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Unveiling the molecular and comorbidity profile among transfusion-dependent and nontransfusion-dependent beta-thalassemic patients in Baghdad city

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Abstract:

BACKGROUND: Beta-thalassemia (BT) is the most common hereditary hemolytic disease in Iraq. The high prevalence rate of this disorder, coupled with, frequently encountered severe clinical course, as well as the life-long burden of comorbidities and complications, have all contributed to its status as one of the most clinically and epidemiologically significant global health issues. This has prompted our efforts to study the molecular map of BT in Baghdad city.

OBJECTIVES: The aims of this study as to identify the molecular map of BT in patients from Baghdad city with investigating the data from several clinical and disease-associated morbidity parameters to establish potential correlation points.

PATIENTS, MATERIALS AND METHODS: The molecular profile of HBB gene of 80 transfusion-dependent (TD) and non-TD BT patients from Baghdad thalassemia centers was examined using multiplex polymerase chain reaction and reverse hybridization technique and direct gene sequencing.

RESULTS: In the current study, 27 different genotypes were characterized. The most predominant displayed genotypes were IVS 1.110 (G>A)/IVS 1.110 (G>A), and IVS 2.1 (G>A)/IVS 2.1 (G>A). Our data also revealed that 70% of the exhibited genotypes were homozygous, and most of those (78.6%) were TD. As for disease comorbidities, cholelithiasis (53.8%), osteoporosis (51.3%), and facial bone deformity (45%) were among the most frequently encountered in our study.

CONCLUSIONS: The compiling clinical and molecular data revealed in the current study, has indicated an aggregation of certain homozygous BT genotypes in Baghdad city that possibly influenced the comorbidity profiles of our patients.

Keywords:

Genotypes, *HBB* gene, hybridization, nontransfusion dependent, transfusion dependent, beta-thalassemia

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Introduction

Beta-thalassemia (BT) syndrome is the most common genetic health disorder across more than 60 countries, with a carrier population surpassing 150 million.[1] It is highly prevalent in countries in the Middle East (including Iraq), India, Southeast Asia, and the Mediterranean.[2] This type of hereditary hemolytic disorder arises from diminished or absent β-globin chains' synthesis, leading to the accumulation of redundant α-chains, culminating in

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ineffective erythropoiesis, of varying severity, that is governed by the type of defect in the β-globin gene, in addition to few other genetic determinants.[3,4]

The genetic architecture of this autosomal-recessive disorder is quite heterogeneous, with more than 400 different variants identified thus far. Yet, it has been reported that in each population, merely 20 distinct BT mutations contributed to more than 80% of the BT etiology, exhibiting geographical allele clustering.[5,6]

Clinically, BT is classified into two main syndromes based on their dependence on blood transfusion; the first is transfusion-dependent (TD) BT, in which the survival of patients is plainly reliant on regular transfusions early on life, and the second is non-TD (NTD) BT, in which patients do not rely on regular transfusions.[7,8]

The spectrum of BT phenotype is broadly diverse, ranging from very mild to extremely severe fatal anemia with an array of disease-associated complications and comorbidities stemming from ineffective erythropoiesis, chronic hemolytic anemia, and iron overload, the hallmarks of BT.[9,10]

Worldwide, this disorder imposes a significant burden due to its frequently encountered severe clinical course, with concomitant comorbidities, generally demanding life-long care and monitoring that can be physically, financially, and psychologically draining.[11-13]

Iraq is not only a high prevalence region for BT, but also the majority of reported cases exhibited a severe phenotype with high complication rate. Baghdad is the most densely populated city in the country, with a population of 7,216,000, accounting for approximately (16%) of total Iraqi population, according to a 2024 elaboration of the latest United Nations data.[14-16] It holds two major thalassemia centers that drain patients from Central Iraq. The relatively high annual cost for thalassemia patients in Iraq has imposed a huge burden exhausting both patients and the health institutions, which cover most of this cost.[15] Thus, introducing a comprehensive preventive program constitutes an absolute necessity. Molecular profiling of BT patients in our country can aid in constructing such a program, and while studies from northern parts of Iraq have provided fairly sufficient data, information from Central Iraq is still scarce. This has prompted our efforts to delve into the intricate realm of the molecular pathology of BT in Baghdad city.

