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Comparison between H63D and G71D gene mutation effects on iron overload in Iraqi patients with β-thalassemia major: A case–control study

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Abstract:

BACKGROUND: Iron overload certainly will develop in β-thalassemia major. Iron homeostasis was mostly regulated by hepcidin that synthesized in the liver and encoded by the hepcidin antimicrobial peptide (HAMP) gene. HAMP and HFE genes, respectively, encode iron-regulating proteins (Hepcidin and HFE). The iron overload's possibility will increase if there is an interaction between β-thalassemia and HAMP-HFE gene mutations.

AIM OF STUDY: In β-thalassemia major patients, we need to identify mutations in iron-regulating genes (HAMP and HFE genes), their impact on the iron overload, and their association with some clinicopathological parameters.

PATIENTS AND METHODS: During a period of 5 months from (November 2020 to March 2021), a case–control study was conducted. It included 80 patients and controls aged ≥14 years and divided into two groups: thalassemic patient group included 40 patients who were diagnosed by complete blood count, blood film, and hemoglobin – electrophoresis as β-thalassemia major and control group included 40 unrelated, apparently healthy controls that were age and gender matched with thalassemic patient group. Complete blood count, liver and renal function tests, serum ferritin, and DNA extraction were performed.

RESULTS: There was a statistically significant difference between study groups by H63D mutations. The proportion of CG genotype was significantly higher among thalassemic patient group than that in controls. There was no statistically significant difference ($P = 0.082$) between study groups by G71D mutations. Serum ferritin and Alanine transaminase (ALT) levels were significantly higher in patients with CG and GG genotypes compared to that in patients with CC genotype of H63D.

CONCLUSION: H63D is associated with iron overload in β-thalassemia patients with unapparent effect on biochemical and hematological data except for ALT and serum ferritin. This could allow early diagnosis and proper treatment to overcome the complications of iron overload in those patients.

Keywords:

G71D, gene mutation, H63D, Iraq, iron overload, β-thalassemia major

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Introduction

Mutations in the β-globin gene clusters on chromosome 11 cause β-thalassemia, which is inherited as an autosomal recessive disease. This disorder in hemoglobin is distinguished by reduced or absence of globin chains synthesis.[1] Depending on the basis of β-globin chain imbalance, severity of anemia, and clinical picture at presentation, β-thalassemia comprises three main categories: major, intermedia, and minor.[2] Thalassemia is one of the top five major birth abnormalities, according to reports.[3]

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In β-thalassemia major, the patients often present with severe anemia that caused by inadequate synthesis of HbA in the first 3–4th months of life. However, depending on the mutation type and HbF production, the transfusion might be delayed up to 2 years of age. If patients do not obtain adequate transfusion therapy, growth retardation, hypersplenism, hepatosplenomegaly, bone changes that occur by bone marrow enlargement, and thalassemia face (frontal bossing, maxillary hyperplasia, depressed nasal bridge) will be developed.[4] Recent studies suggest that each year between 300,000 and 400,000 babies born with a severe hemoglobin disorder (23,000 with β-thalassemia major) and up to 90% of these births occur in low- or middle-income countries.[5] Frequency of β-thalassemia in different parts of Iraq ranged from 3.7% to 4.6%. In Basrah (Southern Iraq), about 4.6% of people are carriers for β-thalassemia gene. There were over 2000 cases of thalassemia in the Kurdistan area and Kirkuk, with around 30000 people were carriers of β-thalassemia. In Mosul, 1028 β-thalassemia cases were registered.[4,6-8] Iron homeostasis was mostly regulated by hepcidin that synthesized in the liver and encoded by the hepcidin antimicrobial peptide (HAMP) gene. The target cells for hepcidin were the intestinal brush border cells, reticuloendothelial macrophages, and hepatocytes.[9] In humans, a deficiency in hepcidin synthesis by a mutation in HAMP gene manifested as multi-organ damage at a young age and was categorized as juvenile hemochromatosis type 2A (an autosomal recessive disease) usually caused an excessive accumulation of iron accompanied by severe organ dysfunction, particularly in heart and endocrine glands.[10] The G71D-HAMP mutation affects 4-8 cysteines of hepcidin structure, causing glycine to be converted to aspartic acid. It considered as one of the causes of iron overload. A reduction in hepcidin expression in the liver is caused by a mutation in the gene encoding hemochromatosis (HFE) protein, according to new research.[11] H63D-HFE mutation acts as a major factor of iron overload, where increased iron absorption in the small intestine and affinity between transferrin and its receptor will be low. Hepcidin regulates iron absorption from the small intestine, a mutation in the HFE gene impacts hepcidin function.[12] The aim of this study in β-thalassemia major patients was to identify mutations in iron-regulating genes (HAMP and HFE genes), their impact on the iron overload, and their association with other clinicopathological parameters (liver and renal function tests [LFT, RFT] and serum ferritin).

