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ORIGINAL ARTICLE / RESEARCH

Prenatal Diagnosis (PND) of β-Thalassemia in the Khuzestan Province, Iran

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ABSTRACT

ß -Thalassemia is present in practically every caste group in Iran. Khuzestan is located in the southern part of the geographical region of Iran. The ß-thalassemia incidence in the Khuzestan province is between 2.6-3.7%, as reported by different researchers. Reverse dot blot hybridization and Amplification Refractory mutation Analysis (ARMS), were used to scan the entire ß -globin gene to localize the mutation, followed by DNA sequencing for characterization. The DNA samples from 254 subjects (127 thalassemia patients and 127 choronic villi samples) had been referred to us at the Research Center of Thalassemia and Hemoglobinopathies of Ahwaz Jondishapur University of medical sciences, Iran. According to our study, CD 36/37 (-T) and IVS 2-1 (G to C) were the most frequent mutation types (14.7 %) in our province, the same as in other geographical regions of Iran. Also, the most predominant mutations which lead to termination of pregnancy of those couples in the first trimester of first pregnancy, CD 6 or HbS (22.5 %), IVS 2-1 (17.5%), CD 36/37 (15%), CD 44(12.5%), and IVSI-110 (12.5 %) mutations, were detected in 40 foetuses, and compound heterozygosity was detected in various combinations for IVSI-110, IVSII-745, IVSI-6, IVSI-1, IVSII-1, IVSI-5, IVSI-130, CD 8. HbS mutations were detected in 13 foetuses, who were later aborted, with the written permission of their families. In conclusion, our preliminary results show the heterogeneity of the ß -thalassemia mutations in the province of Khuzestan. Our data is valuable, in that it includes the mutation screening of patients for prenatal diagnosis in Khuzestan and nearby towns and villages, one of the regions with the highest frequency of ß -thalassemia mutations in Iran. In addition to discovering novel and rare mutations in the rich genetic pool of our region, this study was carried out in order to reduce the frequency of consanguineous marriages and haemoglobinopathies, to educate the population, and to inform the physicians in our region.

Key words: ß- thalassemia, reverse dot blot hybridization, amplification refractory analysis (ARMS), Iran, PCR

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Introduction

Iran, whose area is 1,648,000 km^2, has a large number of thalassemia major patients like many other countries in the region [1]. The gene frequency of β-thalassemia, however, is high, and varies considerably from area to area, having its highest rate of more than 10% around the Caspian Sea and the Persian Gulf. The prevalence of the disorder in other areas is between 4-8%. In Isfahan, a city built around the

river Zayandeh-Rood in the central part of Iran, the frequency rises again to about 8%. In the Fars Province, in southern Iran, the gene frequency is also high, and reaches 8-10% [1]. The region investigated was the province of Khuzestan, located in the southern part of Iran on the Mediterranean Sea, comprising coastal and mountainous areas, with a population of 4.3 million in 10 counties. More than 23 different molecular defects have been identified for β-thalassemia, till date. The case of β-thalassemia, with over 150 various known mutations, is even more perplexing [2]. Each population-at-risk, however, has its own spectrum of common mutations, usually from five to ten, a finding that simplifies mutation analysis and thus determines the origin of mutant genes.

Thalassemia is found in some 60 countries, with the highest prevalence in the Mediterranean region, parts of North and West Africa, the Middle East, the Indian subcontinent, southern Far East and southeastern Asia, together comprising the so-called thalassemia belt. In western countries, thalassemia affects mostly individuals whose ancestry is traceable to high-prevalence areas [3–6]. As an example, there are around 1,000 cases of β-thalassemia major in the United States, most of whom are descendants of Mediterranean, Asian Indian, South Asian or Chinese ancestors [3]. This figure is even less than half of the number of β-thalassemic patients in Fars Province, a region only 120,000 km ^{2} large in southern Iran [7]. As there is no ultimate cure for this hereditary disease, the World Health Organization (WHO) has suggested a control program which comprises of raising public awareness about the disease, screening for carriers, prenatal diagnosis and genetic counseling, which would result in the prevention of the birth of an affected child. Italy, Greece, Canada, the United Kingdom and Cyprus started the program in the 1970's, and achieved success [8–15].