Patients, materials and methods

A cross-sectional study was conducted between November 2022 and February 2024 recruiting 80 adult BT patients aged 18 and above. The subjects were selected using systematic random sampling from the attendee of the only thalassemia care centers in Baghdad (i.e. Ibn Albalady Thalassemia Care Centre and Thalassemia Care Centre at Al Karama General Hospital). Patient data were obtained through a preprepared comprehensive questionnaire form, in addition to reviewing medical files of the selected patients. All relevant data were then collected, evaluated, and cataloged for further analysis.

Five milliliters of pretransfusion venous blood was aseptically aspirated from all patients. Three milliliters of the anticoagulated blood was used to perform hematological and biochemical testing, and the remaining EDTA blood sample (2 ml) was frozen at –20°C for later genetic testing.

Molecular analysis of the *HBB* gene mutations

The extraction of genomic DNA from each blood sample after thawing was done using *GENxTRACTTM Resin* kit, following the manufacturer's instructions. The purity and quality of the isolated DNA were then evaluated via: nanodrop fluorometery, and gel electrophoresis. Mutational screening of *HBB* gene was carried out implementing the *β-Globin StripAssay IME® Test Strips* (ViennaLab Diagnostics GmbH, Vienna, Austria), a reverse-hybridization assay tailored to detect population-specific mutations within the Middle East, Iran, Arabian Peninsula, and India, with the ability to characterize 22 mutations covering the majority (more than 90%) of β-globin genetic defects found in those regions [Table 1].

The extracted DNA samples were then amplified using biotin-labeled primers in multiplex polymerase chain reaction (PCR) reaction. Amplicons were then hybridized to the StripAssay® test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. The labeled PCR products were then identified by means of streptavidin-alkaline phosphatase color reaction. All of our 80 processed test strips showed positive reactions characterizing 13 different mutations distributed among three different genotypes (i.e. homozygous, heterozygous, and compound heterozygous) [Figure 1].

Characterization of beta-globin mutation through direct sequencing for confirmation of the results

Nine samples (10% of DNA templates) were randomly selected to amplify a 428bp targeted long sequence of HBB gene covering the mutational hot spot region for BT, implementing a set of specific primers [Table 2].[17] A reaction mixture was prepared for every sample, with a total volume of 25 µL, implementing *Maxime PCR PreMix kit (i-Taq)* (Intron/Korea) tubes.

After the end of thermocycler program, 5 µl of each amplicon was displayed via gel electrophoresis on two% agarose. Then, a gel purification kit *QIAquick gel extraction kit* (Qiagen/Indonesia) was used to isolate the desired fragments of PCR product in order to ensure the fidelity of the sequencing [Figure 2]. A volume of 20 µl of each amplified sample was subsequently sent for direct sequencing by *Genetic Analyzer System ABI-310/ Macrogen* (Seoul, Korea) using "chain termination method." Once the sequencing results were done, NCBI tools and bioinformatics software were used for their analysis.

Ethical consideration

This study was reviewed and acclaimed approval by the Institutional Review Board of the College of Medicine at Nahrain University. Written informed consent was granted from all study participants. Data confidentiality was preserved in accordance with the revised Helsinki Declaration of Bioethics.[18]

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences version 26. Independent *t*-test and analysis of variance (two-tailed) were also used to compare the continuous variables accordingly. Chi-square test was used to investigate the association between categorical variables, while Fisher's exact test was used instead whenever the expected frequency was <5. A level of $P < 0.05$ was considered statistically significant.

Table 1: β-globin mutations covered by β-Globin StripAssay® IME

Common name	HGVS nomenclature
cap+1 (A>C)	HBB: c.-50A>C
codon 5 (-CT)	HBB: c. 17_18delCT
codon 6 (A>T)	HBB: HbS c. 20A>T
codon 8 (-AA)	HBB: c. 25_26delAA
codon 8/9 (+G)	HBB: c. 27_28insG
codon 15 (TGG>TAG)	HBB: c. 47G>A
codon 16 (-C)	HBB: c. 51delC
codon 22 (7bp del)	HBB: c. 68_74delAAGTTGG
codon 30 (G>C)	HBB: c. 92G>C
mutant IVS 1.1 (G>A)	HBB: c. 92+1G>A
IVS 1.1 (G>T)	HBB: c. 92+1G>T
IVS 1.5 (G>C)	HBB: c. 92+5G>C
IVS 1.6 (T>C)	HBB: c. 92+6T>C
IVS 1.110 (G>A)	HBB: c. 93-21G>A
IVS 1-25 (25bp del)	HBB: c. 93-21_96de
codon 36/37 (-T)	HBB: c. 112delT
codon 39 (C>T)	HBB: c. 118C>T
codon 41/42 (-TTCT)	HBB: c. 126_129delCTTT
codon 44 (-C)	HBB: c. 135delC
IVS 2.1 (G>A)	HBB: c. 315+1G>A
IVS 2.745 (C>G)	HBB: c. 316-106C>G
619bp del	

