Frequency of human hemochromatosis \textit{HFE} gene mutations and serum Hepcidin Level in iron overload \(\beta\)-thalassaemia Iraqi patients

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Abstract

\textbf{Background:} Thalassemia is an important hematological disorder. Iron overload is the main cause of morbidity and mortality in patients with \(\beta\)-thalassemia. The possibility of iron overload development may be increased by Interaction between thalassemia and \textit{HFE} gene mutations. This study aims to investigate the possible association between serum hepcidin level as an indicator of iron concentration and the presence of \textit{HFE} gene mutations or the effects of these gene's polymorphisms on blood hepcidin level and iron overload in the \(\beta\)-thalassemia patients.

\textbf{Methods:} A eighty two of \(\beta\)-thalassaemia (56 BTM and 26 BTI) patients and fifty normal controls were included in this study. Hepcidin levels along with iron parameter were determined. We assessed the frequency of the H63D, C282Y and S65C mutation of the \textit{HFE} gene and the correlation between these mutations and the iron overload in \(\beta\)-thalassemia patients. Genotyping of H63D, C282Y, and S65C of \textit{HFE} variants performed by T-Plex real-time PCR.

\textbf{Results:} Serum hepcidin level was found to be reduced in BTI and BTM patients as compared to controls. Hepcidin level is significantly lower in BTM compared to control (\(P<0.001\)). High serum ferritin level and serum iron are detected in BTM and BTI patients as compared to controls. Among 82 \(\beta\) - thalassaemia (56 BTM and 26 BTI) tested, the observed C282Y, H63D and S65C allele frequency were 4.46\%, 15.1\% and 1.7\% respectively in \(\beta\)-thalassaemia major, 1.9\%, 11.5\% and 2\% respectively in \(\beta\)-thalassaemia intermediate and 2\%, 6\% and 0.0\% respectively in the control group. The S65C was uncommon or rare and we found one subject heterozygous for S65C mutation in BTI and two in BTM. We did not observe any subject with compound heterozygote mutation.

\textbf{Conclusion:} We observed that there was not a statistical significance correlation between the Hepcidin levels and the presence of \textit{HFE} mutations (\(p\) value = 0.10). The controversies about the relation between \textit{HFE} mutations and hepcidin in iron overload \(\beta\) - thalassaemia patients still need more studies considering novel factors and mutations involved in such mechanisms such as ferroportin, hemojuvelin, \(\beta\)-globin, and transferrin receptor-2 (TFR2).

\textbf{Keywords:} \(\beta\)-thalassaemia, \textit{HFE} Gene Polymorphisms, Hepcidin level.

