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Thalassemia Spectrum and Prenatal Diagnosis among Voluntary Couples in Shushtar City, during a Five Year Period

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Abstract

Background: Thalassemia and other hemoglobinopathies are inherited disorders that result from genetic defects causing deficient synthesis of hemoglobin polypeptide chains. This study was done to determine the prevalence and to study the complete spectrum of alpha and beta thalassemia gene mutations in the voluntary hemoglobinopathy carrier couples-to-be referred to health centers of Shoushtar city, located in the north Khuzestan province.

Methods: Subjects referred to health centers of the city of Shoushtar during 2011 - 2016 were evaluated for the presence of thalassemia minor. After that, alpha and beta thalassemia gene mutations were explored in suspected couples. The samples that underwent PND and diagnosing of causative mutational genes by chorionic villus sampling (CVS) through amniocentesis plus direct or indirect genetic testing at first trimester of pregnancy were proved to be a carrier for alpha thalassemia, beta thalassemia, or other hemoglobinapathies.

Results: The mutations for 111/165 (67%) of beta thalassemia chromosomes (19 different mutations) were identified. Of which, IVSII-1 (G > A) and CD 36 - 37 (-T) mutations showed the highest frequency (23.4 for each one) followed by IVSI-110 (G > A) (10%), CD 82-83 (-G) (9%), and -110 (C > T) (7.2%). Among the alpha thalassemia subjects (54/165 (33%)), the -α3.7 mutation was the most common (79/6%) determinant identified in the city of Shoushtar. From 48 voluntary carrier couples who underwent PND, 10 (20.8%) couples were proved to have fetuses with major beta thalassemia; pregnancy termination was done for them.

Conclusions: These data suggest that the spectrum of mutations in the city of Shoushtar differs from those reported from other parts of Iran. Therefore, in order to save the time and cost, it is recommended that for the prenatal diagnosis of thalassemia in the city of Shoushtar, analysis of common mutations should be considered as a front line screening strategy. Prenatal diagnosing in this survey demonstrated that beta thalassemia was the most common accounting for all disorders.

Keywords: Prenatal Diagnosis, Alpha Thalassemia, Beta Thalassemia, Hemoglobinopathy

1. Background

Thalassemia is a hereditary disorder of hemoglobin synthesis and the most common monogenic disease in the world. It commonly causes a reduction in the rate of synthesis of hemoglobin chains (1). Thalassemia was first described in 1925 by a pediatrician named Thomas B. Cooly, in Detroit, in the USA. Although the disease is present worldwide, and in all races, it is prevalent especially from the Mediterranean area to the Middle East, India, and Southeast Asia, and Iran (1). The number of carriers of the genes for this disease is estimated at about 150 million people worldwide, and in Iran, up to 4% of the total population is so afflicted (2).

There are about 25,000 people with thalassemia and more than 2 million carriers that live in Iran. Mazandaran province has the highest number, with 2,559 people, and Zanjan the lowest, with 58 people. For some reason, the prevalence of thalassemia is very high among some family groups; one of the reasons is marriage among relatives. About 25% of marriages in Iran are of this type (3, 4).

Beta thalassemia gene mutations in different regions of our country are different, and with 6 mutations in the beta thalassemia gene causing 80% of the beta thalassemia cases (5). Alpha thalassemia mutations are also different, and clinical phenotypes vary based on the number of mutated genes (6).

The alpha globin gene cluster in a human contains 7 genes; the 2 main genes are 1 alpha and 2 alpha. The mutation in one or both of the alpha globin genes on chromosome 16 is the main cause of the disease, leading to a lack or reduction of alpha globin chains. The prevalence of this

disorder in men and women is the same. Identifying the types of alpha globin gene mutations needed to prevent the onset of HbH is inevitable. For alpha thalassemia, more than 50 point mutations, and many large deletion have been known both in Iran and around the world. Most alpha thalassemia mutations are a type of deletion (7). A significant aspect of alpha thalassemia in the Mediterranean population (including Iran) is the heterogeneity of mutations, and in particular, non-deletion mutations (8).

