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Molecular Characterization of β-Thalassemia Patients in Wasit Province, IRAQ

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Abstract
Background: Hemoglobin-associated disorder is a different group of recessive genetic diseases. which consist of the structural hemoglobin variants and the thalassemia.**Aim of the study: -** to characterize the spectrum of beta globin gene mutations in patients with beta- thalassemia who are registered in thalassemia centers, Wasit, Iraq using PCR - based DNA diagnostic techniques.**Patients and methods:**The genomic DNA was extracted from 106 Iraqi patients with β-thalassemia major and intermedia from unrelated families and (50) healthy numbers as control were collected from Al-Karama Teaching Hospital, in Wasit province, to detect mutation using PCR - based DNA diagnostic techniques. . **Results:** of 106 thalassemia patients, 35 (33%) of patients with no mutation detection, IVS 1.116 [T>G] mutation was detected in 42 patients (39.6%), followed by IVS 1.110 [G>A], which was detected in 11 (10.4%) patients.**Conclusions:** Many mutations recorded a high frequency due to close relative marriage. By analyzing many mutations in β-globin gene leading to defect in beta chains play crucial role in drawing mutation frequency and their distribution in our society.
Keywords: thalassemia, mutation, DNA

Introduction

In genetic diseases, genetic factors play an important role in their etiology. (1) Hemoglobin-associated disorder are a different group of recessive genetic diseases which consist of the structural hemoglobin variants and the thalassemia. These disorders can occur at high carrier incidences in the malaria-endemic regions of the world and are region-specific, due to the distinctive sort of structural variants and thalassemia mutation. (2) Thalassemia's are a group of haemoglobinopathic disorders in which a hemoglobin suffers from defect in β-globin polypeptide chains. (3) The clinical appearances are wide ranged from a symptomatic hypochromic and microcytosis to deep fatal anemias in utero or in early years of life if not got proper medication. Taking in account that about 7% of the world population are carries of a globin gene mutation. (4) Beta (β)-thalassemia is an autosomal recessive disorder initiated solely by alteration in the β -globin gene which is sited in chromosome 11 on the short arm. (5) It is branded by micro-cytosis and hemolytic anemias which result from interruption of the normal synthesis of the β - globin chains of hemoglobin. (6, 7) Recently, more than 1000 mutations have been recognized that have diverse effect on β -subunit and cause β –thalassemia. (5, 8) These mutations have a geographical pattern with racial origin and not eventually distributed. Some mutations maybe a common mutation, others are rare. (9,10,11) Mutations have been reported to interfere with the transcription of the β–globin such as nucleotide substitutions and/or frame shifts of the insertion/deletion gene, splicing procedures and translation of mRNA β -globin gene, resulting in either totally deficiency or reduction of creation of β -globin chains. (7) Modern molecular techniques provide rich information about the nature of mutations and their distribution within world population. (7) The spectra of the β-thalassemia mutations have been determined for the Mediterranean, Asian, Indian, Chinese and many other populations, and approximately, 80 million persons are carrier of the thalassemia trait and the percentage of carriers is increasing all over the world (12).

Aim of the study: - It is to characterize the spectrum of beta globin gene mutations in Wasit in patients with beta- thalassemia who are registered in thalassemia centers, in Wasit province using PCR - based DNA diagnostic techniques.

Patients and methods

This study has involved (106) Iraqi patients with β-thalassemia major and intermedia from unrelated families and (50) healthy numbers as control were collected from Al-Karama Teaching Hospital, in Wasit Province.

These patients previously diagnosed based on standard symptoms and confirmed by Hb