Patients and Methods

Study design, setting, and time

This was a case–control study conducted in Al-Karama Teaching Hospital and private laboratoriess of ASCO learning center, Baghdad, Iraq, during a period of 5 months from (November 2020 to March 2021).

Study population and sample size

This study involved 80 patients and controls (aged ≥14 years) divided into two groups:

- Thalassemic patient group: Included 40 patients who previously diagnosed by complete blood count, blood film, and hemoglobin – electrophoresis as β-thalassemia major on regular blood transfusion at AL-Karama Teaching Hospital
- Control group: Included 40 unrelated, apparently healthy controls that were age and gender matched with case group.

Patients with other types of β-thalassemia and those who refused to participate were excluded from the study. The College of Medicine/Al-Mustansiriyah University's Institutional Review Board examined and approved this research. All study participants gave their informed consent after being fully informed about the study's goals and potential health effects.

Methods and workflow

In an ethylenediaminetetraacetic acid and gel tube, we collected 5 ml of venous blood from both patients and controls.

- A complete blood count using a fully automated hematology auto-analyzer (Mindray BC-10 produced in Korea)
- LFT by spectrophotometer (PD-307 made in Korea)
- RFT by spectrophotometer (PD-307 made in Korea)
- Serum ferritin: By ELISA from 30 µl of serum by ICHROMA I
- DNA extraction: The following steps were used to isolate genomic DNA from a blood sample using Promega, ReliaPrep™ Blood gDNA Miniprep System:
  - Blood sample was mixed for 15 min in a roll mixer at room temperature
  - For each 1.5 ml microcentrifuge tube, 20 µl of proteinase K (PK) solution was distributed, then 200 µl of blood was added and briefly mixed
  - 200 µl of cell lysis buffer was added and for around 10 s mixed by vortex for cell lysis.
  - For 30 min in a water bath at 56°C, all mixtures were incubated
  - During incubation of blood sample, a ReliaPrep™ Binding Column was placed in an empty collecting tube
  - The tube was removed from the water bath after incubation and 250 µl of binding buffer was added and for about 10 s vortex mixed
  - All contents of tubes were transferred to the ReliaPrep™ Binding Column and for 3 min centrifuged at 12000 rpm

- Removed and discarded the collection tube containing flow
- Put the binding column in a fresh collection tube. 500 µl of column wash solution was added For column washing and centrifuged for 3 min at maximum speed, the flow was discarded and we repeated this step for three times
- The column was put in a clean 1.5 ml microcentrifuge tube after the washing step and add 100 µl of nuclease-free water to the column
- Then after 5 min, the 1.5 ml tube with column centrifuge at 5000 rpm for 5e min
- The ReliaPrep™ Binding Column after centrifuge was rejected and eluate kept.

Quantitation of DNA

To identify the quality of samples for downstream applications, a Quantus Fluorometer was utilized to distinguish the concentration of extracted DNA. One micromilliliter of DNA was combined with 199 µl of diluted QuantiFluor Dye. DNA concentration values were observed after 5 min of incubation at room temperature. The concentration of DNA varies between 25 and 60 ng/µl.

