

## *Basic Science In Medicine*

### MOLECULAR STUDIES ON THE DISTRIBUTION OF $\beta$ - THALASSEMIA IN IRAN: THE BASIS FOR PRENATAL DIAGNOSIS

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#### ABSTRACT

By application of modern recombinant DNA technology, especially the polymerase chain reaction (PCR)/dot-blot hybridization techniques, we have investigated the molecular basis of  $\beta$ -thalassemia from four different regions of Iran: central, south-east, south and north. In this study, the DNA samples were isolated from patients and for the identification of the mutations, the 6 oligonucleotide probes for the mutations of IVS.1/nt. 110, IVS.1/nt.6, IVS.1/nt.1, nonsense codon 39, frameshift codon 8 and IVS.2/nt.1 were selected with respect to their relative frequency in the neighbouring country, Turkey. Four mutations accounted for 76.2% and of these, the most frequent was the nonsense codon 39 mutation, which accounts for 60.3% of the  $\beta$ -thalassemia alleles tested. The remainder, in decreasing order of frequency, were frameshift codon 8(9.5%), IVS.1/nt.6 (4.8%) and IVS.1/nt.110(1.6%). No hybridization was observed with the probes corresponding to the mutations of IVS.1/nt.1(G/A) and IVS.2/nt.1(G/A). These results also revealed that the distributions of different types of mutations were different in the four regions. This information and the introduction to the methodology used in this study will facilitate the prenatal diagnosis of the disease in Iran.

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**Keywords:**  $\beta$ -Thalassemia,  $\beta$ -Globin gene mutations, Polymerase chain reaction, Molecular hybridization, Regional distribution.

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## INTRODUCTION

Worldwide,  $\beta$ -thalassemia is one of the most common hereditary autosomal recessive disorders, primarily in regions of the world endemic for malaria, including the Mediterranean basin, West Africa, India, wide parts of Asia and south China.<sup>1-16</sup> This heterogenous genetic disease has mutations that is ethnically specific because of malaria-positive selective pressure.<sup>7</sup>

It has been estimated that 3% of the world population, or 150 million people, carry a  $\beta$ -thalassemia gene.<sup>2,5</sup> The disease is characterized by a deficiency or absence of  $\beta$ -globin production by different mechanisms, resulting in dependence on blood transfusion to sustain life. Indeed, most homozygous patients present with a most severe transfusion-dependent anemia within the first 2 years of life and later suffer from the long-term consequences of iron overload (thalassemia major).

Heterozygous subjects are usually asymptomatic. Their hematology is characterized by a slight to moderate anemia with a marked hypochromia and microcytosis (thalassemia minor).<sup>2</sup>

Even with chelation therapy to remove excess iron stores, the life expectancy in classic  $\beta$ -thalassemia major is shortened to 25 to 30 years on average.<sup>5</sup>

$\beta$ -Thalassemia defects in Mediterraneans (Italians, Greeks and Turks) living in the United States were among the first to be identified<sup>12,17,18</sup> and the results of these studies were later confirmed by others. The mutations described in the first studies have been found to be common in many Mediterranean countries. In 1978, the  $\beta$ -globin gene was one of the first human genes to be cloned in bacteria.<sup>19</sup> It was fortunate that the gene was unusually small and simple for a human gene: it is 1.6 kb in size occurring only once in the haploid human genome (single copy). It is arranged together with the other  $\beta$ -like globin genes ( $\epsilon$ ,  $G_\gamma$  and  $A_\gamma$  and  $\delta$ ) in a 60 kb gene complex on the short arm of chromosome 11 (11p15).<sup>5,7</sup> The coding information of all globin genes is contained on three exons which are divided by two introns of largely unknown function.<sup>20,21</sup>