HGVS=Human genome variation society

Results

Demographic and clinical data

This study included 80 BT patients (56 TD and 24 NTD patients), with a mean age of 27.29 ± 10.8 years (mean ± standard deviation) ranging from 18 to 67 years and a female ratio of 1.1:1. The majority of patients (59 accounting for 73.8% of the study participants) have had a positive family history of BT with one or more affected family members, of those more than two-third were among the TDT group (42), and 17 (28.8%) were among the NTD group. In addition, 85% of our recruited patients (68) reported consanguineous marriages between their parents, with 72.1% (49) of those were TD and 27.9% (19) were NTD patients. Furthermore, our data also demonstrated that 32.5% of the enrolled patients had undergone splenectomy, divided as 29% of NTD and 34% of TD. In regard to disease comorbidities, as detailed in Table 3, cholelithiasis, osteoporosis, and facial bone deformity were the most recurrent disease comorbidities encountered in our study, followed by growth retardation, thyroid dysfunction, and leg ulceration. The earlier and the latter both revealed a statistically significant difference between the studied groups.

Biochemical and hematological parameter

As listed in Table 4, the means of several hematological and biochemical parameters including Hb, HbA_2, HbF, serum ferritin (SF), serum alanine transaminase (ALT), serum aspartate transaminase (AST), total serum bilirubin, serum calcium, blood urea, and serum creatinine levels, were investigated and compared between our two study groups. The inferred data showed significantly higher levels of Hb, HbA_2, SF, ALT, and AST, and lower HbF levels in TD patients than that in NTD patients, while the remaining parameters showed imperceptible difference.

Beta-thalassemia genotypes

In the current study, out of our total eighty genotypes, 70% exhibited homozygous genotypes (most of those were transfusion dependent: 78.6%). The remaining were: 23.7%

Table 2: The specific primer of HBB gene (Integrated DNA Technologies company/Canada)

Primer	Sequence	Temperature (°C)	GC (%)	Product size
Forward	5'-GGCAGAGCCATCTATTGCTTAC – 3'	55.7	50.0	532 base pair
Reverse	5'-CAGGCCATCACTAAAGGCACC – 3'	58.4	57.1	