- Macrogen produced lyophilized forms of the G71D-Fo, G71D-Ro, G71D-Fi, and G71D-Ri gene primers. As a stock solution, lyophilized primers were dissolved in nuclease-free water to a final concentration of 100 pmol/µl. To make a working primer solution of 10 pmol/µl, combine 10 µl of primer stock solution (stored at –20 C) with 90 µl of nuclease-free water
- Macrogen Company provided lyophilized forms of the H63D-F and H63D-R gene primers. As a stock solution, lyophilized primers were dissolved in nuclease-free water to a final concentration of 100 pmol/µl. To make a workable primer solution of 10 pmol/µl, combine 10 µl of primer stock solution (stored at freezer –20 C) with 90 µl of nuclease free water
- To confirm the presence of amplification, after polymerase chain reaction (PCR) amplification, agarose gel electrophoresis was approved. On the extracted DNA criteria, PCR was completely dependent
- Solutions were 1X TAE buffer, DNA ladder marker, and ethidium bromide (10 mg/ml)
- Agarose preparation:
  - Into a flask, 100 ml of 1X TAE was dispensed
  - To the buffer, we add 1.5 g (for 1.5%) agarose
  - Using a microwave, the solution was heated for boiling until the gel particles were completely dissolved
  - Add 1 µl of ethidium bromide (10 mg/ml) to the agarose
  - In order to get mixed and prevent bubbles, the agarose was stirred
  - At 50°C–60°C, the solution was left to cool down.
- Horizontal agarose gel casting: After both ends of the gel tray were sealed with cellophane tapes, the agarose solution was put into the gel tray and allowed to solidify at room temperature for 30 min. The gel was inserted in the gel electrophoresis tank after the comb was carefully removed. 1X TAE-electrophoresis buffer was added to the tank until the buffer level reached 3–5 mm above the gel's surface
- PCR products loading: For the PCR product, 5 µl was directly loaded to well. Electrical power was turned on at 100 volt/50 mAmp for about 60 min. DNA moves from cathode to plus anode poles. The ethidium bromide-stained bands in the gel were be visualized by using a gel imaging system (336 nm UV source).
- Standard sequencing: PCR products for H63D gene were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by E-mail, then analyzed using Generous software.

Statistical analysis

Statistical Package for Social Sciences version 26 - SPSS 26 - (IBM Company, New York, USA) was used to analyze the data. The information is displayed in form of a mean, standard deviation, range, frequencies, and percentages. When the predicted frequency was less than 5, the Fisher's exact test was employed, while Chi square test was used to analyze the connection between study groups and certain information. An Independent *t*-test was used to compare the continuous variables accordingly. $P < 0.05$ was considered significant.

Results

In this study, the mean age was 22.62 ± 3.4 years. There was no statistically significant variance between the study groups ($P \geq 0.05$) in age and gender. There was a statistically significant difference between study groups by H63D mutations. The proportion of CG genotype was significantly higher among the thalassemic patient group than that in controls (35%, $P = 0.027$). There was no statistically significant variance ($P = 0.082$) between study groups by G71D mutations, as shown in Table 1.

Serum ferritin and ALT levels were significantly higher in patients with CG and GG genotypes compared to that in patients with CC genotype of H63D (3871.3 versus 2262.8 µg/l, $P = 0.007$; and 41.88 versus 26.33 U/l, $P = 0.017$, respectively). Other biochemical parameters revealed no significant difference ($P > 0.05$) between genotypes of H63D mutation [Table 2].

There were no significant associations ($P \geq 0.05$) between genotypes of H63D and each of blood group and complications of β-thalassemia [Table 3].

Discussion

β-Thalassemia was generally characterized by the potential development of iron overload and that exacerbated with coinheritance of H63D polymorphism.[13] In β-thalassemia major, there was a rise in serum iron, transferrin saturation, and ferritin level.[14] In this study, proportion of CG genotype was significantly higher among the case group than that in controls (35%, $P = 0.027$), while there was no statistically significant difference ($P = 0.082$) between study groups by G71D mutations.

This result was agreed with studies conducted by Al-Abedy *et al*. in Iraq 2019,[15] Enein *et al*. 2016[16] and Mokhtar *et al*. 2013 in Egypt,[17] Rahmani *et al*. in Iran 2019,[18] and Sharif *et al*., 2019[19] and Selvaraj *et al*., 2021[20] in Pakistan. While other studies performed by Yang J *et al* in China 2018[21], & by Shah M *et al* study in Pakistan 2020 [22] found a significant difference with our study where $P = 0.787$. This might be due to differences in the sample size and genetic distribution according to different geographic areas. In β-thalassemia major, multiple blood transfusions, ineffective erythropoiesis, and increased iron absorption will lead to iron overload and subsequent disorders. The hemochromatosis was often observed in β-thalassemia major, H63D where histidine replaced to aspartate. This will prevent abnormal HFE protein from connecting to β2-microglobulin on cell surface, then excessive iron was absorbed through the crypt of the cells and passed into circulation. The role of H63D mutation polymorphism in β-thalassemia had been studied in areas with a high H63D incidence, such as in southern Europe and Asia.[21] Studies from Italy, Portugal, India, and Egypt suggested that iron overload could be caused by the interaction of-thalassemia with H63D mutations (homozygous or heterozygous). However, Italy, India, Thailand, Brazil, and Spain found that iron status was unrelated to H63D mutation status according to other studies. The difference may be due to the sample size, hereditary variations in different racial populations, the sex ratio, and thalassemia severity. In these studies, patients with β-thalassemia had elevated ferritin levels with H63D mutation.[23] Our study revealed that serum ferritin higher in patients with CG and GG genotype compared to that with CC genotype (wild) of H63D gene and this is agreed with many other studies.[16,18,19,22] Very high ferritin levels that predominantly measured in β-thalassemia patients suggest this analysis as a monitoring means for iron load in these patients, primarily when other methods