Regarding the pathology of  $\beta$ -thalassemia, the expression of the  $\beta$ -globin gene can be inactivated at any step on its way from DNA to protein (transcription, RNA processing, translation) by more than 100 known mutations resulting in  $\beta$ -thalassemia. Although the degree to which these processes are affected frequently varies with the type of mutation, the identification of these mutations, the determination of the severity of each mutation, and the search for other factors that could modify the clinical presentation of the disease (haplotype,  $\alpha$ -thal., high HbF determinants) is one of the prerequisites of a comprehensive prenatal diagnosis program.<sup>12</sup>

Advances in recombinant DNA technology, especially the polymerase chain reaction (PCR)/dot blot hybridization

techniques, have led to the elucidation of the molecular etiology of many types of  $\beta$ -thalassemia.<sup>22-28</sup> To date, there are more than 100 known different  $\beta$ -thalassemia mutations.<sup>5,7,17,29-40</sup> However, in spite of this heterogeneity in the molecular genesis of this disease, a much more limited number accounts for the inactivation of most  $\beta$ -globin genes in many affected populations. In general, each population tends to have a different group of mutations consisting of a few that are very common and a variable number of rare ones,<sup>8,15,17-18,41-55</sup> although exceptions could be found and some mutations were present in several populations.<sup>43</sup>

Regarding the spectrum of mutations which differs considerably between different ethnic groups, many examples could be mentioned. For example, in Sardinia, the codon 39(C/T) nonsense mutation can be found in about 95% of the  $\beta$ -thalassemia genes.<sup>56</sup> In other populations, the genetics are not quite as homogeneous but 10 mutations or less account for more than 90% of all  $\beta$ -thalassemia genes in most ethnic groups. 10 mutations have been described in Asia Indians, of which five have been found to be the most common.<sup>8,25,31,41,43</sup> Also, five mutations appear to be prevalent in Mediterraneans: IVS-I-110(G $\rightarrow$ A), codon 39 (C $\rightarrow$ T), IVS-I-6(T $\rightarrow$ C), IVS-I-1(G $\rightarrow$ A) and IVS-II-745 (C $\rightarrow$ G).<sup>6,9,17,34,36,41,48,50,51,53,56-57</sup> In addition, the screening of large numbers of Mediterranean  $\beta$ -thal. patients during the last several years has resulted in the detection of many other mutations which occur at intermediate and low frequencies.<sup>5,58-62</sup> Since the spectrum of mutations differ in each population, it might be expected that different ameliorating factors would predominate in each group and in general, this appears to be the case. This relative simplicity of the population genetics facilitates the practical approach to genetic screening and to prenatal diagnosis programs.

Mutational data maps prepared on a country and, if possible, regional basis, could facilitate the diagnostic procedure by indicating the screening sequence according to the incidence of each mutation. Country scale frequencies of  $\beta$ -thalassemia mutations have been already determined for several, mainly Mediterranean, countries<sup>10,12,55,63-64</sup> and also for Turkey.<sup>57,65-67</sup> However, in Iran, molecular diagnosis as well as mutational data maps at a regional basis are presently still unavailable. The present study which has been initiated as a first attempt in this direction has allocated the underlying mutation types in 63  $\beta$ -thalassemia cases in four main regions of Iran, among which most of the study cases are obtained from Queshm Island, because this island is one of the biggest islands in the Persian Gulf, with a total population of 70,000 and a high rate of consanguineous marriages. Hence it is worth studying the presence of various kinds of mutations and molecular diagnosis of this genetic disorder among these people. In general,  $\beta$ -thalassemia is one of the major public health problems in Iran, where a total of 15000  $\beta$ -thalassemic patients are living in different regions of the country. Yearly influx of population

growth increases this figure by 1500 patients, which means the growth of  $\beta$ -thalassemia patients is one every 6 hrs. in Iran. The highest number of patients are living in Gilan, Mazandaran and Hormozgan province. In this study, for the first time, national screening for point mutations was initiated. In order to diagnose the type and frequency of  $\beta$ -thalassemia mutations in a large number of patients, considering the fact that these patients were chosen at random and some of them are related, this distribution is expected to reflect the unbiased frequency of  $\beta$ -thalassemia mutations among the Iranian population. For the time being we are doing an extensive, national large scale screening and the results will be published in the near future.