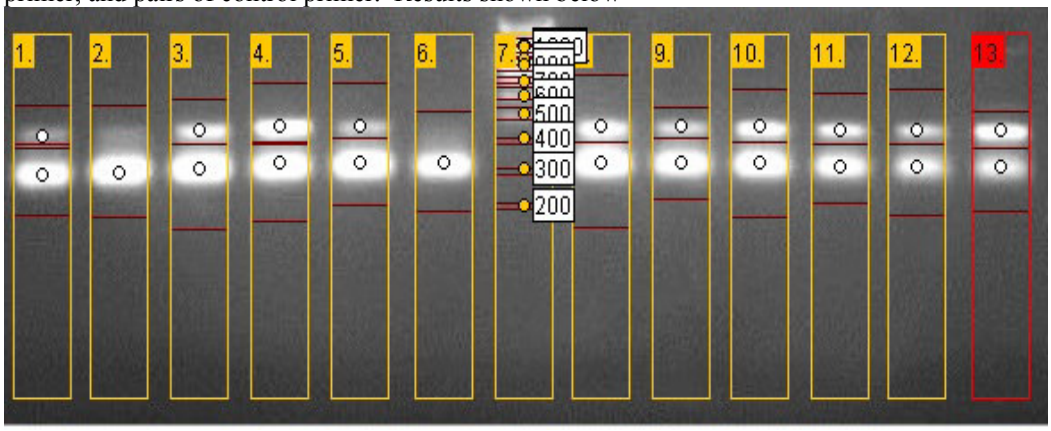
electrophoresis and other parameters include HCV and MCV conducted in the same hospital laboratories.
Venous blood sample (5-10mL) was collected from each patient with β-thalassemia. Each blood sample was divided into two tubes:
- EDTA tubes for molecular studies.
- The serum obtained by placing the blood in gel-plain plastic tube and let clot made at 37C° for 5 minutes then centrifuged. The tubes centrifuged at 5000 rpm for 5 minutes, serum was collected and kept in freezer until used.
The genomic DNA was extracted from the whole blood collected in EDTA anticoagulant tubes were used using AccuPrep® Genomic DNA Extraction Kit.

Primers were supplied by Bioneer Company, lyophilized product at different concentrations. Lyophilized primers were dissolved in a free DNase/RNase water as recommended by the manufacturer and then leave at water bath to dissolve completely. This will give final concentration of (100 pmol/µl), as stock solution. Each primer was diluted to 20 pmol/ µL concentration as aliquots and kept freeze until used as work solution).Statistical analysis used SPSS version 20 software. P value 0.05 or less consider significant.

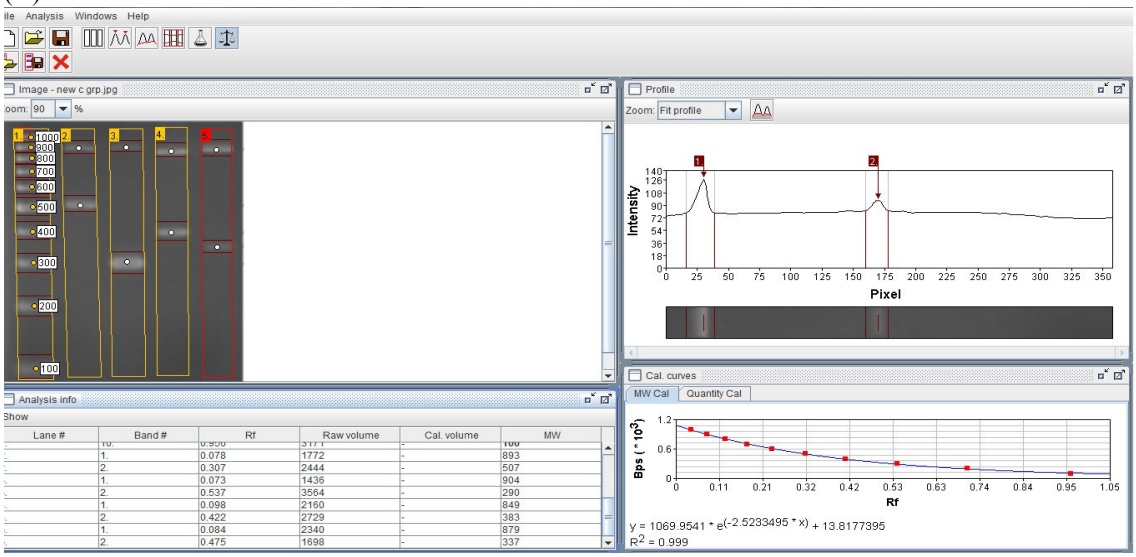
In this work, two methods of mutation detection were adopted to cover most common mutations in the Middle East area. First one is based on the reverse-hybridization of the amplified ß-globin gene to oligonucleotide probes. The second method is multiplex ARMS using different set of primers for normal and mutant with common primer, and pairs of control primer..