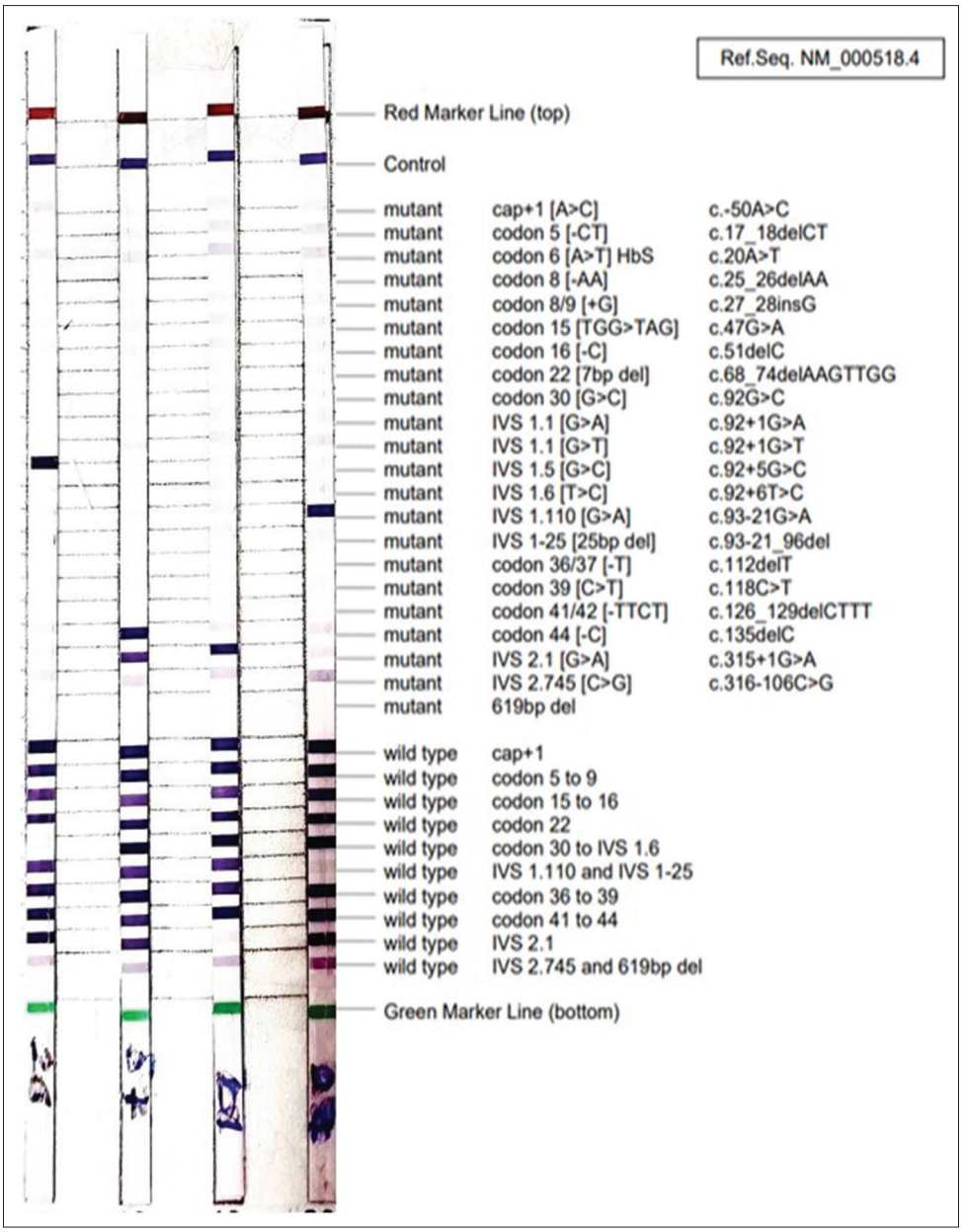

Figure 1: From left to right; homozygous IVS 1.5 (G>C), compound heterozygous Codon 44(-C)/IVS 2.1(G>A), homozygous IVS 2.1 (G>A), homozygous IVS 1.110 (G>A)

compound heterozygotes and 6.3% heterozygotes. The later genotypes were all clustered within NTDT group. A statistically significant difference between the groups was observed, as seen in table Table 5. The above-mentioned genotypes when further examined according to their BT type unveiled the genotypes listed in Table 6. The most common genotype was β^0/β^0 (accounting for 46.3%), followed by β^+/β^+ (accounting for 35%).

Among those, we characterized 27 distinct genotypes, as demonstrated in Table 7, converging from 14 different alleles (13 mutated and one wild type). The most predominant genotypes were IVS 1.110 {G>A}/IVS 1.110 {G>A}, and IVS 2.1 {G>A}/IVS 2.1 {G>A} accounting for 22.5% and 16.3% respectively. While the most frequent BT alleles were IVS 1.110 {G>A} and IVS 2.1 {G>A}, mounting for 27.5%, and 21.9% of the identified alleles respectively.

Beta-thalassemia genotypes and disease comorbidities

As shown in Table 8, generally, patients with β^0/β^0 genotypes had a slightly higher frequency of disease comorbidities, when compared with other genotypes, however failed to reach statistical significance ($P \geq 0.05$).

Discussion

Many studies have shown that despite the quite heterogeneous nature of molecular BT pathology, only

Table 3: Disease comorbidities reported in our study

BT comorbidities	Study group		Total (*n*=80), *n* (%)	*P*
	TD (*n*=56), *n* (%)	NTD (*n*=24), *n* (%)		
Cholelithiasis	28 (65.1)	15 (34.9)	43 (53.8)	0.304
Osteoporosis and/or spontaneous fractures	30 (73.2)	11 (26.8)	41 (51.3)	0.526
Facial bone deformity	26 (72.2)	10 (27.8)	36 (45.0)	0.695
Growth retardation (>2 SD below 3rd percentile)	30 (85.7)	5 (14.3)	35 (43.8)	0.007
Thyroid dysfunction	24 (77.4)	7 (22.6)	31 (38.8)	0.249
Recurrent leg ulceration	16 (53.3)	14 (46.7)	30 (37.5)	0.012
Splenectomy	19 (73.1)	7 (26.9)	26 (32.5)	0.667
DM	9 (81.8)	2 (18.2)	11 (13.8)	0.357
Documented thromboembolic event	4 (50.0)	4 (50.0)	8 (10.0)	0.193

