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Spectrum of β-Thalassaemia Mutations in Bahrain♣

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Objectives : To study the molecular characterization of β-thalassemia defects among Bahrainis.

Methods : We used a variety of polymerase chain reaction (PCR)-based procedures including reverse dot blot (RDB), denaturing gradient gel electrophoresis (DGGE) and DNA sequencing, to study the β-thal mutation in 87 Bahraini individuals from 51 unrelated Bahraini families.

Results : Thirteen different β-thal mutations were identified. Four mutations (Intervening Sequence I (IVSI)-3' end (-25 base pairs (bp)) deletion; Codon (Cd) 39 (C→T) and IVSI-5 (G→C), account for 80% of all β-thal alleles.

Conclusion : We conclude that IVSI-3' end (-25bp) deletion is the major β-thalassemic allele in Bahrain.

Recommendations : Based upon our findings, a preventive approach of β-thalassemia needs to be embloyed for the Bahraini people. This study can be used in implementing a cost effective strategy for screening and diagnosis of Beta thal among Bahrainis.

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The hemoglobinopathies are the most common among genetic disorders in Bahrain, represented mainly by sickle cell disease (SCD) and thalassemias[1]. Among thalassemias both α and β forms are identified. The β-thalassemias are group of inherited anemias characterised by a reduced (β^+) or absent (β°) production of the β-globin chain from the affected allele[2]. This leads to an imbalanced α/non-α globin chain production and subsequent accumulation of the α-chains which is the major pathophysiologic route in the β-thalassemia syndromes. The vast majority of mutations causing β-thalassemia are non-deletional forms of which more than 180 point mutations have been characterized to date[3].

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The incidence of β-thalassemia trait in Bahrain is as low as 2%[2] but it is the most severe amongst other haemoglobinopathies. It is manifested, clinically, in homozygous state, with a life-long blood transfusion dependency phenotype[2]. On the other hand most of the β-thalassemia heterozygote states are clinically asymptomatic with a distinctive hematological phenotype represented by hypochromic, microcytic red blood cells and characteristically raised levels of HbA_2. Exceptions to the later include coinheritance of α-thalassemia which will render HbA_2 into a normal/borderline level. Moreover, the inheritance of β-thalassemia might be masked by coinheritance of sickle cell gene. Thus, molecular diagnosis took an important place as a useful tool to overcome these diagnostic obstacles.

The techniques of denaturing gradient gel electrophoresis (DGGE) and reverse dot blot (RDB) technique as well as direct DNA sequencing were applied in this first study to uncover the molecular basis of β-thalassemia in Bahrain. Moreover, β-haplotypes (patterns of arrangements of the restriction fragment length polymorphisms (RFLPs) in the β-globin gene cluster) were investigated for the uncovered mutations in order to identify possible origin(s) of these mutations.

The aim of this study is to identify the Beta thal mutations among Bahrainis.

METHODS

***Patients**: E*ighty seven native Bahraini individuals representing 51 unrelated families were studied. The patients were divided as follows: 33 clinically homozygote β-thalassemia, 17 S-β^{thal} and 37 simple heterozygotes. All of these individuals are attendees of the genetic and pediatrics departments at Salmaniya Medical Complex, Bahrain. The genetic study was done in the molecular laboratories in Robert Debre Hospital in Paris, France.

Some of the individuals studied were having β-thal major. They were blood transfusion dependent. Others were heterozygous (carrier) for β-thalwith elevated HbA_2, microcytosis and hypochromia.

***Blood Analysis**:* The whole blood samples were collected in EDTA-anticoagulated vacutainers and analysed in automatic cell counter. Hb electrophoresis and HPLC analysis were performed according to established methodologies[2,4].

***DNA Extraction**:* Genomic DNA was isolated from leucocytes by the phenol-chloroform extraction method as described by Dracopli et al[5].

PCR-DGGE and Sequencing: The specific amplification of the different β-globin gene fragments with subsequent DGGE analysis were performed according to previously published procedures[6]. Sequencing protocol was performed according to the dideoxy termination method[7,8] utilizing the Sequenase Version 2.0 DNA sequencing kit (US Biochemical, Cleveland, USA). The same PCR products and primers of DGGE were used for sequencing.

***Reverse Dot Blot**:* The reverse dot blot technique (Fig 1) was used for further searching of explored mutations as described in previous studies[9,10], except for utilising hybridization and washing at temperature of 42°C instead of 45°C.

Figure 1.Representative strips for the reverse dot blot Technique. Strip No.1, homozygote case for Cd 39 (C→T) Mutation. Strip No.2, heterozygote case for Cd 41/42 (-CCCT)

***Haplotype Analysis**:* It is defined by seven polymorphic sites in the β-globin gene cluster, was performed using a PCR-RFLP procedure as described[11-13]. The polymorphic restriction sites studied were *Xmn*I-5'Gγ, *Hin*dIII-Gγ, *Hin*dIII-Aγ, *Hin*cII-ψ β, *Hin*cII-3'ψ β, *Ava*II- β, and *Hin*fI-3' β (Fig 2).