## MATERIALS AND METHODS

### Blood samples

The blood samples ( $n=319$ ) were collected from the thalassemic patients and their families for  $\beta$ -thalassemia combination cases. Some of the samples were related. The localities of the origin of these individuals were determined through careful inquiries.

### DNA isolation

DNA was isolated from each blood sample using the method described by Poncz et al.<sup>68</sup>

### PCR primers and mutation specific probes

All primers for the PCR amplification and the mutation specific oligonucleotide probes used in the hybridizations<sup>55,57</sup> were synthesized with an LKB/Pharmacia Gene Assembler Plus DNA synthesizer.

### Polymerase chain reaction (PCR)

PCR was carried out principally according to the method of Saiki et al.<sup>27</sup> Each amplification tube contained 50 mM KCl, 10 mM Tris-HCl (pH=8.3), 1.5 mM MgCl<sub>2</sub>, 2  $\mu$ M each primer, 0.2 mM each dNTP, 0.1  $\mu$ g of template DNA and 1.0 units of Taq Polymerase (Boehringer Mannheim) in a total volume of 50  $\mu$ l. The PCR cycles were done either manually or by means of a Coy thermocycler. The cycles comprised incubations of 1 min. at 95°C, 1 min. at 60°C and 3 min. at 72°C. In total, 25-30 cycles were performed.

### Dot blotting

Aliquots of the amplified product (5-8  $\mu$ l) were adjusted to 0.4 M-NaOH/25 mM EDTA in a volume of 100  $\mu$ l and applied onto nylon membranes (Nytran-13). DNA was fed to the membrane by UV light (254 nm) for 2 min.

### Mutation analysis by probe hybridizations and ARMS procedure

The oligonucleotide probes was labeled at the 5-end

with <sup>32</sup>P, using [<sup>32</sup>P] ATP (7000 Ci/mmol) (Dupont-NEN) and polynucleotide kinase. The membranes were prehybridized in a solution of 5 $\times$ SSPE/5 $\times$  Denhardt's/0.5% SDS for 30 min. at the hybridization temperature determined for each probe. Hybridization temperatures were determined according to the  $T_m$  values of each probe calculated by the relationship of  $T_m(^{\circ}\text{C})=4(G+C)+2(A+T)$ . Thereafter, the radioactively labeled probe was added to the fresh hybridization solution and hybridization was conducted for 1 hr. at a hybridization temperature. The membrane was washed twice with 2 $\times$ SSPE containing 0.1% SDS at room temperature, and once with 5 $\times$ SSPE containing 0.1% SDS at washing temperature for 10 min. Membranes dried at room temperature were exposed to Kodak X-Omat AR film for 1-12 hr. at -70°C.<sup>28</sup>

## RESULTS AND DISCUSSION

The development of new methodologies has facilitated the identification of  $\beta$ -thal. mutations, the most important being the polymerase chain reaction (PCR) procedure,<sup>22,27</sup> which has expedited the processes of screening for  $\beta$ -thal. defects<sup>69</sup> and sequencing of DNA.<sup>24,25</sup>

The results of the hybridizations of  $\beta$ -thalassemia chromosomes (i.e. the number of the dots hybridizing with at least one control probe from 319  $\beta$ -thalassemia patients, out of which 63 random samples were chosen) with 6 different oligonucleotide probes corresponding to the mutations of IVS. 1/nt. 110 (G/A), IVS.1/nt. 6(T/C), IVS.1/nt. 1 (G/A), nonsense codon 39, frameshift codon 8(-AA) and IVS.2/nt. 1(G/A), are presented in Table I. Four mutations accounted for 76.2%, and the most frequent of these was the nonsense codon 39 mutation, which accounts for 60.3% of the  $\beta$ -thalassemia chromosomes tested. The remainder in decreasing order of frequency were frameshift codon 8 (9.5%), IVS.1/nt.6 (4.8%) and IVS.1/nt. 110 (1.6%). No hybridization was observed with the probes corresponding to the mutations of IVS.1/nt. 1 (G/A) and IVS.2/nt. 1 (G/A).