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Introduction

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Thalassaemia syndromes are the most commonly inherited single-gene disorders worldwide [1]. About 3% of the world population (150 million) carry the β-thalassaemia genes [2]. β-Thalassaemia is an autosomal recessive disorder of hemoglobin synthesis. It is caused by a direct down-regulation in the synthesis of structurally normal β chains. [3]. The clinical manifestations of β-thalassaemia are extremely diverse, spanning a broad spectrum from the transfusion dependant state of thalassaemia major to asymptomatic state of thalassaemia trait. Between these two clinical extremes lies the clinical syndrome of thalassaemia intermedia which comprises a diverse spectrum of phenotypes from a condition that is slightly less severe than transfusion dependence to one that is asymptomatic and often identified through a routine blood test[4]. A common complication of β-thalassaemia involves organ damage from iron overload (hemosiderosis) which is not only an inevitable consequence of prolonged transfusion therapy but also depends on the increased intestinal iron absorption which is proportional to the degree of erythroid hyperplasia[5]. Hepcidin that is mainly synthesized by hepatocytes plays a role in the control of iron absorption and recycling. Hepcidin regulation in hepatocytes is affected by certain factors such as the amount of available systemic iron, hepatic iron reserve, erythropoietic activities, hypoxia, inflammation, and infection. The regulation of hepcidin expression is a complex process that requires coordination among several proteins. Hepcidin dysregulation contributes to developing many diseases such as the anemia of chronic disease, iron-refractory iron deficiency anemia, cancer, hereditary hemochromatosis, and ineffective erythropoiesis such as β-thalassaemia. Iron regulation by hepcidin is clinically important in anemia due to iron overload that occurs in β-thalassaemia[6]. HFE gene mutations have been frequently detected in Hereditary Haemochromatosis (HH). It is an autosomal recessive disorder characterized by increased intestinal absorption of iron and progressive iron overload [7]. The HFE gene is located on the short arm of chromosome 6 at position 21.3; its mutation is a major cause of hemochromatosis[8]. Hereditary hemochromatosis is an iron overload disorder that causes damage to several organs and is often due to HFE dysfunction[9]. The HFE plays its most prominent role in the regulation of cell iron absorption that is accomplished by binding to TFRC (Transferrin Receptor). When a cell has adequate amounts of iron, it competitively binds to TFR and prevents iron absorption via endocytosis[10]. To date, many polymorphisms of the HFE have been identified out of which three missense mutations in the HFE gene are associated with hemochromatosis. About 85% of cases carry cysteine-to-tyrosine substitution at amino acid position 282 in the HFE gene (C282Y) [11]. This mutation is responsible for about 60% of hemochromatosis cases in Mediterranean populations [12], about 15%–20% carry aspartic acid-to-histidine substitution at amino acid position 63 (H63D) [13], and a third mutation results from serine-to-cysteine conversion in amino acid position 65 (S65C) [14]. The last 2 mutations are associated with a milder form of hemochromatosis, but the compound heterozygous state of one of them with the C282Y mutation increases the risk of developing iron overload [5]. The interaction of HFE mutations with the thalassaemias may have a synergistic effect, increasing the iron intake and storage [11].

This study aimed to determine the prevalence of HFE gene mutations (C282Y, H63D and S65C) among b-thalassemia patients and to investigate the effect of these mutations on their hepcidin levels.

Subjects and Methods

Subjects

Fifty controls and eighty two β-thalassemic patients were regularly attending the hematology clinic in Thalassemia hematology center in Al Kut women and children Hospital to follow up of Hb level and iron status either for transfusion and chelation. The diagnosis of β-thalassemia was based on clinical presentation, hematological indices, iron overload, and hemoglobin electrophoresis. (56 BTM and 26 BTI) were randomly selected for this study. This study was conducted in Kut city during the period of February 2018 to April 2019.

Assay methods

Laboratory tests including a 2mL sample of venous blood was withdrawn from each patient and control added to the tubes containing ethylenediaminetra-acetic acid EDTA anticoagulant.. Also, 3 mL sample of venous blood withdrawn from each participant was added to the tubes containing clot activator, serum was separated and preserved at -20°C, to the laboratory to check iron overload parameters, serum hepcidin, and ferritin levels. The ferritin level assay was carried out by Mini VIDAS system (enzyme immunoassay). Transferrin saturation was calculated by dividing the serum iron level by total iron-binding capacity and multiplied by 100. The iron and TIBC were assessed by colorimetric measurement of iron and TIBC was done. Serum hepcidin was determined using commercial ELISA kits SinoGeneClon Biotech, (China)by Human Reader Systems, (Germany) It is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a monoclonal antibody directed towards the antigenic site.
Genomic DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by Promega ReliaPrep genomic DNA purification (USA) Kit. Genotyping of C282Y, H63D and H63D of HFE variants was performed by tetra-primer ARMS PCR assay, SYBR Green I-based real-time PCR, SYBR green-based SNP genotyping methods. This assay is based on the Tm discrimination of the amplified allele-specific amplicons in a single tube. Because different PCR products generally have different melting temperatures (Tm), depending on their GC content, length, and sequence [15]. Primers were designed according to the using Web-based software created by [16](http://cedar.genetics.soton.ac.uk/public_html/ primer1.html). We used the BLAST program at http://www.ncbi.nlm.nih.gov/blast to determine the specificity of the primers. To obtain amplicons with distinct melting points, the hypothetical Tm values were calculated using known software or Web sites (for example, http://eu.idtdna.com/ analyzer/Applications/OligoAnalyzer/) (Table 1).