Several studies in different parts of Iran such as Khuzestan, Mazandaran, and Babolsar revealed that the frequency of -α3.7 mutation is high in these regions (7, 9-12). The other mutations such as Poly A1, Poly A2, - α4.2 and etc... showed with variety of frequency in different populations of Iran. The mutation of αCD192, which was first detected in Iran, causes a change in the reading format and the creation of a premature termination codon (13). The most commonly observed point mutations in the alpha globin gene known in Iran are the frequency (from left to right): αCS Hb constand spring, αCD 19 (αCD 59 Hb Adana, α1c Hb Icaria, α126, α199, α103) (14).

The cause of beta thalassemia is mutation in the HBB gene, which is located on chromosome 11. Beta globin is one of the hemoglobin subunits. This chain is required for the synthesis of hemoglobin alpha. Reducing the beta globin chain leads to early destruction of red blood cells. So far, more than 535 different mutations have been detected in the beta globin gene (15, 16), of which about 40 mutations are responsible for the majority of global beta thalassemia patients detected by population surveyed (17). Some mutations in the HBB gene result in the complete absence of beta globin chains, which are called B0, and in some of the mutations, the beta globin synthesis chains are partially carried out in a small amount, which are called B + (18). About 3% of the world's population, which includes about 150 million people, is the carrier of beta thalassemia mutations (2). Some of mutations that are frequent in different parts of Iran (Qazvin, Khuzestan, Mazandaran, Zanjan) are IVSII-1 (G > A), IVSI-1 (G > A), IVSI-6 (T > C), IVSI- 110 (G > A), Fr 8/9 (+G), IVS1- 5 (G > C), CD 44 (-C) etc... that of which the IVSII-1 (G > A) is the most common mutation in Iran (19-22).

In Iran, each region has its own special mutations (with a number of frequent mutations and rare mutations) (23). Iran is one of regions with the highest prevalence of beta thalassemia in the world. The provinces around the Persian Gulf and the Caspian Sea with a genus frequency of more than 10% include a large area with thalassemia in Iran (11). Due to the psychological, social, and economic losses caused by this disease, it is prevented by identifying carriers and prenatal diagnosis of the disease in couples is a priority in thalassemic countries like Iran (19). Various studies in Iran have shown that the distribution and prevalence of globin gene mutations in different regions of Iran are not completely identical and generally have regional specificity (6, 12).

Since Iran is located within the thalassemia belt and the frequency of thalassemia carriers is high, recognition of the current state of the range of thalassemia mutations in the provinces of the country are helpful in policy-making for the healthcare system. In addition, it also causes the accuracy of the molecular diagnosis of the disease in each area and prevents misdiagnosis due to its similarity to beta thalassemia carriers. In this regard, the present study was conducted to evaluate and identify the type of alpha and beta thalassemia mutations in the city of Shoushtar.

2. Methods

Present study is a descriptive epidemiologic type. In order to investigate the prevalence of thalassemia and its various mutations in the city of Shoushtar (with Latitude: 32.0500 and Longitude: 48.8500), after obtaining permission and coordination with the Shoushtar health department and according to the guidelines of the ethic committee of Ahvaz Joundishapour University of Medical Sciences, all records of suspected cases of thalassemia in health center No. 4 (pre-marriage counseling center) during the years 2011 - 2016 which were referred to pre-marriage counseling centers for pre-marriage examinations were included. Information about age, sex, race, date of referral to the center, as well as cellular indexes, including HCB, HCb, Hb, WBC, HbA2, PLT, MCHC, MCH, and MCV were extracted from the records.

Firstly, all referrals were investigated for diagnosis of thalasemia disease type and rejection of the risk of iron deficiency by the complete blood cell count test (CBC, with cell counter device), hemoglobin electrophoresis (Hb A2 and Hb F), measurement of serum iron level, ferritin, and total iron binding capacity (TIBC). Referrals with iron deficiency anemia were excluded from the study. Finally, all selected patients have low MCV (< 80 Fl), low MCH (< 27 pg), and decreased or normal levels of hemoglobin.