Table 1: Comparison between study groups by certain features

Variable	Study groups		*P*
	Thalassemic patient (*n*=40), *n* (%)	Control (*n*=40), *n* (%)	
Gender			
Male	13 (32.5)	13 (32.5)	0.998
Female	27 (67.5)	27 (67.5)	
Genotype of H63D mutate on			
CC	25 (62.5)	35 (87.5)	0.027
CG	14 (35.0)	4 (10.0)	
GG	1 (2.5)	1 (2.5)	
Genotype of G71D mutation			
GA	30 (75.0)	36 (90)	0.082
AA	3 (7.5)	1 (2.5)	
GG	7 (17.5)	3 (7.5)	
Age (years), mean±SD	23.32±3.9	22.01±3.2	0.101

SD=Standard deviation

Table 2: Comparison between genotypes of H63D mutation by laboratory parameters

Laboratory parameters	Genotype of H63D mutation, mean±SD		*P*
	CG and GG	CC	
Hematological			
WBC (×10⁹/L)	9.18±7.07	8.13±3.35	0.591
RBC (million cells/mcL)	3.32±0.39	3.37±0.41	0.693
Hb (g/dL)	8.83±1.22	8.84±0.81	0.974
PCV (%)	26.27±3.71	25.63±2.95	0.551
PLT (/μl)	387.3±208.6	315.4±162.7	0.232
Biochemical parameters			
Serum ferritin (μg/l)	3871.3±1696.3	2262.8±1463.6	0.007
ALT (U/l)	41.88±21.12	26.33±17.65	0.017
AST (U/l)	37.68±17.97	28.27±14.76	0.08
ALK (U/l)	148.2±72.48	130.62±65.15	0.432
Serum creatinine (mg/dl)	0.56±0.22	0.56±0.16	0.942
Blood urea (mmol/l)	4.54±1.05	4.61±1.30	0.863

WBC=White blood cell, RBC=Red blood cell, ALT=Alanine aminotransferase, AST=Aspartate aminotransferase, Hb=Hemoglobin, PCV=Packed cell volume, PLT=Platelet count

Table 3: Association of genotypes of H63D with blood group and β-thalassemia complications

Clinical characteristics	Genotype of H63D mutation		Total (*n*=40), *n* (%)	*P*
	CG and GG (*n*=15), *n* (%)	CC (*n*=25), *n* (%)		
Blood group				
A	4 (26.7)	11 (73.3)	15 (37.5)	0.596
B	3 (33.3)	6 (66.7)	9 (22.5)	
AB	1 (50.0)	1 (50.0)	2 (5.0)	
O	7 (50.0)	7 (50.0)	14 (35.0)	
β-thalassemia complications				
Osteoporosis	1 (25.0)	3 (75.0)	4 (10.0)	0.252
Osteopenia	2 (50.0)	2 (50.0)	4 (10.0)	
Splenomegaly	2 (100)	0	2 (5.0)	
Combined	10 (33.3)	20 (66.7)	30 (100)	

to measure the iron overload are not available. Moreover, ferritin that was considered as the leading indicator for iron overload.[24] There was frequently an increase in serum iron, transferrin saturation, and ferritin level in thalassemia major. Ferritin was a high molecular weight iron-containing storage protein found mostly in the liver, spleen, bone marrow, and other tissues. Its main actions include removing excess iron from cells, converting it to a harmless soluble form, and providing a mobilizable store of iron that could be relied upon when needed. Serum ferritin levels were discovered to be clinically important; it reflects whole-body iron reserves. It is a reflection of iron storage in health and sickness.[14]

Conclusion

This study found that H63D mutations of HFE gene are common among Iraqi β-thalassemia patients and they possessed a higher level of ferritin in comparison with those who did not possess this mutation. This gives an idea that H63D is associated with iron overload in β-thalassemia patients with unapparent effect on biochemical and hematological data, except for ALT and serum ferritin (this may be due to therapeutic measures). To assess the prevalence and the role of H63D and G71D mutations on iron overload, it is recommended to take a larger sample size and wider range of ages. Hence, the detection of H63D mutation could allow early diagnosis and proper treatment to overcome the complications of iron overload in those patients.

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Conflicts of interest
There are no conflicts of interest.

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