Effect of genetic polymorphism MHFR C677T in some biochemical markers in a group of βT patients


[1] College of Science, Tikrit University, Iraq.
[2],[3],[4] College of Education for Pure Science, Tikrit University, Iraq

*Corresponding author: Ali Nazhat Sabeel (alinazhat1983@gmail.com)

Summary

The current study was conducted as an attempt to know about the prevalence of the mutant genotype TT, caused by substitution mutation C – T, in MTHFR C677T and the effect of resulted genetic polymorphism in the levels of hyc in Iraqi patients of βT registered in Kirkuk public hospital, Kirkuk-Iraq, in addition to a try to reveal if any change have been happened in biochemical parameters in the group of patients compared to healthy control group. The number of patients is 50, 25 males and 25 females, the mean of age and standard deviation 8.32 ± 4.41, and 45 of the healthy control group, 20 males and 25 females, with age, mean and standard deviation 9.51 ± 3.41. The percentage of related parents is 38% in the group of patients, while it is 51.1% in the control group. Data of four of patients and seven of healthy individuals were excluded due to the failing of getting amplified output of blood DNA. Electrophoresis of PCR products after incubation with restriction enzyme Hinfl, showed 198bp band which belong to the normal allele C, and two bands of 175bp and 23bp which belong to mutant allele T. Calculation of allele frequency showed increase in the mutant allele in the group of patients which reached 0.368 compared with 0.046 in healthy control group. On the other hand, the results showed a higher frequency of homozygous genotype of mutant allele TT in the group of patients 10.86% compared to 2.32% in the healthy control group. The test of Chi2 showed a significant difference in numbers of observed and expected genotypes, calculated Chi2 value 0.66 compared to tabulated value 5.99 with 2 degrees of freedom and 0.05 level of significance, while it reached 9.84 for the control group. The results of the biochemical study showed no significant differences in levels of hyc between patients and healthy individuals. The value of mean difference and standard error mean reached (2.33 ± 1.56, t= 1.49), the levels of folate reached (1.31 ± 1.05, t= 1.24. While there were significant differences in the levels of ferritin (-387.18 ± 16.79, t= 23.05) and VB12 (202.99 ± 14.49, t= 14.0).

Key words: Genetic polymorphism, MTHFR C677T, Hyperhomocysteinemia (Hhyc), βT, PCR.

Doi : http://doi.org/10.36295/ASRO.2020.23513

Introduction

β- Thalassemia is an inherited disorder caused by mutations in globin genes that lead to a total or partial loss of synthesis of natural β-globin chains. Thalassemia is a major community health problem in many Middle Eastern societies, including Iraq. For example, the proportion of carriers of mutant genes is between 9-10% in Egypt [1]. Hereditary disorders involve a wide variety of clinical phenotypes, varying in severity from an underlying heterosexual individual to a severe transfusion-
dependent condition called TM [2]. B-TM patients have severe anemia based on frequent blood transfusions and many associated complications, including functional impairment of large arteries and endothelium, and an increase in oxidative stress followed by the development of atherosclerosis and cardiovascular disease [3]. High homocysteine levels in the blood plasma are known to be an independent risk factor for these health problems [4, 24, 25]. It has been shown that many cases of polymorphism in genes that encode enzymes that work within the recombination pathway in homocysteine metabolism, such as methylentetrahydrofolate reductase (MTHFR C677T), which cause high levels of homocysteine, Hyperhomocysteinemia (Hhcy), in patients with folate deficiency, Vitamin B6 and B12 [5]. The substitution mutation CT in nucleotide 677 causes a heat-affected form of the MTHFR enzyme associated with a decrease in its efficacy due to the replacement of the amino acid alanine with valine [6]. According to Abd-Elmawlaa et al [7], the case of HCY in βT patients has not been adequately described till now. A change in the level of homocysteine in plasma patients [8], other studies indicated a decrease in the level of hyc when compared with healthy individuals [9]. Several studies have indicated the spread of the MTHFR C677T gene mutation directly related to hyc in other pathological disorders, but its association with TM-TM is still controversial. Due to the absence of a study to assess the prevalence of the MTHFR C677T gene among β-TM patients in Iraq and its possible effect in the case of high homocysteine (Hhyc) and its relationship in the case of oxidative stress in patients, this study has been conducted.

Materials and Methods
50 samples were collected from patients with β-thalassemia (25 females, 25 males) and 45 healthy individuals. From 10/12/2018 to 7/2/2019, all patients were clinically diagnosed depending on the appearance of the disease and the level of hemoglobin before blood transfusion, and electrophoresis of hemoglobin, Duration of disease, body weight, family history, medication, liver and spleen status, as well as data about each Of serum ferritin, creatinine, liver function tests, and whole blood count. All patients received transfusion every 4 weeks. 5 ml of venous blood was withdrawn by a plastic syringe, and the blood was divided into two parts. 2 ml of blood was kept in Freezer at (-20 °C) to extract DNA from it. While 3 ml were placed in sterile plain tubes made of polystyrene, free of anticoagulant EDTA. Centrifuge at 4000 rpm for 10 minutes and then clear the serum, then the serum was frozen at -20 °C to measure biochemical variables.