Figure 2. Haplotype analysis of the major β-thal mutations in Bahrain. β-globin gene cluster is indicated with the restriction enzymes used as follows : Hf I, Hiunf I ; Av II, Ava II ; Hc II, Hinc II ; Hd III, Hind III and Xm I, Xmn I. The Positive sign indicate presence of the recognition site for the enzyme and hence cutting, while negative sign indicate absence of the recognition site and thus on cleavage.

RESULTS

A total of 70 β^{thal} chromosomes were characterized in this study. The frequency of each mutation is presented in Table I along with previously published frequency data from three neighboring countries. The IVSI-3' end (-25 bp) deletion allele represents the first major mutation in Bahrain with a frequency of 36%, followed by Cd 39 (C→T), a nonsense Mediterranean type mutation, that accounts for 26% of the mutations. The third major mutation is IVSI-5 (G→C) which was found at a frequency of 16%. Thus, four β^{thal} alleles comprised ~80% of all charaterized β-thal mutations. The remaining 20% of the β-thal alleles were distributed among 10 different less frequent or rare mutations.

Table 1. **Spectrum of β-thalassemia mutations in Bahrain and three neighbouring countries.**

	% (No. of Chromosomes)					
Mutation	*Bahrain*	*Kuwait[14] (*	*UAE[15] (*	*Saudi Arabia[16]*	*Western Saudi Arabia[17]*	*Original Description*
IVSI,3' end (-25 bp)	36 (25)	7.3 (7)	9.5 (24)	12.9 (24)	7.3 (3)	Asian Indian
Cd 39 (C→T)	26 (18)	7.3 (7)	4 (10)	12.9 (24)	-	Mediterranean

IVSI,5 (G→C)	16 (11)	18.7 (18)	54.5 (138)	12.9 (24)	22.5 (9)	Asian Indian
IVSII,1 (G→A)	6 (4)	29 (28)	3.5 (9)	12.9 (24)	25 (10)	Mediterranean
Cd 44 (-C)	4 (3)	1 (1)	1.6 (4)	-	-	Kurds
IVSI,1 (G→A)	3 (2)	7.3 (7)	-	-	-	Mediterranean
nt –101 (C→T)	1 (1)	-	0.4 (1)	-	-	Turkish
nt –88 (C→A)	1 (1)	-	-	-	-	Kurds
Cd 8/9 (+G)	1 (1)	3.1 (3)	5.9 (15)	-	-	Asian Indian
Cd 15 (G→A)	1 (1)	-	0.8 (2)	-	-	Asian Indian
IVSI,110 (G→A)	1 (1)	-	2 (5)	26.9 (50)	(17.5 7)	Mediterranean
Cd 35 (-C)	1 (1)	-	-	-	-	Malay
Cd 41/42 (-CTTT)	1 (1)	-	-	-	-	Chinese
Other mutations	-	26 (25)	17.5 (44)	20.4 (35)	21.5 (17)	
Total chromosomes	70	96	253	186	40	

Haplotype analysis of the major and most common β-thalassemia mutations in Bahrain revealed each of them to be in linkage disequilibrium with specific haplotype(s) (Fig 2). However, each single mutation has a common framework background (defined here by the presence or absence of the restriction sites for *Ava* II and *Hinf* I).

In addition to the above findings the following technical features are noteworthy:

1. DGGE profile of homozygous state for codon 44 (-C) was behaving like normal pattern without any alteration in the melting profile. Consequently, this mutation was not possible to be discovered by ordinary DGGE analysis (Fig.3a). However by premixing PCR product of the patient with a homologous normal PCR product succeeded by instant denaturation and annealing steps prior to DGGE analysis, it was possible to characterize the abnormality in DGGE fragment under study through the induction of heteroduplex formation (Fig.3b).

Figure 3. *Heteroduplex analysis in DGGE. (a) Identical melting profiles for both normal* β^{A} *allele and homozygote mutant of Cd 44 (-C)* ($\beta^{Cd\ 44}$). *(b) Induction of heteroduplex formation by mixing normal and mutant alleles prior to DGGE.*

2. Another observation is the very distinct DGGE profile of the 25 bp deletion allele of heterozygote individuals which failed to give any of the expected heteroduplexes and displayed merely as two homoduplexes (Fig 4). This is due to the internal deletion in the mutant allele which prevents formation of encompassing the deleted region with subsequent agarose gel electrophoresis (Fig.5a &5b).