<table>
<thead>
<tr>
<th>HFE gene</th>
<th>primer sequences (5'→3')</th>
<th>(Nucleotide position)</th>
<th>Product Size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dbSNP-ID:rs1800562) C282Y</td>
<td>ACCCCCTGGAAGAGAGAT ATAATTG</td>
<td>10633</td>
<td>196</td>
</tr>
<tr>
<td>Forward inner primer (G allele):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse inner primer(A allele):</td>
<td>ATCCAGCTCCAGGCTGTGTGCTCCTTGT</td>
<td></td>
<td>176</td>
</tr>
<tr>
<td>Forward outer primer:</td>
<td>CCCAGAACATCACCAGTGAAGTGGC TGAA</td>
<td></td>
<td>361</td>
</tr>
<tr>
<td>Reverse outer primer:</td>
<td>CTCAGCCACCCCTAACAAAGACAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(dbSNP-ID:rs1799945) H63D</td>
<td>TGGATGACCGAGCTGTGTGTGCTTCTTCTGC</td>
<td>8617</td>
<td>199</td>
</tr>
<tr>
<td>Forward inner primer (C allele):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse inner primer(G allele):</td>
<td>CGGGGGCTCCACACCGGAGCTCCTACGTC</td>
<td></td>
<td>219</td>
</tr>
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<td>Forward outer primer:</td>
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<td>362</td>
</tr>
<tr>
<td>Reverse outer primer:</td>
<td>CCCCTCTCCACATACCCTTGCTGTGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(dbSNP-ID:rs1800730) S65C</td>
<td>ACCAGCTGTTCGTTCTATGAT</td>
<td>8677</td>
<td>197</td>
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<tr>
<td>Forward inner primer (A allele):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse inner primer(T allele):</td>
<td>GGAGTTGACGGCTCCACACGCGGTCA</td>
<td></td>
<td>224</td>
</tr>
<tr>
<td>Forward outer primer:</td>
<td>TGAAGTATGAACAGGACCTGTGGACCTCA</td>
<td></td>
<td>365</td>
</tr>
<tr>
<td>Reverse outer primer:</td>
<td>AGGCCCTCTCCACATACACCTTGTGCTGTT</td>
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</tr>
</tbody>
</table>

T-Plex real-time PCR.

Tetra-primer ARMS PCR assay, SYBR Green I-based real-time PCR, SYBR green-based SNP genotyping methods generally depend on the allele refractory mutation system–polymerase chain reaction (ARMS PCR) assay adapted to real-time instruments and melting-point analysis with primer design strategies to detect the SNP of interest. This assay is based on the Tm discrimination of the amplified allele-specific amplicons in a single tube, Because different PCR products generally have different melting temperatures (Tm), depending on their GC content, length, and sequence [15]. The specificity, sensitivity, and robustness of the assay were evaluated for common mutations in the HFE genes. Tetra-
Plex (T-Plex) real-time PCR it uses four primers for each genotyping and detected both mutant and wild-type alleles in the same reaction, was performed on Real time PCR Mx3005P QPCR and genotypes were identified based on the characteristic melting temperature of the allele-specific fragments using a melting-curve program.

Real-time PCR method T-Plex

Real-time PCR was performed in a total volume of 20 µl containing 10 µl of 2x GoTaq® qPCR Master Mix Promega, Madison, WI, (USA). 1 µl of each primer per reaction, 4 µl of the genomic DNA dilution (10 ng/µl), and distilled water. The PCR protocol on Real time PCR Mx3005P QPCR Systems Agilent Technologies. It was follows: an initial denaturation step (95 °C for 2 min) was followed by amplification and quantification steps repeated for 30–40 cycles (95°C for 15 s denaturation, 60 °C for 1 min annealing & extension), with a single fluorescence measurement at the end of the elongation step at 72 60 °C), a melting-curve program analyzed the data, and the reaction was terminated by cooling to 40 °C. Melting curves were constructed by lowering the temperature to 65 °C and later increasing the temperature by 0.2 °C/s to 98 °C while continuously measuring the change in fluorescence. Tm values were manually assigned from a plot generated by the Agilent Mx3005P or 2.0 instrument of the negative derivation of fluorescence versus temperature (-dF/dT) of the melting curve for amplification products measured at 530 nm.