Molecular Analysis
The molecular study was carried out including the Extraction of genomic DNA from the preserved blood sample using the method described in the G-spin M total extraction kit. Then measuring the concentration and purity of DNA. For genotyping of the MTHFR gene, polymorphism of the MTHFR gene was detected using the RFLP technique. The gene segment to be studied was doubled using specialized primers by PCR reaction, then the CT mutation was detected using the restriction enzyme Hinfl, which identifies the location of the T-segment. About one package, one large size 175 bp, and the other small size 23 bp. For Amplification of MTHFR C677T by PCR, primers equipped with
Lyophilized powder form from IDT USA, it was re-thawed with a certain volume of sterile distilled water according to the manufacturer’s instructions to obtain the required concentration of 100Pmol / µL. Concentrations were prepared to obtain a storage solution for each initiator. The prefixes were kept in special test tubes prepared for this purpose at room temperature, the storage solution was kept at -20 ° C, and then the working solution was prepared using the dilution law. 1, as explained in the table 1.

Table 1: main parameters of molecular analysis for Extraction of genomic DNA

<table>
<thead>
<tr>
<th>Annealing Temperature</th>
<th>Final concentration of primer</th>
<th>Length (pb)</th>
<th>Primer sequences</th>
<th>Primer Name</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>10 Pmol/µL</td>
<td>23</td>
<td>5’TGA AGG AGA AGG TGT CTG CGG GA 3’</td>
<td>MTHFR F</td>
<td>MTHFR</td>
</tr>
<tr>
<td></td>
<td>10Pmol/µL</td>
<td>20</td>
<td>5’AGG ACG GTG CGG TGA GAG TG 3’</td>
<td>MTHFR R</td>
<td>MTHFR</td>
</tr>
</tbody>
</table>

Digestion of PCR Amplification Product by Restriction Enzymes (RFLP)

The restriction enzyme *HinfI* was used to detect the polymorphism of the PCR products of the MTHFR C677T. 10 units of the enzyme were added 1-5 µl of the PCR reaction product, and the mixture was incubated for 24 h at 37 ° C. The resulting array was investigated as the normal model C gives 198 bp, while the mutant model gives 175 and 23 bp.

Genotype Frequency:
Frequency of genotypes = number of observed genotype / total number of genotypes The recurrence of the expected genotype Expected Genotypes is based on the calculation of the extraction of alleles replication and then apply the law of Hardy- Weinberg, (P2 + 2pq+ q2= 1) whereas, P2 Represents the expected replication of the Normal Homozygous, 2pq represents the expected replication of the Heterozygous asymmetric genotype, and q2 represents the expected replication of Mutant Homozygous [10].

Allele Frequency:
The frequency of mutant MTHFR is calculated by applying the following equation Allelic frequency (q) = 2 (No. of homozygote TT) + No. of heterozygote CT / total number x 2 [10].

Measurement of homocystein (Hcy) concentration in serum:
The serum Hcy concentration was estimated by the Spin 120 chemical self-analysis device using the diagnostic kit supplied by the American Elab Science Company.
The basic principle: Serum Hcy concentration was estimated using a diagnostic kit supplied by a company Elabs Science American. The principle of the test is based on measuring the amount of absorption at the wavelength of 340 nm. The reduced absorbance resulting from the decrease of NADH corresponds to the homocysteine concentration in the model.

Setting Parameter
Table 2 shows the setting parameters of the primers

<table>
<thead>
<tr>
<th>Rate method</th>
<th>Method</th>
<th>37°C</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>240 s</td>
<td>Reaction time (Serum + R1)</td>
<td>Down</td>
<td>Reaction Direction</td>
</tr>
<tr>
<td>120 s</td>
<td>Reaction time (Serum + R1+R2)</td>
<td>Linear</td>
<td>Calibration method</td>
</tr>
<tr>
<td>340 nm</td>
<td>Dominant wavelength</td>
<td>13 µL</td>
<td>Sample volume</td>
</tr>
<tr>
<td>405 nm</td>
<td>Auxiliary wavelength</td>
<td>240 µL</td>
<td>Reagent I(R1)</td>
</tr>
<tr>
<td>120 s</td>
<td>Detection time</td>
<td>65 µL</td>
<td>Reagent II (R2)</td>
</tr>
</tbody>
</table>

Elisa Technology: For the purpose of extracting the concentration of folic acid and vitamin B12 Elisa technique was used, and for this purpose was used Mw-12 AMindray streamer and Mindray reader MR-96A.