In this study, the PCR was only performed in samples with low MCV and MCH values. This kind of sample selection obviously increased the frequency of the thalassemia carrier detection. Minor thalassemia was classified as MCV less than 80, MCH less than 27, and HBA2 electrophoresis above 3.5. Other indices of peripheral red blood cells have been detected. If the couples were abnormal for the above mentioned indices, for complementary tests and sequencing the alpha and beta genes were referred to the genetic lab. Iron deficiency anemia was excluded from this group

based on the ferritin levels within the reference range. The cases that did not have the necessary information were excluded from the study, and only the cases with complete information were reviewed.

In this study, 12,068 couples with different ethnicities that had referred to the pre-marriage counseling center of the city of Shoushtar were studied. A total of 10,190 couples were both healthy, 1,774 individuals where only 1 person in the relationship was healthy, 15 have been affected in both alpha and beta, and 20 and 47 have both been affected in Alpha and beta, respectively. Also, 1 couple was proved to be the carrier for sickle cell.

After genetic counseling, genomic DNA was extracted from peripheral leukocytes of the patients (83 couples) according to the routine salting out method. Then, multiplex- PCR reaction performed on samples according to the Tan method (24) in order to recognize prevalent deletion mutations in the alpha cluster gene. The PCR products were electrophoresed on a 1% agarose gel using 1X TBE buffer and lastly, the gel was colored with ethidium bromide color.

For the beta cluster gene, extracted DNA from each subject was amplified by PCR by using primers as described by Galehdari et al. (20), which are listed in Table 1. To control the accurate size and specificity, the PCR products were visualized using staining with ethidium bromide color after electrophoresis on 1.5% agarose gel. Next, the amplified PCR products were cycle sequenced with the described primers in Table 1 in 2 separate reactions for each amplified PCR product by the ABI PRISM big dye primer cycle sequencing ready reaction kit (Applied Biosystems) as described in the company manufacture. All equipment's that were used for this study were calibrated.

Table 1. The Sequences of Primers That Were Used in This Study are Listed Below

Primer Name	Primer Sequence	Applied in
BT-NF	5-AACTCCTAAGCCAGTGCCAGAAGA-3	PCR FORWARD
BT-NR	5-CACTGACCTCCCACATTCCCTTTT-3	PCR REVERSE
BT-seq-1F	5-AGGTACGGCTGTCATCAC-3	SEQUENCING FORWARD
BT-seq-501F	5-CATGGCAAGAAAGTGCTC-3	SEQUENCING FORWARD
BT-seq-683R	5-AGGTACGGCTGTCATCAC-3	SEQUENCING FORWARD
BT-seq-2F	5-ATCTCTTTCTTTCAGGGC-3	SEQUENCING FORWARD

After data collection, the frequency and type of mutation of alpha and beta genes in couples referring to software 16 were then calculated and descriptive statistics (frequency) were used to analyze the data.

Fetal sampling was done at weeks 10 - 11 of age of the gestation during 1st trimester of pregnancy. Chorionic villus sampling (CVS) was carried out under ultrasonography guidance. Authorized pregnancy termination was done in the 1st trimester, if direct or indirect genetic testing methods demonstrated serious disorders.

3. Results

In this study, 83 couples (165 individuals) have been analyzed; from these, 44 individuals were with Arabian, 85 with Bakhtiary, and 27 with Fars background. In total, we detected 25 different mutations. A total of 19 (76%) were confirmed to have beta thalassemia and 6 of them (24%) were alpha thalassemia mutation.

The most frequent mutation in alpha thalassemia with 79.6% was the -α3.7 mutation. In Table 2, all the detected mutations are listed. We detected 9% of Arab subjects, 12.7% of Bakhtiary subjects, and 4.2% of Fars subjects to have -α3.7. The PolyA 2 mutation was detected only in Bakhtiary subjects.

Results of beta thalassemia mutational gene analysis showed the frequency of CD 36-37(-T) and IVSII-1 (G > A) mutations to be 23.4% (Table 3). Our data showed that the frequency of CD 36 - 37 (-T) and IVSII-1 (G > A) mutations are first in the Bakhtiary background.