TABLE I. Frequencies of 6  $\beta$ -thalassemic mutations in Iran

Mutation Type	No. of mutations	Frequency of mutations (%)
Nonsense Codon 39(C/T)	38	60.3
Frameshift Codon 8(-AA)	6	9.5
IVS.1/nt. 6(T/C)	3	4.8
IVS.1/nt. 110(G/A)	1	1.6
IVS.1/nt. 1(G/A)	-	0
IVS.2/nt. 1(G/A)	-	0

The distribution of the mutation types in heterozygotes, compound heterozygotes and homozygous cases are shown

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in Table II. The nonsense codon 39 mutation is present in all 5 compound heterozygous cases, in 75% of the homozygous cases and finally 90% of the heterozygous cases.

**TABLE II. The distribution of the mutation types in homozygous, compound heterozygous and heterozygous cases.**

Mutation Type	No. of samples
Fsc.8/Nonsense codon.39	3
IVS.1/nt.6/Nonsense codon.39	1
IVS.1/nt.110/Nonsense codon.39	1
IVS.1/nt.6 homozygote	1
Nonsense codon.39 homozygote	3
Nonsense codon.39 heterozygote	27

Table III shows the distribution of  $\beta$ -thalassemic mutations among the DNA samples from Iran.

**TABLE III. Distribution of  $\beta$ -thalassemic mutations among DNA samples from Iran.**

DNA Sample Number	Mutation Type	Molecular Diagnosis
3	Fsc.8/Codon.39	Double Heterozygote
1	IVS.1-nt.110/Codon.39	Double Heterozygote
1	IVS.1-nt.6/Codon.39	Double Heterozygote
3	Codon.39	Homozygote
1	IVS.1-nt.6	Homozygote
3	Fsc.8	Heterozygote
27	Codon.39	Heterozygote

In this study, the DNA samples were isolated from patients from four different regions of Iran, and for the identification of the mutations, the 6 oligonucleotide probes for the mutations of IVS.1/nt.110, IVS.1/nt.6, IVS.1/nt.1, nonsense codon 39, frameshift codon 8 and IVS.2/nt.1 were selected with respect to their relative frequency in the neighbouring country, Turkey.<sup>16</sup> The nonsense codon 39 (C/T) mutation which was the most frequent mutation in this study, is in agreement with previous studies in Sardinia,<sup>50,56</sup> Spain,<sup>53</sup> France<sup>48</sup> and Tunisia,<sup>52</sup> corresponding to a rather Western Mediterranean prototype.

Although these results appear to display some degree of heterogeneity, only a few mutations appear to be prevalent. Nearly 76% of  $\beta$ -thalassemia mutations have been identified with 4 probes in general, consistent with the findings of several other research groups,<sup>50,53</sup> and support the general observation that in most populations a few mutations (five or less) are prevalent and account for most of the  $\beta$ -thal. alleles. Nonsense codon 39 was found to be the most frequent mutation in Iran. On the other hand, unlike findings of other research groups<sup>16,57,65,67</sup> in which IVS.1/nt.110 (G/A) and

IVS.1/nt.6(T/C) were found as the most frequent mutations in Turkey and a few other countries, these two mutations accounted for only 1.6% and 4.8% respectively in Iran. However, the frequency of IVS.1/nt.6(T/C) is relatively near the frequency of this mutation in Greece,<sup>12</sup> Lebanon<sup>34</sup> and France.<sup>48</sup> Likewise, the frequency of IVS.1/nt.110 (G/A) in Iran is roughly in agreement with that reported in other investigations<sup>50</sup> in the Sardinian population, but shows great differences with Cyprus (Turkish)<sup>9</sup>, Lebanon,<sup>34</sup> Greece<sup>12</sup> and Turkey.<sup>69</sup>