Results

In this work, two methods of mutation detection were adopted to cover most common mutations in the Middle East area. First one is based on the reverse-hybridizationof the amplified ß-globin gene to oligonucleotide probes. The second method is multiplex ARMS using different set of primers for normal and mutant with common primer, and pairs of control primer. Results shown below



(A)



(B)

Figure (1) represent agarose gel electrophoresis of PCR amplification using MARMS-PCR 1000bp molecular weight marker. (A) represent the first set of MARMS, (B) represent gel analyzer software screen for different product of MARMS-PCR groups in one gel.

Of (**106**) patients with thalassemia who treated in thalassemia uniting ALKut Hospital, gene mutation done as well as complete blood picture. Same investigation were done to **50** normal health condition. The mean age of the patient is **13.9** years with range (**2-52)** years. Table No. One shows the descriptive values of the patients with thalassemia regarding blood indices. The mean of hemoglobin was **8.8** g/dl, mean of RBC was **3.5** $\times 10^{12}$. The mean of MCV (mean corpuscular volume) was**55**fL, mean of MCH (mean corpuscular hemoglobin was **21** pg , MCHC (mean corpuscular hemoglobin concentration was **28** g/dl.

Table No. 1: descriptive data of (106)thalassaemia patients.

Item	WBC ($\times 10^9$)	RBC($\times 10^{12}$)	Hb(g/dl)	PCV(L/L)	Platelet($\times 10^9$)	MCV(fL)	MCH(pg)	MCHC(g/dl)
Mean	11.9	3.5	8.8	25	308	55	21	28
Median	8.2	3.1	9.0	26.3	303	65	24	29
Std. Deviation	11.6	0.5	2.0	32.259	159	4.8	3.5	1.2
Minimum	3.2	1.4	3.1	11	32	65	20	26
Maximum	81.1	3.6	11.1	32	1030	78	29	30

Table No. (2): shows the statistical difference between patients and controls regarding blood indices, there is highly statistical difference between two groups regarding Hb, RBCs, MCV and MCH (p. value **0.001**).

And statistical difference in PCV and MCHC (p. value **0.01, 0.02**) respectively. There is no statistical difference in WBC and platelets (p. value **0.4, 0.5**) respectively

Table No. 2: comparison between patients and control in hematological values.

Item	**Patients mean± SD (106)**	**Controls mean±SD(50)**	**P. value**
WBC ($\times 10^9$)	**11.9± 11.6**	**10.2±12.1**	**0.4**
RBC($\times 10^{12}$)	**3.5 ± 0.5**	**4.6 ± 0.7**	**0.001**
Hb(g/L)	**8.8 ± 2.0**	**11.2 ±2.9**	**0.001**
PCV(L/L)	**25 ± 32.2**	**36 ±0.07**	**0.01**
Platelet($\times 10^9$)	**308 ±159**	**290±150**	**0.5**
MCV(fL)	**55 ±4.8**	**79.2 ±5.3**	**0.001**
MCH(pg)	**21±3.5**	**24.4 ±5.3**	**0.001**
MCHC(g/dl)	**28±1.2**	**30.5 ±11.934**	**0.02**

Male constitute about 58% of the thalassemia patients, the family history of thalassemia in analyzed thalassemia patients, about three quarters of thalassemia patients had family history of thalassemia 74%.

Table No (3):shows demographics data regarding thalassemia types, consanguinity, gender, family history of thalassemia and blood group of analyzed thalassemia patients. More than two third of the patients were thalassemia major (**69%**), majority of the patients (**85%)**had consanguine marriage.

Table No.3: demographic data of thalassemia patients

Items	**Frequency**	**Percentage**
Thalassemia type		
Major	**73**	**68.9**
intermedia	**33**	**31.1**
consanguinity		
Yes	**90**	**84.9**
No	**16**	**15.1**
Sex		
Male	**61**	**57.5**
Female	**45**	**42.5**
family history		
Yes	**78**	**73.6**
No	**28**	**26.4**
Total	**106**	**100.0**

For all control group, no mutation was detected during mutation detection process, while for 106 thalassemia patients, many types of mutation were detected as shown in table number five. IVS 1.116 [T>G] mutation was detected in 42 patients (about 40%), IVS 1.110 [G>A] mutation was the second one in frequency and was detected in 11 patients (10.4%). No mutation was detected in 35 patients. -30 [T>A], Codon 39 [C>T], IVS 1.5 [A>C] mutation were detected in one patient for each. Because of low frequency and statistical purpose, all of the mutation with 1% consider as others. Table No. 4.