DM=Diabetes mellitus, TD=Transfusion dependent, NTD=Nontransfusion dependent, BT=Beta thalassemia, SD=Standard deviation

Table 4: Hematological and biochemical parameters among transfusion-dependent and nontransfusion-dependent patients

Lab parameters	Study group (mean±SD)		*P*
	TD	NTD	
Hematological parameters			
Hb (g/dL)	6.5±1.9	7.75±1.8	0.031
HbA2 (%)	2.44±1.6	3.57±1.5	0.004
HbF (%)	89.19±12.0	70.54±13.6	0.001
Biochemical parameters			
SF (μg/L)	4043.7±2161.9	3019.5±2018.6	0.048
ALT (U/L)	59.41±63.4	31.95±18.5	0.004
AST (U/L)	64.1±73.7	41.83±16.2	0.036
TSB (μmol/L)	40.59±23.7	36.71±20.4	0.461
Serum calcium (mmol/L)	2.29±0.3	2.2±0.2	0.152
Blood urea (mmol/L)	3.45±0.9	3.85±1.1	0.117
Serum creatinine (μmol/L)	56.1±15.6	56.23±15.5	0.973

TD=Transfusion dependent, NTD=Nontransfusion dependent, HB=Hemoglobin, SF=Serum ferritin, ALT=Alanine transaminase, AST=Aspartate transaminase, TSB=Total serum bilirubin, SD=Standard deviation

several mutations are accredited to the vast majority of cases in certain population, denoting an evident geographical variation.[19] In the current study, efforts were made to discern the genetic map of BT in Central Iraq, thence to comprehend its possible implications on disease course and comorbidities.

Our data have revealed that more than half of our enrolled patients have suffered from cholelithiasis and osteoporosis representing the most frequent complications documented in our study. We noticed that the most common complications among TD patients were osteoporosis and growth retardation that was significantly higher than that in the transfusion nondependent group (53.6% and 20.8%, respectively), followed by fascial bone deformities, gallstones, and thyroid dysfunction. This was consistent with data by Amin *et al*. which has showed that osteoporosis and bone deformity were the most recurrent thalassemia associated morbidities among transfusion dependent patients, followed by endocrinopathies (as growth retardation, and thyroid dysfunction.[20]

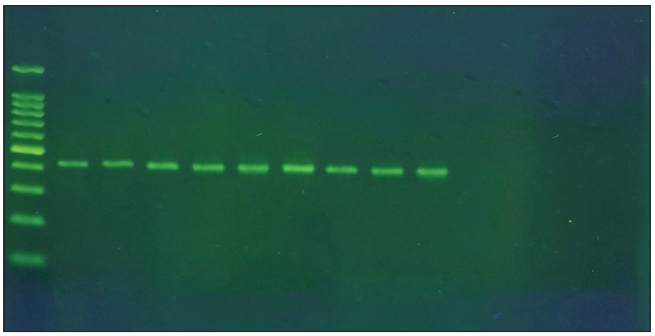

Figure 2: Gel electrophoresis of polymerase chain reaction products following gel purification showing the desired fragments of 428bp size

Furthermore, Abdullah *et al*. had reported even higher rates of growth retardation, with 79% of the TD patients having short stature.[21] Osteopathy was also the most presented complication reported by Bonifazi *et al*. which seems to increase with advancing age.[22]

The high rates of skeletal changes seen in thalassemic patients as; osteoporosis, growth retardation and delayed bone age can be attributed to several aetiological factors as; bone marrow expansion, iron overload (due to iron direct toxicity on osteoblasts), hypothalamic-pituitary-gonadal axis dysfunction, and even iron chelators that may in turn cause osteoporosis and osteopenia.[23,24]

On the other hand, cholelithiasis and recurrent leg ulceration were the most recurrent comorbidities registered among our NTD group as data have suggested that NTD patients are more prone to develop gallstones than TD patients because of more prominent peripheral hemolysis.[25] In addition, our data have shown that leg ulceration was significantly higher among our NTD patients which probably reflect the transfusion practices for those patients who have chronic undertreated anemia, as the majority of patients were transfused on demand rather than on need. Since chronic anemia and endothelial cell injury are the main risk factors that prompt ulcer development in BT patients,[26] this may provide a rationalization to our findings.