Frameshift codon 8 (-AA) which accounts for 9.5% of our  $\beta$ -thal. alleles tested, is not among the few common  $\beta$ -thal. mutations in several Mediterranean countries.<sup>5,9,12,34,41,48,50,52,53,56,58,69</sup>

The results of the remaining two probes corresponding to the mutations of IVS.1/nt.1(G/A) and IVS.2/nt.1(G/A) which show no hybridizations in our tested samples, are consistent with the findings of other research groups in the Sardinian<sup>50</sup> population, whereas the former is also in agreement with results obtained from Lebanon<sup>34</sup> and the latter is consistent with reports from Sicily<sup>70</sup>, Tunisia<sup>52</sup> and Spain.<sup>53</sup> Meanwhile, they are present among  $\beta$ -thal. mutations in Greece and Italy,<sup>12,41,56</sup> Yugoslavia,<sup>63</sup> and Turkey<sup>58,69</sup> populations in different frequencies.

**TABLE IV. Regional distribution of  $\beta$ -thalassemic mutations in Iran**

Region	Nc39 ( $\theta$ )	Fsc8 ( $\Omega$ )	IVS.1 nt.6	IVS.1 nt.110	IVS.1 nt.1	IVS.2 nt.1
A	9	2	3	1	-	-
B	27	3	-	-	-	-
C	1	-	-	-	-	-
D	2	1	-	-	-	-
Total	39	6	3	1	0	0

A, Central part of Iran (Tehran, Ghom, Saveh)

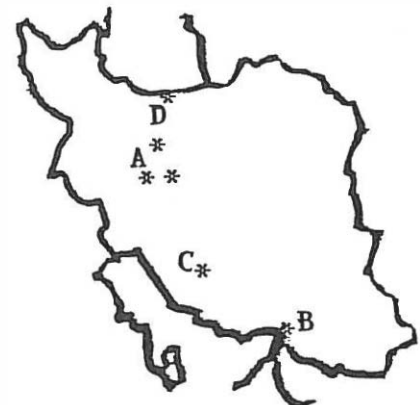
B, South-east (Queshm Island)

C, South (Shiraz)

D, North (Ramsar, Tonekabon)

( $\theta$ ) Nonsense codon 39

( $\Omega$ ) Frameshift codon 8(-AA)



Of the six  $\beta$ -thalassemic mutations detected, the nonsense codon 39 (C/T) mutation appeared to be the most frequent on a regional basis (Table IV). The obtained samples from four Iranian regions comprises the following cities: Tehran, Ghom, Saveh, Shiraz, Ramsar, Tonekabon and Queshm Island.

Prevention of  $\beta$ -thalassemia by genetic counselling and prenatal diagnosis is an important health issue. Techniques for prenatal diagnosis of monogenic disorders by DNA analysis have progressed such that today, following DNA amplification by PCR, prenatal diagnosis by direct detection of relevant mutations is possible in most cases in the first trimester of pregnancy. The technique of allele-specific priming of the PCR used for carrier screening in this study has also been successfully used for over 100 cases of prenatal diagnosis.<sup>71</sup> As it detects the mutations directly, this method overcomes the labour-intensive process of studying the DNA polymorphisms in family members.

The results of this first attempt have potential important implications and may suggest that in Iran, as in many other countries (such as Greece)<sup>12</sup>, it will be possible to perform prenatal diagnosis in most patients, especially in regions like Queshm Island by searching for a few mutations (although countries such as Tunisia<sup>52</sup> and Turkey<sup>16,69</sup> do not appear to follow this rule).

An extensive evaluation of the data with respect to possible region-dependent mutational differences will contribute to a better understanding of these mechanisms. Further studies including detailed family histories from patients and their relatives can possibly help to trace the precise geographical origins of the various alleles identified in this study. Also, further and more extensive large scale studies of regional distributions of  $\beta$ -thal. mutations, along with different types of this disease in Iran and their incidence, will undoubtedly contribute great insight to a more rational application for the planning of programs of  $\beta$ -thalassemia prevention.

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