Statistical analysis

IBM Statistical package for social science (SPSS) version 23 (USA) was performed to determine all the statistical relations between the categorial group. Parametric variables were designated as mean and standard error of mean (SE). Genotype / allele frequencies were calculated by direct gene counting. Hardy-Weinberg equilibrium (H-WE) equation was computed to determine the differences between observed and expected genotype/allele frequencies using Chi-square goodness-of-fit test. Association analysis was conducted using WinPepi software program to determine Pearson Chi-square (with Yates correction), odds ratio (OR), confidence interval (C · I) of odds ratio at 95%, etiological / preventive fraction (EF/PF) [17]. The differences for all relations were based on two-tailed P-value at significant level≤0.05.

Results

Biochemical Results

Serum hepcidin (µg/L) was detected in both groups of patients and controls. There was a highly significant decrease in BTI and BTM patients when compared with healthy controls, with a mean ±SD of 45.3±25.27, 49.8±39.1 and 85.8 ±56.7, respectively (P < 0.001). As regards serum iron (µg/dl) in both groups patients and controls, there was a highly significant decrease in healthy controls when compared with BTM and BTI patients, with a mean ±SD of 84.6 ±29.4, 136.8 ±34.2, and 168.6 ±45.2 respectively (P < 0.001). As regards serum ferritin (ng/ml) in both groups patients and controls, there was a highly significant increase in BTM patients when compared with BTI patients and healthy controls, with a mean ±SD of 4044±2394, 1758 ±1136 and 127.7 ±40.29, respectively (P < 0.001). Serum TIBC (µg/dl) was significantly higher in controls than in both groups patients and controls, with a mean ±SD of 321±53.1, 255.8±33.3 and 241.7±51.68, respectively (P < 0.001). Transferrin saturation (TS%) in both groups patients and controls, there was a highly significant increase in BTM patients when compared with BTI patients and healthy controls, with a mean ±SD of 70.3±17.23, 54.4±16.6and 24.3±7.6, respectively (P < 0.001), as shown in (Table 2).

Table 2: Iron parameter for Iraqi BTM and BTI patients and controls.

| Hepcidin µg/L | 49.83 ±39.18 | 45.3 ±25.27 | 85.8 ±56.75 | 0.001 |

T-Plex real-time PCR genotyping of HFE gene

When T-Plex real-time PCR was performed with all the primers, the melting-curve analysis revealed unique melting-point curve shapes for each of the combination of the co-amplified allele-specific products and nonallelic gene-specific product. Thus, screening of the mutation with the known DNA samples revealed different melting-point curve shapes for wildtype, heterozygous, and homozygous samples. All the amplicons have different Tm values. The T-Plex real-time PCR assay was tested to genotype the 845G→A, 187C→G, and 193A→T mutation respectively in the HFE gene. For the 845G→A allele (C282Y) wild type and homozygous mutant samples had melting peaks with Tm values of 86.85 and 84.20 °C, respectively. Heterozygous mutant samples had two melting-point peaks with Tm values of 84.30 and 86.60 °C (Figure-1). The genotype for (H63D) 187C→G wild type and homozygous mutant samples had melting peaks with Tm values of 84.40 and 80.60 °C, respectively. Heterozygous mutant samples had two melting-point peaks with Tm values of 84.35 and 80.65 °C (Figure-2). Finally, The genotype for (S65C) 193A→T wild type and homozygous mutant samples had melting peaks with Tm values of 85.85 and 81.75 °C (Figure-3).