Table 5: Association between study groups and genotype

Genotype	Study group		Total (*n*=80), *n* (%)	*P*
	TD (*n*=56), *n* (%)	NTD (*n*=24), *n* (%)		
Homozygous	44 (78.6)	12 (21.4)	56 (70.0)	0.001
Compound heterozygous	12 (66.7)	6 (33.3)	18 (23.7)	
Heterozygous	0	6 (100.0)	6 (6.3)	

TD=Transfusion dependent, NTD=Nontransfusion dependent

Table 6: BT genotypes in study groups

BT genotype	Study group		Total (*n*=80), *n* (%)
	TD (*n*=56), *n* (%)	NTD (*n*=24), *n* (%)	
β^0/β^0	31 (83.8)	6 (16.2)	37 (46.3)
β^+/β^+	18 (62.1)	11 (37.9)	29 (36.3)
β^+/β^0	7 (87.5)	1 (12.5)	8 (10.0)
(β^+/β) and/or (β^0/β)	0	6 (100)	6 (7.5)

TD=Transfusion dependent, NTD=Nontransfusion dependent, BT=Beta thalassemia

Table 7: BT genotype identified in this study

BT genotype	*n*=80, *n* (%)
β^0/β^0	
IVS 2.1 (G>A)/IVS 2.1 (G>A)	13 (16.3)
Codon 36/37 (-T)/Codon 36/37 (-T)	5 (6.3)
Codon 44 (-C)/Codon 44 (-C)	3 (3.7)
Codon 41/42 (-TTCT)/Codon 41/42 (-TTCT)	3 (3.7)
Codon 8 (-AA)/Codon 8 (-AA)	3 (3.7)
Codon 5 (-CT)/Codon 5 (-CT)	2 (2.5)
IVS 1-25 (25bp del)/IVS 1-25 (25bp del)	2 (2.5)
IVS 2.1 (G>A)/Codon 41/42 (-TTCT)	1 (1.3)
Codon 39 (C>T)/Codon 39 (C>T)	1 (1.3)
IVS 1-25 (25bp del)/Codon 15 (TGG>TAG)	1 (1.3)
Codon 5 (-CT)/IVS 1-25 (25bp del)	1 (1.3)
Codon 44 (-C)/IVS 2.1 (G>A)	1 (1.3)
IVS 1-25 (25bp del)/Codon 44 (-C)	1 (1.3)
β^+/β^+	
IVS 1.110 (G>A)/IVS 1.110 (G>A)	18 (22.5)
IVS 1.6 (T>C)/IVS 1.6 (T>C)	3 (3.7)
IVS 1.5 (G>C)/IVS 1.5 (G>C)	3 (3.7)
IVS 1.6 (T>C)/IVS 1.110 (G>A)	2 (2.5)
IVS 1.110 (G>A)/IVS 2.745 (C>G)	1 (1.3)
IVS 1.5 (G>C)/IVS 1.6 (T>C)	1 (1.3)
IVS 2.745 (C>G)/IVS 1.6 (T>C)	1 (1.3)
β^+/β^0	
IVS 1.110 (G>A)/Codon 44 (-C)	5 (6.3)
IVS 1.6 (T>C)/IVS 2.1 (G>A)	1 (1.3)
IVS 1.110 (G>A)/IVS 2.1 (G>A)	1 (1.3)
IVS 2.745 (C>G)/IVS 2.1 (G>A)	1 (1.3)
(β^+/β) or (β^0/β)	
IVS 2.1 (G>A)/wild type	4 (5.0)
IVS 1.110 (G>A)/wild type	1 (1.3)
Codon 44 (-C)/wild type	1 (1.3)

BT=Beta thalassemia

Other complications, such as diabetes mellitus and TE disorders, were less frequent and only reported in < 15% of our study population.

Furthermore, several hematological and biochemical parameters have been investigated among our TD patients along with those of our NTD patients. The first group exhibited significantly higher levels of ALT and AST than the latter group, which can be attributed to iron-induced liver injury imposed by the accompanying significantly higher SF of this group, as data have shown that liver enzyme levels positively correlate with SF levels in BT patients especially when levels mounted above 2000 ng/ml.[27-29] The results in this work lie in accordance with data observed in other Iraqi studies in which the majority of BT patients have also displayed a significantly elevated SF in addition to elevated levels of AST, and ALT enzymes. A study by Bashi *et al*., on 80 transfusion dependent BT patients from Mosul city,[30] and another by Owaid *et al*., in Diyalah,[31] in addition to a further study by Ali *et al*., on 70 β-thalassemic patients from Erbil city,[32] all exhibited a similar trend.