Although historically, the prevention program, abortion, was considered unacceptable in Iran, intensive consultation led to the clerical approval of induced abortion in cases with β-thalassemia major in 1997, and a nationwide prevention program with screening, counseling and prenatal diagnosis (PND) network has been developed [16]. Due to the high consanguinity rate and high frequency of β-globin gene mutation carriers, a prenatal diagnosis program was needed, and we set up a laboratory to screen the globin gene mutations, at The Research Center of Thalassemia and Hemoglobinopathies of Ahwaz Jondishapur University of Medical Sciences, Iran. Two renowned Islamic clerics who were consulted before the service was introduced, ruled that a pregnancy can be terminated if the foetus is affected by serious genetic disorders, and if termination is before 90 days (12 weeks) of gestation. The prenatal diagnosis is performed by several molecular methods, by using samples like chorionic villi (CV), amniotic fluid, and cord blood.

Materials and Methods

We received a total of 381 DNA samples from patients (254 thalassemia traits) and prenatal cases (127 choronic villi samples), from various geographic areas and ethnic groups within the Khuzestan province, between 2002 -2006. Blood was collected into EDTA, and red cell indices were obtained from a Coulter AcT. In cases with a mean corpuscular volume (MCV) of ≤80 fl, HbA2 and HbF were determined by column chromatography and alkali denaturation, respectively. Haemoglobin electrophoresis was carried out on cellulose acetate and agar gel. Cases with MCV<80 fl, HbA2≥3.5%, and HbF≥2%, were considered to have the β-thalassemia trait. Those cases with normal HbA2 with MCV<80 fl, were sent for further evaluation to haematology clinics. If the couples were both carriers of an abnormal haemoglobin or β-thalassemia gene, they were informed confidentially about their test results, and were counseled about the reproductive options and about obtaining prenatal diagnosis.

As our first approach, the samples were screened for a panel of 22 relatively common β- globin mutations using an assay based on the polymerase chain reaction (PCR) and reverse-hybridization, by using oligonucleotide arrays immobilized on test strips [17]. They were asked to come to the center in the case of pregnancy, and the PND test was done for the fetuses, by choronic villus sampling. Foetal DNA is usually and preferably, obtained through choronic villus sampling in the first trimester of pregnancy (10-12 weeks) [16]. Chorionic villus sampling provides a good yield of DNA, which is isolated using the conventional method of phenol chloroform extraction, after careful microscopic dissection to remove any contaminating maternal deciduas. The CV sample was used for

screening β-globin gene mutations. The reverse dot blot hybridization (RDBH) kit (22 mutations for Beta-globin Strip Assay, VienLab), **Amplification Refractory mutation Analysis (ARMS),** and dideoxy termination chain of DNA sequencing, were used to determine the mutations of the β-globin gene [18]. When the mutation found in the foetus was the same as found in her/his mother, a variable number of tandem repeat (VNTR) markers (ApoB, MCT, IgJH, and D4S95) were used to avoid maternal contamination [19].

[Table/Fig 1] ß-Globin mutations identified by reverse- hybridization and DNA sequencing. 254 thalassemia patient and prenatal DNA sample were initially tested for 22 common mutations by reverse – hybridization, and if negative further analyzed by DNA sequencing of the entire ß-globin gene. Total 18 different mutations discovered by this two step approach are listed, including number of chromosomes, heterozygous and homozygous cases.

Mutation	Type	Ethnic origin	Heterozygous cases	Homozygote cases	No. of chromosomes (%frequency)
Fr 36/37(-T)	*β0*	Kurd, Iranian	16	6	28(14.7)
IVS 2-1(G to C)	*β0*	Iranian	14	7	28(14.7)
CD 6 (Hb S)	*βs*	----------	4	9	22(13)
IVS 1-110 (G to A)	*β+*	Mediterranean	5	5	15(8.8)
IVS 1 –6 (T→C)	*β+*	Mediterranean	0	5	10(5.89)
CD 44 (-C)	*β0*	Kurdish	4	2	8(4.7)
IVS 1-5 (G to C)	*β0*	Asian Indian, SE Asian, Melanesian	2	2	6(3.5)
CD 39(C to T)	*β0*	Mediterranean	4	0	4(2.35)
Fr 8/9 (+G)	*β0*	Asian Indian, Japanese	2	1	4(2.35)
IVS 2-745 (C to G)	*β+*	Mediterranean	4	0	4(2.35)
IVS 1-1(G to A)	*β0*	Mediterranean	2	1	4(2.35)
IVS 1(3' end)-25bp	*β0*	Asian Indian, UAE	2	1	4(2.35)
CD 8 (-AA)	*β0*	Mediterranean	1	1	3(1.8)
CD 5 (-CT)	*β0*	Mediterranean	2	0	2(1.17)
-88(C to A)	*β+*	Kurds	2	0	2(1.17)
CD 82/83(-G)	*β0*	Czech, Azerbaijan	1	0	1(0.59)
IVS 2-2,3(+11, -2)	*β+*	Iranian	1	0	1(0.59)
IVS 1-130 (G to A)	*β0*	Egyptian	1	0	1(0.59)
Unknown			23	0	23(14.1)
Total			90	40	170(100)