(B)

Figure-1: T-Plex Real-time PCR with appropriate primers was performed to amplify the nonallelic gene-specific fragment and each allele in separate reactions. Then melting-curve analysis was performed to show the melting point of each allele-specific and nonallelic gene-specific amplicon. The genotyping can be performed based on the Tm of specific amplicons and unique shape of the melting peaks. T-Plex real-time PCR assay for HFE 845G→A genotyping. (A) Melting-curve analysis for the allele-specific and nonallelic gene-specific amplicons. Briefly, during the amplification reaction, a 316-bp region of the HFE gene was amplified with the outer primers, and the allele-specific amplification produced 196- and 176-bp PCR products specific for the 845G→A alleles, respectively. (B) Separation of the real-time PCR products by 2% agarose gel electrophoresis stained by ethidium bromide to distinguished the three PCR products showing C282Y genotype (wild type) (GG)in lane 1, 2, 5, 6, 8 (196 bp), (heterozygous) (GA) in lane 3 (176 bp, 196 bp), lane 7 carrying homozygous mutant (AA) (176 bp) allele and lane 4 NCT.

Figure 2: T-Plex real-time PCR assay for HFE 187C→G genotyping. (A) Melting-curve analysis for the allele-specific and nonallelic gene-specific amplicons. Briefly, during the amplification reaction, a 362-bp region of the HFE gene was amplified with the outer primers, and the allele-specific amplification produced 219- and 199-bp PCR products specific for the 187C→G alleles, respectively. (B) Separation of the real-time PCR products by 2% agarose gel electrophoresis stained by ethedium bromide to distinguished the three PCR products showing H63D genotype (wild type) (CC) in lane1,2,3,4 (199 bp), (heterozygous)(CG) in lane 6,7,9,10,11,12,13,14,15 (199 bp, 219 bp), lane 5 carrying homozygous mutant (CC) allele (219bp) and lane 16 NCT.

Figure 3: T-Plex real-time PCR assay for HFE 193A→T genotyping. (A) Melting-curve analysis for the allele-specific and nonallelic gene-specific amplicons. Briefly, during the amplification reaction, a 362-bp region of the HFE gene was amplified with the outer primers, and the allele-specific amplification produced 219- and 199-bp PCR products specific for the 193A→T alleles, respectively. (B) Separation of the real-time PCR products by 2% agarose gel electrophoresis stained by ethedium bromide to distinguished the two PCR products showing S56C genotype (wild type) (AA) in lane1,6,7, (197 bp), (heterozygous)(AT) in lane 4,5 (197 bp, 224 bp) and lane 2 NCT.
Genotyping of *HFE* gene analysis.

**Table 3:** Genotype/allele frequencies and epidemiological parameters for Iraqi BTM patients & controls

<table>
<thead>
<tr>
<th>HFE SNP</th>
<th>Controls (50)</th>
<th>Epidemiological parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observe N (%)</td>
<td>Expected N (%)</td>
</tr>
<tr>
<td>H63D- 8617 (rs179994)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>CC</td>
<td>45(90)</td>
<td>44.18(88.3)</td>
</tr>
<tr>
<td>CG</td>
<td>4(8)</td>
<td>1.29(2.3)</td>
</tr>
<tr>
<td>GG</td>
<td>94(94)</td>
<td>5.64(11.3)</td>
</tr>
<tr>
<td>C</td>
<td>5(10)</td>
<td>0.18(0.36)</td>
</tr>
<tr>
<td>G</td>
<td>1(2)</td>
<td>0.03</td>
</tr>
<tr>
<td>S282Y- 10633 (rs180056)</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>GG</td>
<td>48(96)</td>
<td>1.96(3.92)</td>
</tr>
<tr>
<td>GA</td>
<td>2(4)</td>
<td>0.02(0.01)</td>
</tr>
<tr>
<td>AA</td>
<td>107(95.5)</td>
<td>0.02(0.01)</td>
</tr>
<tr>
<td>G</td>
<td>1(1.78)</td>
<td>0.00</td>
</tr>
<tr>
<td>C</td>
<td>5(4.46)</td>
<td>0.00</td>
</tr>
<tr>
<td>S65C- 8766 (rs180073)</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>AA</td>
<td>54(96.4)</td>
<td>4.63</td>
</tr>
<tr>
<td>AT</td>
<td>2(3.57)</td>
<td>0.00</td>
</tr>
<tr>
<td>T</td>
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<td>0.49</td>
</tr>
<tr>
<td>A</td>
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<td>0.00</td>
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<tr>
<td>G</td>
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| HWE=Hardy-Weinberg equilibrium, OR: Odds ratio, EF: Etiological fraction, PF: Preventive fraction, CI: confidence interval, ND=Not determined, N: Number.