In our study, we directed our efforts toward examining the diverse spectrum of BT genotypes and its impact of comorbidity profile in patients, as a result, the genotype study revealed that the genotypic map of our enrolled patients is largely composed of homozygous and compound heterozygous genotypes accounting for 70% and 23.7%, respectively. Homozygosity was the dominant trend among TD patients, while heterozygosity was only exhibited among the NTD group. The recessive nature of inheritance which dictates the presence of two mutated alleles can explain such findings as our study population is consistent mainly of TD patients (70%), and the remaining 30% is NTD. The presence of heterozygotes among 25% of the NTD group, however, can be attributed to the co-inheritance of other genetic determinants capable of exacerbating the α/non-α chain imbalance, thereby fueling α chain precipitation,[33] as α-globin gene triplication.

Out of 56 patients who exhibited homozygous genotypes, 37 were β^0/β^0 representing the most common BT genotype accounting for 46.3%. Interestingly, more than one-third of our patients exhibiting this genotype were homozygous IVS 2.1. As it would be expected, β^0/β^0 genotype was more prevalent among TDT patients. This genotype also generally had a slightly higher rate

Table 8: Comparison between genotypes according to disease comorbidities

Disease comorbidities	β^0/β^0 (%) (*n*=37)	β^+/β^+ (%) (*n*=29)	β^+/β^0 (%) (*n*=8)	β^+/β and/or β^0/β (%) (*n*=6)	*P*
Facial bone deformity	14 (37.8)	16 (55.1)	5 (62.5)	1 (16.7)	0.154
DM	6 (16.2)	2 (6.9)	2 (25.0)	1 (16.7)	0.613
Gall bladder stone	19 (51.4)	19 (65.5)	3 (37.5)	2 (33.3)	0.448
Recurrent leg ulceration	13 (35.1)	11 (37.9)	3 (37.5)	3 (50.0)	0.87
Osteoporosis and/or spontaneous fractures	17 (45.9)	14 (48.3)	5 (66.7)	5 (83.3)	0.261
Documented thromboembolic event	4 (10.8)	2 (6.9)	1 (12.5)	1 (16.7)	0.899
Thyroid dysfunction	17 (45.9)	11 (37.9)	1 (12.5)	2 (33.3)	0.228
Growth retardation (>2 SD below 3rd percentile)	15 (40.5)	14 (48.3)	5 (66.7)	1 (16.7)	
Splenectomy	12 (32.4)	10 (34.5)	3 (33.3)	1 (16.7)	0.845

DM=Diabetes mellitus, SD=Standard deviation

of disease comorbidities, when compared with other genotypes, however not at significant levels. These findings agree with a study from northeastern Iraq done on 242 beta-thalassemia patients, which similarly reported higher rates of comorbidities among the above-mentioned genotype.[20]

In addition, β^+/β^0 genotype accounted for 10% of the studied genotypes, with compound heterozygote IVS 1.110/Codon 44 representing the main contributor to this genotype.

The presence of null β^0 mutations possibly explains the clustering of most patients exhibiting this genotype among the TD group. Similar results were also deduced in a study of 60 TD patients from Karbala, Iraq, in which inheritance of null mutations in homozygous (β^0/β^0) or compound heterozygous (β^+/β^0) was the most frequently encountered mechanism in more than 88% of the patients.[34]

Furthermore, β^+/β^+ was the most prevalent genotype among our NTD patients contributing to 45.8% of NTD genotypes, with dominance of homozygous IVS 1.110. As it would be presumed, inheriting two mild β^+ mutations results in less severe phenotype as a result of relatively lesser excess of unmatched α chains.[35,36] These figures were close to those by Al-allawi *et al*., who reported that β^+/β^+ and β^0/β^+ genotypes had contributed to 49% of BT intermedia genotypes in Baghdad.[37] Whereas, they were comparable to some extent to figures reported from Sulaymaniyah (40.9%), yet less than those described by studies from other parts of Kurdistan with 60.2%, and 54.9% in Erbil, and Dohuk respectfully.[38-40]

Interestingly, 25% of our NTD patients exhibited the severe β^0/β^0 mutation. This genotype–phenotype discrepancy can be attributed to concomitant inheritance of other genetic determinants that can ameliorate the plethora of unbound α chain such as co-inheritance of α-thalassemia and/or polymorphisms within the three major quantitative loci that boost HbF production.[36,41] A study on 102 BT intermedia patients from Baghdad revealed that the main affecters modifying phenotype severity were the co-inheritance of XmnI, and the BCL11Agene polymorphism (that can potentiate γ-chain expression), rather than AT.[37]