From 48 voluntary carrier couples who underwent PND, 10 (10.3%) carrier couples were proved to have fetuses with major beta thalassemia (Tables 4 and 5), and pregnancy termination was done for them. Prenatal diagnosing in the present study revealed that beta thalassemia was the most common disorder accounting for all.

4. Discussion

The present study was carried out to identify the prevalence and to survey the complete spectra of gene mutations that are responsible in alpha and beta thalassemia in our region. Shoushtar is situated in the north of Khuzestan province, and is one of the majorities of the population in Khuzestan province. The present study was performed as a prospective survey in a 5-year period (2011 - 2016) with 83 voluntary carrier couples from Shoushtar city, who were confirmed to be carriers for alpha thalassemia, beta thalassemia, or other hemoglobinapathies.

In the present study, we identified that the frequency of alpha thalassemia carrier with deletion type was 79.6%. Alpha thalassemia carrier with -α3.7 mutation was the most frequent and occurred in high frequency in Shoushtar area compared to other ethnic groups. The prevalence of other mutations of alpha thalassemia was low in the city

Table 2. Mutation Prevalence of the Alpha Globin Gene Cluster in Different Ethnic Groups

Mutations	Race			Total[a]
	Arab	Bakhtiary	Fars	
-α3.7	15	21	7	43 (79.6)
Poly A2	0	1	0	1 (1.8)
Med	0	1	1	2 (3.7)
cs	0	0	3	3 (5.5)
Poly A1	2	0	0	2 (3.7)
a.a a (anti-3.7 trilication)	0	3	0	3 (5.5)
Total	17	26	11	54

[a]Values are expressed as No.(%).

Table 3. Type and Distribution of Observed Mutations of Beta Globin Gene Cluster in Different Ethnic Groups of Shoushtar Area

Mutations	Race			Total[a]
	Arab	Bakhtiary	Fars	
CD 36-37 (-T)	0	23	3	26 (23.4)
IVSII-1 (G > A)	8	12	6	26 (23.4)
IVSI-110 (G > A)	5	4	2	11 (10)
IVSI-5 (G > C)	1	0	0	1 (0.9)
IVSI-6 (T > C)	3	0	0	3 (2.7)
CD 44 (-C)	0	2	2	4 (3.6)
CD 5 (-CT)	0	0	1	1 (0.9)
CD 82-83 (-G)	0	8	2	10 (9)
-88 (C > A)	0	1	2	3 (2.7)
CD 39 (C > T)	0	0	1	1 (0.9)
IVSI (-del25nt)	2	3	0	5 (4.5)
cd22-23-24 (-AAGTTGG)	0	0	1	1 (0.9)
-110 (C > T)	4	4	0	8 (7.2)
5UTR + 20 (C > T)	0	1	0	1 (0.9)
20UTR (C > T)	0	1	0	1 (0.9)
CD 30 (G > C)	0	1	0	1 (0.9)
IVSI-130 (G > C)	0	4	1	5 (4.5)
CD 8 (-AA)	0	1	1	2 (1.8)
IVS II-745 (C > G)	0	1	0	1 (0.9)
Total	23	66	22	111

[a]Values are expressed as No.(%).

of Shoushtar. In the surveyed of other researchers in other regions of the country, frequency of -α3.7 mutation is reported as 9.1%, 32%, 35.71%, 55.2%, and 42.1%, and between suspect people (9, 10, 25-27).

Prevalence of mutations in alpha cluster gene in this study is different of another study in Ahvaz city due to the different population (7). Detecting people with alpha thalassemia mutations is very crucial in order to obstacle giving birth to babies with defects such as hydrops fetalis and hemoglobin H disease plus social, financial, intellectual,

Table 4. The Type Mutation of Couples of Alpha Globin Gene Cluster That Have Been PND in Different Ethnic Groups of Shoushtar Area (3 Couples)

Mutations	Race			Total[a]
	Arab	Bakhtiary	Fars	
.a.3/7-	0	1	0	1 (25)
med	0	0	1	1 (25)
cs	0	0	1	1 (25)
a.a a (anti-3.7 trilication)	0	1	0	1 (25)
Total	0	2	2	4

[a]Values are expressed as No.(%).