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To investigate C282Y, H63D, and S65C SNPs, T-Plex real-time PCR technique was performed to determine the genotypes and their corresponding alleles analysis of the wild type and mutant type positions of C282Y, H63D and S65C SNPs. However, there was a departure from HWE for C282Y SNP genotypes in both patients and controls. In a recessive model analysis of the (CC vs. CY + YY) C282Y locus showed the genotype distributions of the CC, CY, and YY genotypes were 92.8%, 5.3%, and 1.7% in BTM, 96.1%, 3.8%, and 0.0% in the BTI patients and 96%, 4%, and 0.0% in the healthy controls, respectively. The Y(A) allele frequency was significantly higher in the BTM group than in the BTI and control group (4.46%, 1.9%, and 2%), and the results also showed that there was an equal significant distribution of (CC)GG genotype/ G allele in in control and BTI compared to BTM patients group was decreased (96%

Discussion

In our study the Serum ferritin was significantly lower in control and thalassemia intermedia than in those with thalassemia major. This result was compatible with Origa et al, who reported that Serum ferritin was significantly lower in patients with thalassemia intermedia than in those with thalassemia major, despite the liver iron concentration being comparable. Lower serum ferritin in thalassemia intermedia reflects iron accumulation predominantly in hepatocytes rather than in macrophages, as observed by Perl’s stain. This phenotype is similar to that occurring in hereditary hemochromatosis and is likely the consequence of hepcidin deficiency [18]. As in previous studies [19], we also found very low or undetectable hepcidin levels in thalassemia intermedia. As a consequence of this difference in cellular iron distribution, serum ferritin levels were much lower in non-transfused patients, and did not adequately reflect the patients’ liver iron load [18]. It is known that in patients with BTI, there is also increased risk of iron overload due to increased intestinal iron absorption triggered by chronic anemia, ineffective erythropoiesis and decreased serum hepcidin [20]. The difference in hepcidin levels between thalassemia intermedia and major is almost certainly due to transfusion therapy [20]. In our study, hepcidin level in BTI and BTM patients is markedly reduced compared to both control and patients group (19.6% vs. 8%, p=0.05, OR=2.81, EF=0.1, C • I 95%=0.84–9.37), (15.3% vs. 8%, p=0.05, OR=2.09, EF=0.09, C • I 95%=0.49–8.95). Additionally, the homozygous (CC) genotype increased significantly in BTM patients as compared to controls (15.9% vs. 8%, p=0.05, OR=2.77, EF=0.03, C • I 95%=0.29–26.9; 3.8% vs. 2%, p=0.05, OR=1.96, EF=0.06, C • I 95%=0.12–31.4). Also by using a recessive model analysis of the (SS vs. SC + CC) S65C locus showed the genotype distributions of the SS, SC, and CC genotypes were 96.4%, 3.57% and 0% in BTM and 96%, 3.8%, and 0% in the BTI patients and 100%, 0%, and 0% in the healthy controls, respectively. The C (T) allele frequency was significantly equal in the BTI group than in the BTM and higher than control group (2%, 1.7% and 0%), and the results also showed that there was a significant distribution of (HH/CC genotype/ C allele in control than in two patients group (90% vs. 75%, P=0.07, OR=0.33, PF=0.6, C • I 95%=0.11–0.99; 90% vs. 80.7%, p=0.05, OR=0.47, PF=0.4, C • I 95%=0.12–1.78, respectively). The (D) allele frequency was significantly higher in the BTM group than in the BTI and control group (15.1%, 6% and 11.5%), and the results also showed that there was a increased significant distribution of (HH/CC genotype/ C allele in control than in two patients group (90% vs. 75%, P=0.07, OR=0.33, PF=0.6, C • I 95%=0.11–0.99; 90% vs. 80.7%, p=0.05, OR=0.47, PF=0.4, C • I 95%=0.12–1.78, respectively). The (D) allele frequency was significantly higher in the BTM group than in the BTI and control group (15.1%, 6% and 11.5%).