Table No. 4: mutation frequency of 106 thalassemia patients

Mutations	Frequency	Percent
IVS 1.116 [T>G]	42	39.6
IVS 1.110 [G>A]	11	10.4
IVS 1.5 [G>C]	7	6.6
Codon 8/9 (+G)	3	2.8
c.315+1G>A	3	2.8
codon5[-CT]	3	2.8
c.17_18delCT	2	1.9
-30 [T>A]	1	.9
Codon 39[C>T]	1	.9
IVS 1.5 [A>C]	1	.9
ND	35	33
Total	106	100.0

Table No five shows the association between family history of thalassemia and mutation, about three quarters of patients with IVS 1.116 [T>G], All patients with others mutation had family history 100%). In spite of P. value statistically not significant, but this need attention.

Table No 5: association between mutations types and family history of thalassemia

Family history of thalassemia	'IVS 1.116 [T>G]	IVS 1.110 [G>A]	IVS 1.5 [G>C]	'Codon 8/9 (+G)	'codon5[-CT]	Others	P. value
no	10 (23.8)	4 (36.4)	2(28.6)	2(66)	1 (33.3)	0(0)	
yes	32(76.2)	7 (63.6)	5(71.4)	1(33.3)	2 (66.7)	5(100)	0.9
Total	42 (100)	11(100)	7 (100)	3 (100)	3 (100)	5(100)	

Table No six shows the Association between consanguine state of patient's father and mother and mutation types. IVS 1.5 [G>C] and others mutation were detected in (100%) of patients with consanguinity marriage. More than (80%) patients with mutation IVS 1.116 [T>G], and IVS 1.110 [G>A], mutation were had consanguine marriage, with P. value 0.7.

Table No 6: association between mutations types and consanguinity

consanguinity	IVS 1.116 [T>G]	IVS 1.110 [G>A]	IVS 1.5 [G>C]	codon5[-CT]	Codon 8/9 (+G)	Others	P. value
no	5(11.9)	2(18.2)	0 (.0)	1 (33.3)	1 (33.3)	0(0)	
yes	37(88.1)	9(81.8)	7(100)	2 (66.7)	2 (66.7)	5(100)	0.7
	42 (100)	11(100)	7(100)	3 (100)	3 (100)	5(100)	

Table No (7) describes the Association between thalassemia types and mutation. More types of mutations are more common in thalassemia major, all analyzed patients whom had IVS and 1.5 [G>C] mutations were thalassemia major (100%). While more than (70%) patients with IVS 1.116 [T>G] mutation were thalassemia major. All patients with c.315+1G>A mutation were thalassemia intermedia (100%). With P. value 0.01.

Table No 7: association between mutations types and thalassemia types.

Thalassemia types	'IVS 1.116 [T>G]	IVS 1.110 [G>A]	IVS 1.5 [G>C]	'codon5[-CT]	'Codon 8/9 (+G)	Others	P. value
Intermedia	11(26.2)	5(45.5)	0(0)	1 (33.3)	0 (0)	3 (60)	
major	31(73.8)	6(54.5)	7(100)	2 (66.7)	3 (100)	2(40)	0.01
Total	42 (100)	11(100)	7(100)	3 (100)	3 (100)	5(100)	

The relationship between genders and mutation types were describe in table No (8) 'IVS 1.116 [T>G] mutation which is more common in male gender (66.7%). While 'codon5[-CT] mutation more common in female.

Table No 8: association between mutations types and gender

Gender	'IVS 1.116 [T>G]	IVS 1.110 [G>A]	IVS 1.5 [G>C]	'codon5[-CT]	'Codon 8/9 (+G)	Others	P. value
female	14(33.3)	6(54.5)	4(57.1)	2 (66.7)	2 (66.7)	1(20)	
male	28(66.7)	5(45.5)	3(42.9)	1 (33.3)	1 (33.3)	4(80)	0.6
Total	42 (100)	11(100)	7 (100)	3 (100)	3 (100)	5(100)	

Discussion

The gene analysis play important role In diagnosis as well as treatment of thalassemia. For 106 beta thalassemia patients with mean age 13.9 years, complete blood picture was done, which shown mean of blood indices (Hb,

RBCs, MCV, MCHC, MCH) as well as WBC and platelets mean. The mean of WBC and platelets are ranged with normal limits, while others parameters are lower than whatever mention in textbooks, ([13]).