Measure the concentration of iron storage protein Ferritin in serum:
The serum Ferritin concentration was estimated by the Spin 120 chemical self-analysis device using the diagnostic kit supplied by the Spanish company SPINREACT.

The basic principle: The concentration of Ferritin in serum was determined using the diagnostic tool provided by the Spanish company SPINREACT. The principle of the test work is based on measuring the amount of turbidity formed in the serum.  

Reagents:
Table 3: show the details of the reagents under study.

<table>
<thead>
<tr>
<th>FERR-CAL</th>
<th>R2 Latex</th>
<th>Concen.</th>
<th>R1 Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator. Ferritin concentration is stated on the vial label.</td>
<td>Latex particles coated with rabbit IgG anti-human ferritin, PH 8, 2. Preservative.</td>
<td>20 mmol/L</td>
<td>Tris buffer, pH 8.2. Preservative</td>
</tr>
</tbody>
</table>

Statistical Analysis: The results of the study were analyzed statistically using the Statistical Package of Social Sciences (SPSS) program, for the purpose of detecting the data under normal distribution, the test of homogeneity, and the application of t-test for independent samples to identify the significant differences between the control group and the patient group. The Chi² test was used to detect if the two populations were in Hardy-Weinberg equilibrium.
Results and discussion:

Research community:

Table 4 and shows the nature of the healthy group (control group) and the group of patients with βT for age, gender and consanguinity

<table>
<thead>
<tr>
<th>Group</th>
<th>Individual No.</th>
<th>Age M ± SD</th>
<th>Gender</th>
<th>Consanguinity</th>
<th>M</th>
<th>F</th>
<th>Relative</th>
<th>Non-relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>45</td>
<td>9.51 ± 3.84</td>
<td>25</td>
<td>20</td>
<td>22</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>50</td>
<td>8.32 ± 4.41</td>
<td>25</td>
<td>25</td>
<td>31</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M: mean; SD: standard deviation, M: male; F: female

Results of Molecular study:

The purity of DNA extracted from (45) blood samples from healthy group (control group) and (50) blood samples from individuals with βT was tested using concentration measurement as described in [12], before starting the MTHFR C677T amplification of forward primer '5’TGA AGG AGA AGG TGT CTG CGG GA 3' And reverse primer '5’AGG ACG GTG CGG TGA GAG TG 3’. The results revealed the success of DNA amplification in 43 healthy subjects (95.55%) and 46 patients (92%).

Genetic polymorphism:

Figure 2 and Figure 3 show the results of the treatment of the C677T amplification products with the restriction enzyme *Hinf1* and after migration on the agarose gel. No amplification products were found for sample 4 and sample 22 in the control group. In the patient group, the amplified products that did not appear were 20, 33, 39 and 49. The absence of one of the two mutant alleles T was due to technical defects and the presence of one of the two bands indicated the presence of the allele, as a result of the action of the enzyme cutting in the specified position.

Figure 2.Electrophoretic array of amplified DNAand incubation with *Hinf1* in the control group.C: 198bp, T: 175bp, and 23bp.
Figure 3. Electrophoretic array of amplified DNA and incubation with HinfI in the patient group. C: 198bp, T: 175bp, and 23bp.

The results of incubation with the restriction enzyme HinfI showed that the replication of the mutant genotype MTHFR C677TT was higher in the patient group at 10.86% versus 2.32% in the control group. The frequency of mutant allele of the MHFR gene was 0.368 higher in the patient group than in the healthy control group 0.046. Tables 5 and 6 show the observed and expected genotypes in both healthy and patient groups.

Table 5 Observed and expected genotypes frequency in the control group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>40</td>
<td>39.05</td>
</tr>
<tr>
<td>CT</td>
<td>2</td>
<td>3.81</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>42.95</td>
</tr>
</tbody>
</table>

The total value of X² was 9.84 = 2 with a degree of freedom = 2, while the value of the tabulated X² at P≤ 0.05 for the degree of freedom 2 = 5.99. Since the calculated value of X² is higher than its tabulated value, this leads to the conclusion that the differences between the observed values and the expected values are due to chance.

