8.8, 200 mM (NH₄)<sub>2</sub>SO₄, 0.1% Tween 20) | 6           | 2.5                               |                                |                 |
| 25 mM MgCl<sub>2</sub>                                  | 7           | 1.5                               |                                |                 |
| dNTPs (1 mM)                                            | 8           | 5.0                               |                                |                 |
| Betaine (5 M)                                           | 9           | 3.75                              |                                |                 |
| DMSO (10%)                                              | 10          | 1.25                              |                                |                 |
| Platinum Taq (5 U/mL)                                   | 11          | 0.1                               |                                |                 |
| DNA template (100 ng/mL)                                | 12          |                                    |                                |                 |
| Water                                                   | 13          |                                    |                                |                 |

Gel electrophoresis conditions

Run PCR products out on 1.5% (1:1 Nusieve: agarose) gel for 2–3 h

Interpretation of results

| PCR fragment size (bp) | Genotype                        | Product of primers |
|------------------------|--------------------------------|--------------------|
| 2020                   | α-thalassaemia: −α<sup>37</sup> | 1 + 2              |
| 1800                   | Normal (αα)                     | 1 + 3              |
| 1628                   | α-thalassaemia: −α<sup>42</sup> | 4 + 5              |

DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; T, temperature.

β-Globin gene mutations were first characterized using an M-ARMS to detect common mutations in Southwest Iranian populations, including IVS-II-1 (G → A), IVS-I-1 (G → T), IVS-I-110 (G → A), CD36/37 (−T), IVS-I-5 (G → C), cd5, IVS-I-36 (−C), and cd39 (C → T), as previously described [8]. Uncommon β-thalassemia genes were further characterized by direct DNA sequencing of all coding regions and exon–intron boundaries to detect uncommon point mutations, as described previously [9]. α- and β-Thalassemia mutations were subsequently screened by gap-PCR to detect entire globin gene deletions, as previously described [10–13].

2.5. Gap-PCR

Gap-PCR techniques (amplification using oligoprimers flanking deletion breakpoints) are used to detect different types of globin gene deletions, such as common α-thalassemia deletion mutations and α-gene duplication [11]. A typical gap-PCR test is illustrated for the diagnosis of α-thalassemia, and the primers can be multiplexed [14,15], as shown in Tables 2 and 3. The 3.7- and 4.2-kb α<sup>+</sup>-thalassemia deletions can be detected in one assay [11,12], and the Mediterranean thalassemia deletion (−<sup>MED</sup>) in the
Table 3  Multiplex gap-PCR protocol for the diagnosis of \(-\text{MED}\) thalassemia deletion

| Primer | Name | Sequence | Annealing T (°C) |
|--------|------|----------|-----------------|
| 1      | MED(F) | CGATGAGAACATAGTGAGCAGAATTGCAGG | 60 |
| 2      | MED(R) | ACGCGCAGGTGCTGCCCAGCTTCTTCCAC | 60 |
| 3      | \(\alpha\)-R | AGACCAGGAAGGGCCCGTG | 64 |
| 4      | 4.2-R | CCCGTTGGATCTTCTCATTTC | 64 |

**PCR mix**

| Component | µL |
|-----------|----|
| MED(F) (10 \(\mu\)M) | 0.4 |
| MED(R) (10 \(\mu\)M) | 0.4 |
| 10× buffer (750 mM Tris–HCl pH 8.8, 200 mM (NH\(_4\))\(_2\)SO\(_4\), 0.1% Tween 20) | 2.5 |
| 25 mM MgCl\(_2\) | 1.5 |
| dNTPs (1 mM) | 4.0 |
| Betaine (5 M) | 3.75 |
| DMSO (10%) | 1.25 |
| Platinum Taq (5 U/mL) | 0.1 |
| DNA template (100 ng/mL) | 1.0 |
| Water | 6.2 |

**Gel electrophoresis conditions**

Run PCR products out on 2% (1:1 Nusieve:agarose) gel for 1–1.5 h

**Interpretation of results**

| PCR fragment size (bp) | Genotype | Product of primers |
|------------------------|----------|--------------------|
| 1010                   | Normal (\(\alpha\alpha\)) | 3 + 4 |
| 875                    | \(\alpha\)-thalasaemia: \(-\text{MED}\) | 1 + 2 |

PCR, polymerase chain reaction; T, temperature.

second assay [12]. Specific primer details are listed in Tables 2 and 3 for the multiplex diagnosis of the common \(\alpha\)-thalassemia deletion genotypes.

### 2.6. Statistical Analysis

Statistical analysis of hematologic indices and the results of molecular tests and mutation types among carrier couples was performed by one-way analysis of variance with SPSS software (SPSS Inc., Chicago, IL, USA).

### 3. RESULTS

Among all volunteers, 995 (5.6%) had \(\beta\)-thalassemia minor (435 female and 560 male). Of these, 695 cases (69.85%) belonged to the city of Abadan and 300 (30.15%) to Ahvaz. Average value of HbA2 among female and male populations in the city of Abadan was 4.97% and 5.23%, respectively. In Ahvaz, the average value of HbA2 was 4.3% in women and 5.01% in men. The mean hematological indices among TTs are shown in Table 4.