As thalassemia syndrome as one cause of hypochromic microcytic anemia all blood indices are below normal ranges. As comparison in blood indices between 106 analyzed thalassemia patients and 50 controls there is significant statistical difference in RBCs, Hb ,MCV, MCH. P value (0.001) and P. value is also statically different (0.01, 0.02) for PCV and MCHC respectively. These differences are compatible with what written in literatures, ([13]).

Although the thalassemia is autosomal recessive disease, the sex disturbed is equally in both male and female, but in this study the Male to female ratio is 1.3:1 which may reflect the community nature. As our culture is Eastern community, Since inbreeding is a high percentage of our society, this explains the high proportion of patients who have a family history of the disease, as consanguinity marriage percent (84%), and family history of thalassemia (73.6%).

Thirty three patients considered as thalassemia intermedia on clinical base (as they received first blood transfusion older than two years of age as well as their needs to blood transfusion is less than major).

Mutations:

IVS 1.116 [T>G] mutation:

This mutation was detected in 42 patients (39.6%), which is highest percent. More than three quarter of the patient were have family history of thalassemia and consanguinity marriage. As this mutation detected more in thalassemia major, it was detected in (73.8%). And mainly in male gender (66.7%).

In spite of this mutation is Mediterranean mutation and one of mutation that frequently occur in Iranians peoples, ([14]). But unfortunately most studies which done in Iraq didn't chose IVS 1. 116 as panel for screening thalassemia mutation. And didn't reported in north Iraq study ([15]).

This mutation was reported in Turkey thalassemia patients in (1.6%), ([16]). And in Syria (1.4%), also detected in Egyptian patients in (1.2%), ([17]). Which is lower than what reported in this study.

IVS 1.110 [G>C] :

This mutation was reported in this study in 14 (13.2%) patients, (64%) of them were have family history of thalassemia, more than 80% of them have consanguinity marriage, and thalassemia intermedia was reported in more than one half (57%). This mutation was reported equally in females and male (50%).

IVS 1. 110 [G>C] mutation was reported in about 18% thalassemia patients in Baghdad study, [(18)] and 27% in Ninawa study [(19)]. which is more than what reported in this study. This mutation was detected in 25% of Jordanian thalassemia patients [(20)] which is high to what reported in our study. And reported 10% percent in Iranian patients [(22)]. Which is lower than what reported in this study.

IVS 1.5 [G>C] mutation:

This mutation was reported in seven patients (6.6%), most of them were have family history of thalassemia, all of them were have consanguinity marriage and thalassemia major. This mutation have been equally reported in both gender.

IVS 1.5 [G>C] Mutation was reported in 2% in Ninawa study [(19)],which is lower than what reported in this study. While in Al muthana study was reported in 10% [(18)]. And reported in 2% in Turkey study [(21)].

Codon5[-CT] mutation :

This mutation was reported in five patients (4.7%). Eighty percent of the patients with this mutation were have family history of thalassemia, consanguinity marriage and thalassemia major, this mutation were reported more in female (60%).

This mutation was not reported in Baghdad, Mothanna, and north Iraq studies, but reported in Iran study in (3.8%) of thalassemia patients, [(22)]. And reported in 0.5% in Turkey study [(21)].

This mutation was detected in 3.8 % of Jordanian thalassemia patients [(20)].

Codon 8/9 (+G) mutation:

This mutation was reported in three patients (2.8%). All patients with this mutation were thalassemia major (100%). This mutation were not reported in north Iraq, Mothanna, Ninawa and Iran studies. But in other study in Baghdad done by Al-Azzawi the percent is (7.7%)[(23)]. In Turkey study the percent is (2.1%) [(21)].

Others mutation which reported in one patients for each, -30 [T>A], Codon 39 [C>T], IVS 1.5 [A>C] as -30 [T>A] mutation were reported in Turkey patients in 4.6%, [(21)].Codon 39 [C>T] mutation were reported in Turkey patients in 3.6%, [(21)].And 4.9% in Jordanian patients, [(20)].15% in Al Muthana study, [(18)] . And 1% in Baghdad study, [(18)].

Conclusion : Conclusions: Many mutations record a high frequency due to close relative marriage. By analyzing many mutations in β-globin gene leading to defect in beta chains play crucial role in drawing mutation frequency and their distribution in our society.

β-thalassemia is very common in Iraqi society, due to high consanguinity rates.

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