Table 6 Observed and expected genotypes frequency in the patients group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>17</td>
<td>18.37</td>
</tr>
<tr>
<td>CT</td>
<td>24</td>
<td>21.40</td>
</tr>
<tr>
<td>TT</td>
<td>5</td>
<td>6.23</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>46</td>
</tr>
</tbody>
</table>

The value of the total X² = 0.66, while the tabulated X² value with P≤ 0.05 for the degree of freedom 2 = 5.99, and since the calculated value of X² is smaller than the tabulated value, this leads to the
conclusion that the differences between the observed and expected values are not attributed to chance, though another factor may cause them. In reviewing Table 5 regarding the type of marriage between parents, we find that inbreeding constitutes 62% in the patient group compared to 48.8% in the control group. When looking at the genotype of the patients, the frequency of heterozygotes CT was 0.52, which is high compared to 0.046 in the control group. The high proportion of the heterozygote associated with the high rate of inbreeding leads to the expectation of increased tendency toward homozygous of the mutant allele, as demonstrated by the present study. The study conducted by [7], showed that the recurrence of the genotype MTHFR677TT was higher in the β-TM patients with 12% compared with 3% in the control group healthy.

Results and discussion
Levels of HYC, Ferritin, Folate, VB12 in control and βT patients:
Figure 4 shows the levels of HYC, Ferritin, Folate, and VB12 in the control group and the β-thalassemia patients group. The results of the present study are inconsistent with the findings of Nienaber-Rousseau et al [13], which showed a combination between the genotype MTHFR677 TT and high levels of HYC,

Figure 4 Levels of HYC (A), Ferritin (B), Folate (C), VB12 (D) in the control group (1) and the βT patients group (2).

Figure 4-B shows the levels of Ferritin in both the control group and β-thalassemia patients. The mean values in the patient group were 433.12 ± 15.0 and 45.93 ± 5.92 in the healthy group and showed a significant difference in the mean values. The study of Essa and El- Gamal [14], showed a significant
increase in the proportion of fermentation in male patients, it is assumed that the increase in iron ratios due to blood transfusions and inefficient process of red blood cells and increased absorption of iron from the digestive channel \[^{15}\]. Folate levels in the β-thalassemia group were lower compared to the control group. The mean values in the β-thalassemia group were 15.79 ± 5.69, and 17.11 ± 4.45. The difference in VB12 levels in the control group compared with that of β-thalassemia patients. The mean values in the healthy group were 583.17 ± 10.56 compared to the patient group averaging 380.18 ± 9.93. The homocysteine conversion pathway to amino acid methionine involves the action of the MTHFR gene required by the interaction between folate and cobalamin VB12 \[^{15}\]. Significant differences in Ferritin, and VB12 mean values in control group and βT patients. Table 5 shows the significance of differences in mean values of healthy and patient groups Ferritin, and VB12 levels. The mean difference between hyc values showed no significant differences of 2.33 ± 1.56. This is consistent with a previous study by Rahimi et al \[^{17}\] and Mustafa et al \[^{18}\]. The results of the current study differ with the Asadi study \[^{19}\], which showed a significant increase in the level of hyc in serum of patients with βT compared with healthy people. Talat \[^{20}\] indicated a significant rise of hyc in βT patients compared to healthy people, He suggested that the rise of hyc in patients may lead to complications in the kidneys and other vital organs in the body. It is well known that the kidney removes the excess of hyc in the body, so the kidney dysfunction is associated with Hhyc in the blood. As for the mean differences between the values of the average ferritin level in the two groups, it was found that there were significant differences (387.18 ± 16.79, Table 5). This result is in agreement with the expectation of higher ferritin levels in the βT patient group. One of the diagnosed manifestations in βT patients is that the increased intestinal absorption of ferritin occurs in all stages of the disease. Jubouri, in his study \[^{21}\] stated a significant increase in serum level of Ferritin in patients with βT. The results of the current study are consistent with (Abd-Elmawla et al., 2016) and Idan \[^{22}\], which assumes that the increase in ferritin level is attributable to frequent blood transfusions in patients with βT. The mean differences in the healthy group and patient group for Folate levels were (1.31 ± 1.05, Table 5). This result is consistent with the expectation of low levels of folate in the βT group, which is involved in the construction and formation of hypoxic DNA. It plays an important role in the formation of new red blood cells continuously to compensate for the lack of red blood cells that accompany people with βT throughout their life, and this constant decomposition of RBCs causes depletion of folate, which leads to its lack. This result is consistent with the findings of Mohammed et al \[^{23}\] and Abd-Elmawla et al \[^{7}\] which indicated a decrease in the level of folate in βT patients. The mean differences between the values of vitamin B12 in the two groups were found to have a significant decrease, reaching(202.99 ± 14.49, Table 6) This result is consistent with the expectation of lower levels of B12 in the group of patients with βT, as vitamin B12 has a key role in the formation and production of DNA, and the formation of new red blood cells continuously to compensate the deficiency of decaying red blood cells, which is associated with increased levels in patients with βT, as shown by the study (Mohammed et al., 2013, and Abd-Elmawla et al., 2016)\[^{22, 7}\]. Table 6 mean values and mean difference HYC, Ferritin, Folate, and VB12 in control group and βT patients.
References:


