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β-Thalassemia Mutations among Thalassemia Major Patients in Basrah Province - Iraq

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ABSTRACT

Keywords

Mutation, βthalassemia, ARMS-PCR, Basra, Iraq.

Article Info

Accepted: 18 April 2016 Available Online: 10 May 2016 β- thalassemia is one of the important health problems in Basrah, Southern Iraq were the frequency of carriers for β - thalassemia gene reaches 4.6%. The study aimed to define the types of mutation among patients with B- thalassemia major (B-TM) registered at the Center for Hereditary Blood Diseases (CHBD) and geographical distribution of these mutations in different districts of Basrah. Blood samples were collected from 100 known patients with β-TM (53 males and 47 females), their ages ranged from 1-31 year. The amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique was used for molecular diagnosis of seven types of β -thalassemia mutations codon15 (G-A), IVS1nt -5(G-C), codon 8/9(+G), codon 30(G-C), -88(C-T), codon41/42(-TCTT) and codon 8(-AA). The most frequent β - thalassemia mutations are codon-15(G-A) and IVS 1nt-5(G-C) in 31 (37.3%) and 18(21.7%) of patients respectively, followed by codon 8/9 in 14(16.9%) and codon 30 in 8 (9.6%). In addition -88 (C-T) and Codon 41/42(-TCTT) were reported in 10(12.1%) and 2(2.4%) of patients respectively. Codon 8(-AA) was not reported in this study. The frequencies of these mutant genes differ from that of other parts of Iraq and neighboring countries. The study confirms a heterogeneity of diagnosed mutations within different districts of Basrah which have been studied for the first time in this province and signifies the need for further future studies to detect mutations that were not identified in this study.

Introduction

β-Thalassemia is a hereditary blood disorder caused by mutations that affect the synthesis of the β-globin chain, the protein component of the hemoglobin (Hb), it is an important public health problem in many parts of the world (Vichinsky *et al.*, 2005).

In Basrah, Southern of Iraq, 4.6% of population are carriers for β -thalassemia gene, with a gene frequency of 0.023(Hassan *et al.*, 2003).

Thalassemia is characterized by microcytosis and hemolytic anemia, and results mainly from either a decrease (β +) or absence (β °) of the expression of β -goblin gene (Weatherall and Clegg, 2001).

 β -Thalassemia major(β -TM) is the most severe and important type causing severe transfusion-dependent anemia with reduced life expectancy if untreated properly(Mok *et* *al.*, 2011). More than 95% of the genetic disorder responsible for β -globin genes are base substitution mutations, while a minority of the mutations corresponds to gene deletion (Weatherall, 2005).

The severity of the clinical syndrome of β -Thalassemia depends on the type of mutation in the β -globin gene. More than 400 different mutations have been reported and identified in the β -globin gene which are responsible for the development of the β -Thalassemia (Pan, 2010). In addition to the direct effects of altered or reduced β-globin synthesis, many of the clinical features of this disorder appear to be consequence of the resulting cytotoxic buildup of free α globin chains (Vok et al., 2007; Khandros et al., 2012). The relative excess of unbound α globin chains that precipitate in erythroblast precursors in the bone marrow, will lead to their premature hemolysis and hence to ineffective erythropoiesis (Weatherall and Clegg, 2001). Blood transfusion is the mainstay in treating patients with β -TM. However, humans have a very limited ability to excrete iron, so regular blood transfusions inevitably lead to iron overload (Luangasanatip et al., 2011), which is the most relevant problem associated with transfusion therapy(Toumba et al., 2007).

In Iraq, several studies were conducted in order to identify the types of mutations that cause thalassemia depending on the geographical distribution of these mutations in different parts of the country (Al-Allawiet al., 2006; Jalal et al., 2010; Omer et al., 2010; Katta et al., 2013; Al-Fartosi and Aziz 2014). However, these studies didn't focus on mutations causing thalassemia in Basrah. Therefore, the current study was done to determine the molecular basis of the most common types of mutations associated with β -TM in Basrah province and also to map Basrah according to these mutations.

Materials and Methods

The present study was conducted during the period from October 2014 through June 2015. One hundred patients with β -TM (53 males and 47 females) registered at the Center for Hereditary Blood Diseases (CHBD) at Basrah Maternity and Children Hospital were recruited, their ages ranged from 1- 31 year. All of them were attending the CHBD for blood transfusion and/ follow up.

Three ml of venous blood were collected from each patient in K3-EDTA tubes before blood transfusion.

A written consent was obtained from patients and/ one parents before enrollment in the study. The work was also approved by the College of Science, University of Basrah, and Basrah Health Directorate.

Genomic DNA isolation from the whole blood was done by using the automated nucleic acid purification instrument "ExiPrepTM16 Plus Blood Genomic DNA (Bioneer, Korea). Agarose kits gel electrophoresis was used, after genomic DNA extraction to confirm the completion and presence of the extracted genomic DNA.

Primers Selection

A-Primer sets that were used for amplification refractory mutation system (ARMS) diagnosis of mutations are shown in Table (1), (Talmaci *et al.*, 2004; Baig *et al.*, 2005; Basak 2007; Mirasena *et al.*, 2008; Sarookhani *et al.*, 2009; saleh-Gohari *et al.*, 2010).

B- Oligonucleotides of primers which were selected for ARMS technique are:

The internal control primers that used for all ARMS-PCR reactions:

Primer (Forward):5-CAA TGT ATC ATG CCT CTT TGC ACC -3-

Primer (Reverse):5-GAG TCA AGG CTG AGA GAT GCA GGA- 3-

Common primer C: 5-ACC TCA CCC TGT GGA GCC AC3'

Common primer D: 5-CCC CTT CCT ATG ACA TGA ACT TAA3-

ARMS-PCR Programmes

A total of 20 µl final PCR reaction volume was used which composed of 5µl of master mix (Bioneer, Korea), 0.5 µgm of DNA template, 10 pmol of each primers (2 internal control, 1 common, and 1 mutant or normal ARMS primers for the reaction). The thermal cycling for IVS1-5, codon 30, codon 8/9, and Cd41/42 consist of 30 cycles; denaturation at 94oC for 1 minute, primers annealing at 65oC for 1 minute and extension at 72oC for 90 seconds with final extension at 72oC for 3 minutes. While for codon 8, codon 15 and -88 mutations, the same program was used except the annealing temperature was 61oC. Then PCR products was analyzed on 2% agarose gel electrophoresis.

Results and Discussion

In the current study, ARMS-PCR technique was used for molecular screening and detection of seven types of β -thalassemia mutations using a specific set of primers for each mutation and a set of internal control primers.

ARMS-PCR Screening

The internal control PCR-product of 861 bps molecular weight was observed in all

successful ARMS-PCR reactions. the ARMS-PCR products for normal, homozygous and / heterozygous cases for any type of the studied mutations are observed. For diagnosed patients, the amplification refractory mutation systempolymerase chain reaction (ARMS-PCR) products was found only in mutant primer reactions in homozygous cases and within both normal and mutant reactions in heterozygous cases while in normal cases the ARMS-PCR products was found only within the normal primer reactions.

The pattern of β -thalassemia mutations in this study is shown in Figure 1.

Genotypes of Patients according to the Identified Variants

Table (2) shows that 15(18.1%) of the patients are homozygous genotype and 68(81.9%) are heterozygous genotype for both single and double mutation. Homozygous IVS1nt-5 for mutation represent 3 (3.61%) of cases, codon-15 mutation 3 (3.61%), - 88 mutation 2(2.4%) and codon-30 mutation also was detected in 2(2.4%) of the patients, while 5(6.1%) of patients were homozygouse for codon-8/9 mutation.

Heterozygosity for codon-15 was present in 28(33.7%) of patients, and for IVS1nt-5 and codon 8/9 mutations in 15(18%) and 9(10.8%) of patients respectively. In addition, heterozygosity for - 88 mutation and codon-30 mutation were detected in 8(9.6%) and 6(7.2%) respectively. Codon 41/42 was found in only 2 (2.4\%) of the patients.

The geographical distribution of diagnosed mutations among β -TM patients revealed that the highest frequency of Codon 15 mutation was in Southern and Northern areas of Basrah, comprising around 77.8%

and 42.9% respectively. Whereas, frequency of IVS-1nt-5 mutation was higher in of Basrah (33.3%) Western districts compared with other areas of Basrah. The highest frequency of codon 8/9 mutation (25%) was reported in the Eastern parts of Basrah. While codon 30 mutation and codon (-88) mutation were higher in southern of Basrah with frequency of (11.1%) and (22.2%) respectively. In addition, the results showed that the codon 41/42 mutation was only observed in the center of Basrah (3.6%). However, codon 8 was not found in all the thalassemic patients investigated in our study

Basrah is the ssecond largest and most populous Iraqi city after Baghdad, located on the extreme south of Iraq between Kuwait and Iran. Despite the relatively high carrier rate of β - thalassemia gene, still no any preventive program has been implemented yet.

Thalassemia is common in Arab countries and many neighboring countries, with variable frequencies. The frequency of β thalassemia in different areas of Iraq range from 3.7%- 4.6% ((Hassan *et al.*, 2003; Al-Allawi and Al-Dousky, 2010). Consanguineous marriages are common in most communities of the Middle East. In Iraq, consanguineous marriages range from 40 – 49%, and the percentage of first cousin marriages is around 28 % (Alwan and model, 1997).

The genotype of patients according to the identified variants has revealed that more than two thirds have a single mutation, i.e., have one type of mutation for all studied locus of β -globin gene, whereas (7%) of cases (alleles) have compound or double mutations (combined heterozygote conditions); these compound mutations are located in different alleles, this combination between different mutations is due to that

the ethnic background of Basrah population is different from that of other provinces of Iraq, which have resulted in a heterogeneous genetic admixture, this compound mutations have not been mentioned in other parts in Iraq. The remaining patients (24 %) were not identified, indicating the presence of other mutations that have not been studied in the current study.

The presence of single mutation as a homozygous condition (two alleles) or two different (compound) mutations as a heterozygous condition is sufficient for the induction of β -TM; the severity and signs of the disease based on the clinical picture and phenotypic characteristics of the causative mutation result from homozygosity of β + and β o-Thalassemia which may be improved by coinheritance of mutations in the gene encoding the α -globin chain associated with α -Thalassemia. On the other hand, in the double heterozygous or homozygous state, β-Thalassemia can be lethal, especially in areas where health services are poor or lacking (Cao and Galanello, 2010).

The type of β -globin mutation(homozygous versus compound heterozygous) can be used as a prognostic biomarker of thalassemia-free survival after hematopoietic stem cell transplantation in β -TM patients, with improved thalassemia-free survival among patients with homozygous mutations compared to those with heterozygous mutations (Hamidieh *et al.*, 2014).

The distribution of β -Thalassemia alleles varies within each district and among different districts of Basrah. This is in agreement with what was reported that β -Thalassemia mutations were very heterogeneous, and there are no specific distribution patterns that would aid in the identification of any ethnic background (Weatherall, 2000; Lahiry, 2008).

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Mutation	Oligonucleotides Sequence(5'-3')	Common	Product
		primer	size
IVS- I-5(G –C)	5'-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG-3'	С	285bp
Normal	5-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC-3'	С	285bp
Codon 8 \ 9(+G)	5'-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC-3'	С	225bp
Normal	5'-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT-3'	С	225bp
- 88 (C-T)	5-TCA CTT AGA CCT CAC CCT GTG GAG CCT CAT-3'	D	684bp
Normal	5'-TCA CTT AGA CCT CAC CCT GTG GAG CCT CAC-3'	D	684bp
Codon 8 (-AA)	5'- ACA CCA TGG TGC ACC TGA CTC CTG AGC ACG-3'	D	520bp
Normal	5'-ACA CCA TGG TGC ACC TGA CTC CTG AGC AGA-3'	D	520bp
Codon 15 (G-A)	5'-TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA-3'	D	500bp
Normal	5'-TGA GGA GAA GTC TGC CGT TAC TGC CCA GTG-3'	D	500bp
Codon 41/42(- TCTT)	5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3'	С	439bp
Normal	5'-GAGTGG ACA GAT CCC CAA AGG ACT CAA AGA-3'	С	439bp
Codon 30 (G-C)	5'-TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG-3'	С	280bp
Normal	5'-TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC-3'	С	280bp

$\begin{array}{c} \textbf{Table.1} \ \text{Nucleotide Sequences of the Primers used for } \beta \text{- globin Gene Mutations,} \\ \text{Normal and Mutant Primer} \end{array}$

Table.2 Genotype of Patients according to the Frequency of β -Thalassemia Mutation

β-thalassemia mutation	Туре	Homozygous N. (%)	Heterozygous N. (%)	Total N.(%)
Codon-15(G-A)	β°	3(3.61)	28(33.7)	31(37.3)
IVS1-nt-5(G-C)	β°	3(3.61)	15(18)	18(21.7)
Codon 8/9(+G)	β°	5(6.1)	9(10.8)	14(16.9)
Codon 30(G-C)	β°	2(2.4)	6(7.2)	8(9.6)
- 88(C-T)	β°	2(2.4)	8(9.6)	10(12.1)
Codon 41/42	β°	0	2(2.4)	2(2.4)
Total		15 (18.1)	68 (81.9)	83(100)



Fig.1 ARMS-PCR Products on 2% Agarose Gel Electrophoresis at 60 Voltages for One Hr.