Patients of β-thalassemia major have decreased concentrations of hepcidin due to opposing influences of ineffective erythropoiesis and concomitant iron overload [23]. This results also agreed with Wrighting and Andrews who reported that the erythropoietin includes a mechanism to decrease hepcidin production [24]. Accordingly, low hepcidin levels have been reported in patients with thalassemia and other disorders with ineffective erythropoiesis. This result was in agreement with Rund and Rachmilewitz who reported that hepcidin levels were found to be low in patients with thalassemia intermedia and thalassemia major [25]. Toledano et al. reported that Several diseases with chronic iron overload such as hereditary hemochromatosis and β-thalassemia major are characterized by low hepcidin expression in the liver. The low hepatic hepcidin in these patients is probably responsible for the intestinal absorption of iron [26]. In our study the serum iron levels was a highly significant increase in BTM patients when compared with BTI patients and control. Our result that agreement with Kaddah, 2011 who reported serum iron level in BTM which is significantly higher in BTM compared to BTI and in both groups compared to control. Nyoman explained that the blood transfusion of thalassaemia patients leads to increase of serum
Iron metabolism in the body is regulated by several genes, including hepcidin antimicrobial peptide (HAMP) and human hemochromatosis (HFE, high Iron Fe) genes[6,9]. Hepcidin dysregulation contributes to developing many diseases such as the anemia of chronic disease, iron-refractory iron deficiency anemia, cancer, hereditary hemochromatosis, and ineffective erythropoiesis such as β-thalassemia. Iron regulation by hepcidin is clinically important in anemia due to iron overload that occurs in β-thalassaemia[6]. Hereditary hemochromatosis is an iron overload disorder that causes damage to several organs and is often due to HFE disfunction[9]. The HFE plays its most prominent role in the regulation of cell iron absorption that is accomplished by binding to TFRC (Transferrin Receptor). H63D mutation is much more frequency than C282Y and S65C mutation. We analyzed the frequency of three point mutations (C282Y H63D, S65C) in HFE gene related to beta-thalassaemia patient in Iraqi population. We studied the relationship between HFE gene mutations and hepcidin status in BTM and BTI found significantly higher allelic frequencies of the H63D and C282Y mutations among BTM (15.1% and 4.4%) and BTI (11.5% and 1.9%) respectively compared the controls without hemoglobinopathies(6% and 2% respectively). H63D, S65C and C282Y heterozygote genotype frequencies among BTM were 19.6%, 3.5% and 5.3% ,and BTI were 15.3%, 3.8% and 3.8% respectively while in the controls they were 8%, 0.0% and 4% respectively. The allelic frequencies obtained for HFE gene mutations C282Y, H63D and S65C in the BTM was presented in Table 1. There were significantly high allelic frequencies of the H63D, S65C and C282Y mutations among BTM 5.3%, 0.0% and 1.7% and BTI were 3.8%, 0.0% and 0.0% respectively compared with the controls (2%, 0.0% and 0.0%) respectively. In agreement with our results the allele frequencies in thalassemia patients in Brazil were 0.29%, 13.70% and 0.60% for the C282Y, H63D and S65C mutation respectively [11]. However, the role of HFE gene mutations on developing iron overload was previously studied in 41 beta-thalassaemia carriers in Egypt and it was found to be more common among beta-thalassaemia carriers compared with normal controls (H63D, S65C, and C282Y) allele frequencies were 30.5%, 13.4% and 7.3% respectively in beta-thalassaemia carriers and 10.0%, 2.5% and 0.0% respectively in the control group). The results of HFE allele frequencies in our study were similar to Panigrahi et al. [33].The results of our study also agreed with Oliveira et al. who reported allelic frequencies of the H63D, S65C and C282Y mutations among β-thalassaemia carriers of 13.7%, 0.6% and 2.4% respectively compared with 9.5%, 0.9% and 0.3% respectively among controls; the heterozygote genotype frequencies among their patients and controls were not significantly different [7].