In a molecular study of 50 individuals (5%) randomly selected using M-ARMS, eight codons from the \(\beta\)-globin defects accounted for 90% of the total \(\beta\)-thalassemia mutations: IVS-II-1 (G → A; 26%; \(n = 13\)), IVS-I-1 (G → T; 16%; \(n = 8\)), IVS-I-110 (G → A; 14%; \(n = 7\)), CD5 36/37 (-T; 10%; \(n = 5\)), IVS-I-5 (G → C; 8%; \(n = 4\)), IVS-I-6 (T → C; 6%; \(n = 3\)), cd5 (6%; \(n = 3\)), and cd39 (C + T; 4%; \(n = 2\)); the most common mutation was IVS-II-1 (Fig. 1). Prevalence of \(\alpha\)-TTs was 6.65% (\(n = 1169\)). Also, 50 individuals were tested for \(\alpha\)-thalassemia gene mutations based on a gap-PCR assay. Genotype frequencies of \(\alpha\)-globin mutations were \(-\alpha\)-3.7 kb (50%; \(n = 25\)), Med/\(\alpha\alpha\)thal (12%; \(n = 6\)), and \(-\alpha\)-4.2/\(\alpha\alpha\) (10%; \(n = 5\)), which were the most frequent deletion mutants (72% in total). The \(-\alpha\)-1.7 kb deletion was the most common deletion (Fig. 2).

Prevalence of SCA, IDA, and silent thalassemia was 1240 (7.05%), 911 (5.18%), and 1134 (6.45%) respectively, as shown in Table 5 and Fig. 2. Distribution of SCA haplotypes among people in Southwest Iran is shown in Table 6. Genotype and hematological characteristics of subjects with silent \(\alpha\)- or \(\beta\)-thalassemia are presented in Table 1.

### 4. DISCUSSION

Thalassemia is common in Iran due to a tradition of inbreeding, a conservative religious culture, and a large number of ethnic groups in different areas of the country. Genetic prevention programs based on hospital-based screening and prenatal diagnosis were started in 1997, and Iranian laws were modified between 1998 and 2005 to permit abortion of affected fetuses [16]. Some research on the clinical and laboratory presentations of \(\beta\)-thalassemia
The α-thalassemia deletions are common in Asia, where a high prevalence of α-thalassemia has been observed.

Recent studies have revealed the presence of >47 different β-globin gene mutations responsible for β-thalassemia in Iran. IVS-II-1 (G → A) mutation followed by IVS-I-5 (G → C), codons 8/9 (+G), IVS-I-110 (G → A), IVS-I-1 (G → A), 25-bp deletion, IVS-I-6 (T → C), codon 5 (−CT), and codon 39 (C → T) mutations were the most frequent mutations, which accounted for 85% of the total β-thalassemia defects in Iran [19,22].

Rezaee et al. [23] attempted to study the origin of β-thalassemia mutations in different parts of Iran. They demonstrated that β-thalassemia mutations in different regions have different distribution patterns. These β-thalassemia mutations are indicative of the ancestral origin of the people who migrated to Iran from other regions of the world. In this study [23], in the southwest near the Arabian Peninsula, codons IVS-I-1 (G → A), 8/9 (+G), IVS-I-110 (G → A), IVS-I-1 (G → A), IVS-I-6 (T → C) and IVS-I-5 (G → C) were common mutations that caused 86% of cases of β-thalassemia. Codon IVS-I-1 is the most frequent mutation in Southwest Iran.

Karimi et al. [24] carried out a prospective study demonstrating that the IVS-II-1, IVS-I-110, IVS-I-1, and FSC 8/9 mutations were the most prevalent in the country, and IVS-II-1 was the most frequent in Southern Iran (having the highest rate of 24%).

In our study, the thalassemia syndromes (α and β-thalassemia mutations, sickle cell disease) were assessed using M-ARMS, direct DNA sequencing of α and β-globin genes, and gap-PCR among the 17,581 volunteer couples living in Khuzestan Province. Eight codons out of the β-globin defects accounted for 90% of the total β-thalassemia mutations: IVS-II-1 (G → A; 26%; n = 13), IVS-I-1 (G → T; 16%; n = 8), IVS-I-110 (G → A; 14%; n = 7), CDs 36/37 (−T; 10%; n = 5), IVS-I-5 (G → C; 8%; n = 4), IVS-I-6 (T → C; 6%; n = 3), cd5 (6%; n = 3), and cd39 (C + T; 4%; n = 2. IVS-II-1 (G → A).
mutation was the most frequent (26%) mutation for β-thalassemia in Khuzestan Province. Our study has confirmed the previous studies.

Prevalence of α-TTIs was 6.65% (n = 1169) and $\alpha^{3.7}$/αα ($\alpha^{3.7}$/αα) was the most common α-thalassemia deletion (50%; n = 25), which was consistent with Zandian et al. [25]. It indicates the importance of identification of this gene in couples to prevent the occurrence of hemoglobin H (HbH) disease and hydrops fetalis.

Our study demonstrated the distribution of SCA haplotypes among different ethnic groups in Southwest Iran. The Arab ethnic group has the highest frequency of SCA traits among all ethnic groups, confirming the results of Zandian et al. [26].

5. CONCLUSION

The genetic basis and clinical severity of α- and β-thalassemia are heterogeneous among Iranians due to the presence of multiple ethnic groups in the country. The β-thalassemic IVSII-1 (G $\rightarrow$ A) mutation had the highest frequency in Southwest Iran. As a Mediterranean mutation, it might reflect its independent origin, genetic admixture, and or gene flow from neighboring countries. A broad spectrum of α-thalassemia alleles has been detected among Iranians and $\alpha^{3.7}$/αα was the most prevalent thalassemia mutation. The results of this study could be useful to upgrade the program for prevention of neonatal thalassemia in Iran.

CONFLICTS OF INTEREST

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