A- Codon 15 mutation (500 bp.), B- IVS-1-5(G-C) mutation (285 bp.)

C- Codon 8/9 (+ G) mutation (225 bp.), D- Codon 30(G-C) mutation (280 bp.)

E- -88 (C-T) mutation (684 bp.) and F- Codon 41/42(- TCTT) mutation (439 bp.)

Marker: ladder marker (100 bp) DNA. N: Normal; M: Mutant.

Fig.2 Basrah Governorate Map and Geographical Distribution of the Diagnosed Patients according to the Type of Mutation.



The frequency of codon 15(G-A) mutation in thalassemic patient's in Basrah was 37.3%. This result is in agreement with Saud et al, who reported that IVS-I-5 and codon-15 were more prevalent in the middle and south of Iraq (Saud et al., 2013). On the other hand, Saud et al, didn't report codon 41/42 and codon 30 mutations among thalassemic patients in their study (Weatherall, 2000), while in this study codon-30 and codon 41/42 were present in 9.6% and 2.4% of patients with β - TM respectively. However, codon 8 was not found in all the thalassemic patients investigated in our study.

Al-Allawi *et al.*, carried out a study to determine the molecular mutation of β -thalassemia in northern Iraq. The frequent

mutations were IVS-1nt-5 (G-to-C) and codon 8/9 (+G) in 6.7%, 7.7% respectively. Less frequent mutations were: codon-30 (G-C)in 1.0% and codon-8 (-AA) among 2.9% of studied patients(Al-Allawi *et al.*, 2006). These results are similar with the findings of our study where the commonest mutations are IVS-I-5 and codon 8/9 in Basrah population.

Codon-15 (G-to-A), and codon 8/9(+G) is the mutations of Asian- Indian origin which located in exon I, mutation in codon-15 (TGG \rightarrow TGA(stop codon)), nonsense mutations in the human β -globin gene lead to unexpected levels of cytoplasmic mRNA accumulation and result β° -Thalassemia (Romao *et al.*, 2000). The IVSI-5, is the widespread and most frequent severe mutation of Asian- Indian origin, and it is very common in a belt comprising the region of the Indian subcontinent, it is the most common mutation in the Oman (62%), United Arab Emirates (UAE) (55%), and was frequent in neighboring Saudi Arabia and Kuwait 17% -19% (Al-Sultan et al., 2011). This reflects the ancient trade between India and the Arabic peninsula and the influence of the Arabic culture. It is of highest frequency in countries of the Arabian Peninsula, while it is less frequent in Western Asian Arab countries which might be explained by migration and invasion which have important roles in genetic admixtures.

The codon 41/42(-TTCT) is located on exon 2, and it is the severe β° mutation among those of Chinese and southeast Asian origin (Mirasena *et al.*, 2008). This mutation have not been studied previously in β -TM patients of Iraq, except in Wasit governorate, where it was detected in 2(8%) of the total 25 thalassemic patients studied (Katta *et al.*, 2003). The frequency of this mutation in this study was only 2.4%.

The Arab countries occupy large areas including the Western Asia, Northern Africa, Arabian Peninsula, and Nile Valley. The heterogeneity of the Arab people was about 52 β-Thalassemia reflected in mutations detected, and were mainly of Asian and Mediterranean origin. In each Arabian country, admixtures from different populations throughout history has been experienced, in addition to geographical migration differences. Furthermore. between Arab countries has been common until recently, and although some countries have unique mutations, no specific mutation seems to be confined to the Arabs (Zahed et al., 2001; Akhavan-Niaki et al., 2011).

The study confirms a heterogeneity of β thalassemia mutations within different districts of Basrah. In addition the frequency of these mutations differ from those detected in other parts of Iraq and neighboring countries and signifies the need for future studies to detect mutations that were not identified in this study.

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