Our results also agreed with a screening study for haemochromatosis mutations in β-thalassaemia minor patients from the Islamic Republic of Iran, which indicated significant differences in the frequencies of C282Y and H63D mutants in relation to control individuals, where the H63D and C282Y allele frequencies were 12.9% and 1.6% in patients compared with 8.7% and 0% in controls respectively [8]. our study agreed with a screening study for the frequency of H63D mutation in Syrian population was of 12.3%, the allele frequency which is very close to the values seen in the Arab countries (17.5% to 11.25%).The H63D mutation was more common in the Syrian population[34].Our results also agreed with a screening study for H63D mutation in the Jordanian population with 93 heterozygotes (21.1%), three homozygote mutants (0.68%) and an allele frequency of 11.25%. Our results showed that the H63D allele frequency higher than that found in Saudi Arabian population (8.5%) [35].on the other hand, a recent report in the Saudi population estimated allele frequency to be as high as 17.7% [36]. C282Y mutation was reported to be responsible for 60% of hereditary hemochromatosis cases in the Mediterranean population [37].There are not enough studies for the S65C mutation and we did not have adequate information about its prevalence.The S65C variant has not been investigated much and there is little information about its genetic frequency[38].In our study, we found only one individual carrying this mutation in form heterozygous mutation, and there were no homozygous mutation. In our study that only one person is a carrier of S65C mutation in heterozygous pattern. There were no S65C homozygotes in our study and heterozygotes frequency was 3.57% of our population with an allele frequency of 1.7%.The allele frequency was reported in a
Jordanian Arab population S65C was found only in one case with an allele frequency of 0.11%. S65C was detected with 0.23% heterozygous and no homozygous. [38]. Recently, S65C variant was found to have a lowering effect on iron status markers [39]. A different finding was observed in previous studies by Mellouli et al. who suggested that the inheritance of H63D mutation does not influence the severity of iron overload in beta-thalassemia patients [40]. Also, Garewal et al. suggested that the presence of H63D mutation does not increase body iron as measured by serum ferritin in beta thalassemia [41]. Previous studies have yielded conflicting results: some proposed iron overload may emerge from the association of the β-thalassemia with homozygosity or even heterozygosity for HFE mutations and others claimed that no relation exists between total body iron and HFE genotypes [42]. As a second explanation, mutations in the HFE gene other than those were tested in this study may be responsible for the iron overload and to higher serum ferritin. HFE gene mutations could be involved in increasing iron storage possibly through interaction with other genetic determinants of β-thalassaemia. This can lead to iron overload with secondary tissue harm in multiple body organs creating most of morbidity and mortality in thalassemia patients [43]. In accordance with this study Piperno et al. [44] found that mutations of HFE lead to hepcidin deficiency and the adult form of hereditary hemochromatosis.

Conclusion

This work shows that as there is a correlation between HFE mutation and hepcidin levels the presence of HFE mutation may be a predictor of susceptibility to iron overload due to deficiency of hepcidin in Beta thalassemia patient. Our results confirm the hypothesis that in some way the HFE gene mutations could be implicated in increasing iron storage when interacting with other genetic determinants of β-thalassaemia [45].

Conflicts of interest

None of the authors have any conflicts of interest relevant to this research subject.

Ethical consideration

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee.

References