Results

In the study period, 127 subjects and 127 foetuses were screened for haemoglobinopathies in the province of Khuzestan. All subjects (254 samples) involving thalassemia patients and prenatal DNA samples, were initially tested for 22 common mutations by reverse–hybridization, and if negative, further analyzed by DNA sequencing of the entire β-globin gene. A total of 18 different mutations discovered by this two-step approach, are listed, including the number of chromosomes, and heterozygous and homozygous cases ([Table/Fig 1]). The results are listed in [Table/Fig 1] (170 affected chromosomes and 84 normal chromosomes). Our study showed that 77 patients out of 127, and 53 foetuses out of 127, were affected. CD 36/37 (-T) and IVS 2 (G to C) were the most frequently observed β-thalassemia mutations (14.7 %) for each type, followed by CD 6 or HbS (13 %) , IVSI-110 (8.8 %), IVS I -6(T to C) (5.89%), CD 44 (-C) (4.7%), IVSI-5(G to C) (3.5%), CD 39 (C to T) (2.35%), Fr 8/9 (+G) (2.35%), IVS II-745 (C to G) (2.35%), IVS I-1(G to A) (2.35%), IVS 1(3' end) -25bp (2.35%), and CD 8(-AA) (1.8%). Homozygosity for IVSI- 110, CD 36/37, IVSI-1, IVSI-6, CD 44, CD 8, CD 8/9, IVSI-5, and IVS 1(3' end)-25bp mutations ([Table/Fig 2]), were detected in 40 foetuses, and compound

heterozygosity was observed in various combinations for IVSI-110, IVSII-745, IVSI-6, IVSI-1, IVSII-1, IVSI-5, IVSI-130, CD 8. HbS mutations ([Table/Fig 3]) were detected in 13 foetuses, who were later aborted, with the written permission of the families. Twenty-three foetuses were found to be heterozygous for the above mentioned mutations, and the remaining foetuses were found to have the normal genotype for the β-globin gene.

[Table/Fig 2] 40 of 53 affected fetuses have compound homozygous mutations for beta globin gene (31 beta thalassemia and 9 HbS) including related genotypes

Mutation	No. of chromosomes (frequency %)	genotype
CD 6 (Hb S) /CD 6 (Hb S)	*18(22.5)*	*βS / βS*
IVS 2-1(G to C) / IVS 2-1(G to C)	*14(17.5)*	*β0 / β0*
Cd36/37(-T) / Cd36/37(-T)	*12(15)*	*β0 / β0*
IVS 1-110 (G to A) / IVS 1-110 (G to A)	*10(12.5)*	*β+ / β+*
IVS 1 –6 (T→C) / IVS 1 –6 (T→C)	*10(12.5)*	*β+ / β+*
CD 44 (-C) / CD 44 (-C)	*4(5)*	*β0 / β0*
IVS 1-5 (G to C) / IVS 1-5 (G to C)	*4(5)*	*β0 / β0*
Fr 8/9 (+G) / Fr 8/9 (+G)	*2s(2.5)*	*β0 / β0*
IVS 1-1(G to A) / IVS 1-1(G to A)	*2(2.5)*	*β0 / β0*
IVS 1(3' end)-25bp / IVS 1(3' end)-25bp	*2(2.5)*	*β0 / β0*
CD 8 (-AA) / CD 8 (-AA)	*2(2.5)*	*β0 / β0*
Total	*80(100)*	