Figure 4. DGGE analysis showing failure of heterozygote cases of the 25 bp Deletion ti give heteroduplexes. Samples 1,2,3 and 6 are normal controls for Framework (FW) : ½, 1/3, 1/3a and 3a/3a, respectively. Samples 4,5,and 7 are Del/del (homozygote sample for deletion), del/3a and del/1, respectively.

Figure 5a. Sequencing gel of the 25 bp deletion allele. 'A' Stands for adenin, 'G', guanin ; 'C', cytosin and 'T', thymin.

Figure 5b. A simple diagnostic strategy of the 25 bp deletion. Sample 1 and 5 normal alleles ; 2, 3 and 6 heterozygotes and sample 4 is homozygote for the 25 bp deletion allele. M is molecular weight marker No. VIII (Boehringer Mannheim, Germany).

DISCUSSION

Thirteen different β^{thal} alleles have been discovered in the Bahraini population throughout this study. However, three mutations, IVSI-3' end (-25 bp) deletion; Cd 39 (C→ T) and IVSI-5 (G→ C), comprised ~80% of all β^{thal} alleles. Further three mutations, IVSII,1 (G→ A); Cd 44 (-C) and IVSI,1 (G→ A), found to be less frequent with a total frequency of 13 %, though the remaining seven mutations are rare.

Our results revealed quite similarities in type of mutations with published spectrums of β^{thal} mutations from other neighbouring countries, and in particular for the first four mutations

found in Bahrain, albeit their different frequencies (Table I). This may reflect a genetic admixture among people of these countries.

However, the IVSI-3' end (-25 bp) deletion seems to be the outstanding feature of this region with the frequency in Bahrain being the highest ever reported. This mutation was originally described in an Asian Indian individual[18]. However, it was reported as a rare β^{thal} defect in the Indian subcontinent at a frequency of 0.4%[8]. Adding all these observations together, this study proposes a unicentric origin of this mutation somewhere around the Arabian Gulf including the island of Bahrain.

Moreover, haplotype analysis indicate that IVSI-3' end (-25 bp) deletion is in linkage disequilibrium with two different haplotypes, both haplotype I and IX[19]. However both of these haplotypes have a common framework background (Fig 2). This finding along with the observed higher frequency of the mutant allele that is in linkage disequilibrium with haplotype IX more than haplotype I may suggest emergence of this mutation being exclusively on haplotype IX with subsequent dispersion of the deletion toward haplotype I by recombination event in the presumed hot spot 5' of the β-globin gene[20].

Haplotype analysis of Cd 39 (C→T), a typical Mediterranean mutation, may exclude the possibilitiy of introduction this mutation into Bahrain via Mediterranean people. The majority of Cd 39 (C→T) alleles in the Mediterranean basin are in linkage disequilibrium with haplotypes I and II[21]. However, this study found it to be in linkage disequilibrium with haplotype VII, that lied on a different framework background[19]. This observation has been previously described in the Kurdish people[22]. Nevertheless, the mentioned study showed all of the major haplotypes bearing Cd 39 (C→T) mutation (haplotypes I and II as well as haplotype VII) are sharing a common identical $(ATTT)_n$ tandem repeats and $(At)_x$ $(T)_y$ motif in the 5' highly polymorphic region of the β -globin gene[22]. Thus, they concluded the unlikliness of recurrent mutational event of this mutation on haplotype VII versus haplotype I and II.

Haplotype analysis of the third major mutation in Bahrain, IVSI-5 (G→C), revealed this mutation to be in linkage disequilibrium with haplotype I. This is identical to a previously reported haplotype analysis from the United Arab Emirates[23] but in contrast to haplotype VII found in Asian Indian by Varawalla, et al[24]. The possibility of introducing this mutation via the Indian subcontinent cannot be excluded, however this conclusion is based upon realising that Varawalla's study did not include all of the Indian ethnic groups such as the Baluchi ethnic group, for example. In this group (Pakistan) this mutation has been described previously with very high frequency of 76.2%[8]. The Baluchis are living in the vicinity of the Arabian Gulf region. Hence the possible introduction of this mutation via Baluchistan. Haplotype analysis is needed to assess this possibility. Alternatively, the mutation might be reoriginated in the Arabian Gulf region as was proposed previously[23] and introduced subsequently into the neighboring people by gene flow through population migration and genetic drift.

Regarding technical aspects of this study, the DGGE analysis despite being a powerful and potent technique to detect any nucleotide variation on the DNA level should be utilized with a caution. In particular with the suspected homozygote states. As it was noticed in the case of Cd 44 (-C) (Fig 3a & 3b), the diagnosis may not be reached without the deliberately induced heteroduplex formation prior to DGGE (Fig 6).

Figure 6. A suggested molecular diagnostic approach for B-thalassemia in Bahrain

CONCLUSION

We conclude that IVSI-3' end (-25bp) deletion is the major β-thalassemic allele in Bahrain.

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