Table 5. The Type Mutation of Couples of Beta Globin Gene Cluster That Have Been PND in Different Ethnic Groups of Shoushtar Area (45 Couples)

Mutations	Race			Total[a]
	Arab	Bakhtiary	Fars	
cd 36-37 (-T)	0	9	1	10 (20)
IVSII-1 (G > A)	6	6	2	14 (28)
IVSI-110 (G > A)	3	2	0	5 (10)
IVSI-6 (T > C)	1	0	0	1 (2)
cd44 (-C)	1	0	0	1 (2)
cd5 (-CT)	1	0	1	2 (4)
cd82-83 (-G)	1	2	0	3 (6)
-88 (C > A)	0	1	1	2 (2)
cd39 (C > T)	0	0	1	1 (2)
IVSI (-del25nt)	2	0	0	2 (4)
-110 (C > T)	1	1	0	2 (4)
5UTR + 20 (C > T)	0	2	0	2 (4)
IVSI-130 (G > C)	0	2	1	3 (6)
cd 8 (-AA)	0	0	1	1 (2)
IVS II-745 (C > G)	0	1	0	1 (2)
Total	16	26	8	50

[a]Values are expressed as No.(%).

and sanitary topics that these babies' families and society will be opposite. Additionally, those mothers who are having embryos with hydrops fetalis, are at 80% risk of some difficulties such as getting high blood pressure in the period of pregnancy, vaginal bleeding, preterm birth, and depression during pregnancy. Emotionally, carrying a dead embryo till the time of birth is very difficult for these mothers (28).

We could detect a complete spectrum of mutations in the beta globin gene from beta thalassemia diagnosed patients. From the molecular point of view, beta thalassemia is a disease with restricted mutations (4 to 10 mutations) in the populations (29). However, due to the composed and multi ethnic population in Iran, especially in southwest Iran, the distribution of mutations in Iran's ethnic groups is very different (30, 31). The detection of 19 mutations with a broad scale of frequency confirms the mentioned hypothesis. On the other hand, we must consider that just 2 of the 19 detected mutations in this work represented more than 50% of the total mutations and therefore, belong to the common nucleotide changes within the beta globin gene in Shoushtar area (Khuzestan province).

The heterogeneity of mutations may be explained with the closeness of Khuzestan province with many Arab coun-

tries in the Persian Gulf and the frequent traveling between the nations in this region.

For example, one of the most frequent mutations in this study, the IVSII-1 (G > A), is with 15.2%, 13.3%, and 26% in other reports from Iran (6, 20, 21). The mentioned mutation has originated from the Mediterranean and its frequency seems to increase from the east to west of Iran (29). This mutation is observed in all other Arab countries as well, however, in Algeria and Tunisia, it is the less common mutation (30). Recently, there are reports that the 6 mutations: IVSI-110 (G > A), IVSII-1 (G > A), IVSI-1 (G > A), IVSI-5 (G > C), CD 36 - 37 (-T), and IVSI (-del 25 nt) with 50% are the common mutations in southwest of Iran (31).

The other common mutation in the present study was the CD 36-37 (-T) with 23.4%, being the frequent mutation in the southwest of Iran, which was not reported in most Arabic countries, however, it possesses a high prevalence in Saudi Arabia (30). It seems also that Khuzestan province holds a wide spectrum of mutations in the HBB gene.

Around 15 patients have been affected in both alpha and beta cluster gene; these patients could not get married with each other.

Also, our study revealed that 20.8% of the 48 CVS samples carriers had affected fetuses. The results of mutational genes screening revealed that beta thalassemia major is the most common mutational gene. This is in line with the other study in the Khuzestan province (mostly in Ahvaz) (25).

We conclude that our data will be very important as serving a basis for further prenatal and postnatal molecular diagnosis, at least in the Shoushtar area.

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