[Table/Fig 3] The data showing here consists of 13 out of 53 affected fetuses that have compound heterozygous mutations for beta goblin. Also it is including related genotypes

Mutation	No. of chromosomes(frequency)	Genotype
IVS 2-1(G to C) / Fr 36/37(-T)	4(15.4)	*β0 / β0*
Fr 36/37(-T) / CD 39(C to T)	4(15.4)	*β0 / β0*
CD 44 (-C) / Fr 36/37(-T)	2(7.7)	*β0 / β0*
IVS 1-1(G to A) / IVS 1-110 (G to A)	2(7.7)	*β0 / β+*
-88(C to A) / IVS 2-2,3(+11, -2)	2(7.7)	*β+ / β+*
IVS 2-1(G to C) / CD 44 (-C)	2(7.7)	*β0 / β0*
IVS 2-1(G to C) / IVS 1(3' end)-25bp	2(7.7)	*β0 / β0*
IVS 2-1(G to C) / IVS 1-110 (G to A)	2(7.7)	*β0 / β+*
CD 8 (-AA) / CD 39(C to T)	2(7.7)	*β0 / β0*
IVS 2-1(G to C) / IVS 2-745 (C to G)	2(7.7)	*β0 / β+*
IVS 1-130 (G to A) / IVS 1(3' end)-25bp	2(7.7)	*β0 / β0*
Total	26(100)	

Discussion

Haemoglobinopathies are a major public health problem, causing both severe socio-economic and psychological debilitation in the population due to symptomatic care and hospitalization. Therefore, since 2002, we have been performing prenatal diagnosis for β-thalassemia, which is seen frequently in the Khuzestan Province of the country, in our molecular genetic laboratories. In recent years, there has been an increase in the number of β-thalassemia cases, as was presented in conferences and seminars by experts to the public. As a result, 127 CV samples for prenatal diagnosis were analyzed at 9-12 weeks. All of the CV samples could evaluate for beta-globin gene mutations. There was no miscarriage after CV sampling. All 40 affected foetuses were aborted according to the decision of the parents. The results of the molecular prenatal diagnoses were available within 4+1 days after sampling. To eliminate the risk of maternal contamination, VNTR analysis using ApoB, MCT, IgJH, and

D4S95 alleles was performed, when the genotype of the foetus was found to be identical to that of the mother [25]. There has been no misdiagnosis in our laboratory, till date. A 15-20 minute genetic counseling seems insufficient for those who are informed of these diseases just before phlebotomy, or when they receive the test results. These data show that the public is not well informed about these genetic diseases. Enlightenment of the public and the support of government, are the leading criteria for the preventive measures. The meaning of the carrier status should be made well known to the public, long before the age of marriage. As noted by Modell, Cao, Mitchell, and Loutradi-Anagnostou, co-operation of the government and non-government organizations, leaders in the community, parent organizations at schools, religious leaders, and local health personnel, are the prerequisites for the education of the public in this regard. With the help of these organizations, along with premarital screening programs, fewer affected births were observed in recent years in the United Kingdom, Italy, Canada, and Cyprus [20–24]. Scriver's group achieved almost a 90-95% success in informing high school students about such genetic diseases, utilizing similar organizations, voluntary screening, and genetic counseling over a 20-year span in Canada [23]. In the United Kingdom, the utilization of prenatal diagnosis, which was 20% from 1974-1994, had risen to 80% by 2000 [20],[21]. In this screening survey, 53 homozygous babies would possibly not have been born, if the couples had been well informed, had health insurance coverage, and had been given the opportunity for undergoing prenatal diagnosis in all pregnancies. Our results show similarities with those of other groups that study the prenatal diagnosis of β-thalassemia and sickle cell anemia [26–28]. In conclusion, our data is valuable, in that it includes the screening for mutations in patients who were selected for prenatal diagnosis in Khuzestan and nearby towns and villages, one of the regions with the highest frequency of beta-thalassemia mutations in Iran. Also, we aim to record all the carriers and patients for β-thalassemia, using a program via the Research Center of Thalassemia and Hemoglobinopathies of Ahwaz Jondishapur University of Medical Sciences, Iran, in addition to discovering novel and rare mutations in the rich genetic pool of our region, in order to reduce the frequency of consanguineous marriages and haemoglobinopathies, to educate the population, and inform the physicians in our region.

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