On the other hand, our data have shown that genotypes harboring the wild-type alleles (i.e. β^+/β, β^0/β) represented the least frequent genotypes in our study, and they were exclusively expressed among the NTD group. The co-inheritance of an extra α gene can explain our findings as it would further exacerbate the α/non-α chain disparity and lead to moderately severe anemia that may require transfusion therapy in an otherwise typically asymptomatic BT carrier.[42,43] These findings corroborate with data from previous study on 83 BT intermedia patients in Erbil province which revealed that (6%) of patients had co-inherited α-globin gene triplication with heterozygous genotype.[39] Similar findings were also encountered in a study from Baghdad city in which α-globin genotyping has les led to the detection of $\alpha\alpha\alpha^{anti\text{-}3.7}$ gene triplication in all BT patients carrying the heterozygous genotype.[37]

As pertaining BT alleles, our work has revealed that three alleles accounted for approximately 60% of the total alleles. These are IVS 1.110 (G>A), IVS 2.1 (G>A), and Codon 44 (-C). IVS 1.110(G>A) allele, which was represented mainly as a homozygote in 22.5, has the highest recorded allele frequency in our study, comprising 27.5% of total number of alleles. In other provinces of Iraq, the frequency of this allele was relatively high across, displaying the highest documented rates to date being that of 34% in Nineveh Province.[44] While in Kirkuk the rate was:16.25%,[45] and in Karbala: 15.83% of alleles.[34] It has been reported that it is the most widespread mutation among Arabs with higher rates ranging from 12%–38%, in Arabs from the Eastern-Mediterranean region[46]

IVS 2.1 (G>A) allele appeared as homozygous in 16%, as compound heterozygous in 6.3%, and as heterozygous in 5% of the identified genotypes, and it was the second most frequent allele, which was exhibited in

approximately 22% of the total number of studied alleles. This is consistent with findings from another study in Baghdad, in which the predominant alleles were IVS 1.110 in 30.1% and IVS 2.1 in 18.4%.[47] Furthermore, IVS 2.1 mutation was the highest documented mutation in Karballa[34] and in the northeastern provinces of Iraq.[20,48] In Kirkuk, however, the earlier mutation represented the second highest, accounting for 20% of documented alleles.[45] Interestingly, this allele was the most common one related to BT in all regions of Iran, with a prevalence of more than 60%, which is the highest recorded prevalence in the world.[49-51]

The third-ranking allele in the current study was Codon 44(-C) that accounted for 8.8% of alleles, concentrating primarily among the TDT group. It is worth noting that Codon 44 frameshift mutation is the most common mutation involving coding sequence encountered in our study. It is caused by deletion of a single nucleotide (C), hindering normal translation and creating a severe β^0 phenotype.[52] This explains the aggregation of such allele among our TDT group. This mutation of Kurdish origin was also documented in other studies from Iraq. The closest to our finding is that of Al-Fatlaw and Hameed, who reported a frequency of 8.33%.[34] While previous molecular data from Baghdad city were quite heterogeneous, with one characterizing this allele in only 4.9%,[47] and the other exhibiting 14.5% in Baghdad,[53] generally our result lies in between these two values. The frequency of this mutation was higher in Kurdistan, as it would be expected. It has fluctuated between 23.2% in Koya city, and 12.5% in Duhok, representing the second most common allele there.[54]

The remaining identified alleles were less frequent and exhibited more sporadic frequencies. The reasons behind such allele clustering could be the high consanguinity rates, as Iraq is among the countries with the highest reported rates of consanguinity in the world, echoing cultural and tribal mentality that favors marrying first degree relatives.[55] On top of that, years of genetic processes as; natural selection and genetic drift, have potentially led to dominance of certain allelic variants amid dilutions of others which have slowly become obsolete.

Conclusions

Finally, our study has identified two predominating genotypes, namely homozygous IVS 1.110 (G>A) and IVS 2.1 (G>A), that have consistent frequencies across Iraq, indicating allele aggregation. Nevertheless, we strongly advocate for the induction of larger sample-sized studies, as this may reveal less frequent alleles that remain elusive with smaller sample sizes to provide a broader understanding of the molecular background of this disorder. In addition, our study has highlighted the most recurrent comorbidities, underscoring skeletal abnormalities and cholelithiasis as the major contributors. It is our belief that the true picture of BT comorbidities in Iraq is far dimmer than what has been described, hence we recommend for the construction of a specifically tailored comprehensive preventive program to evade such complications.

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Conflicts of interest
There are no conflicts of interest.

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