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Screening of C677T polymorphism in the methylenetetrahydrofolate reductase gene in Anbar, Iraqi patients with β-thalassemia major

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ABSTRACT: The synthesis of proteins, DNA, and RNA as well as the metabolism of methionine and folate, depend on the enzyme MTHFR, a crucial regulator of the one-carbon cycle. This enzyme is necessary for the remethylation of homocysteine to methionine and transforms 5,10-methylenetetrahydrofolate into its active form, 5-methyltetrahydrofolate. Determining the degree of polymorphism in the gene for the enzyme methylenetetrahydrofolate reductase (MTHFR) in patients with beta-thalassemia major and comparing it to the control group, as well as sequencing, analyzing, and detecting mutations in the DNA nucleotides of the gene for the enzyme MTHFR, were the objectives of the study. 90 samples were taken from patients at the Al-Ramadi Teaching Hospital for Women and Children, both male and female. The study included 60 samples of individuals with β-thalassaemia major. The patients were between the ages of 3 and 52. The control sample consisted of 30 samples of each sex, ages ranging from 16 to 65. For every sample in the investigation, the MTHFR C677T gene band was amplified using the polymerase chain reaction (PCR). When compared to the 100 bp marker, all samples had a band size of 198 bp. The CHROMAS was used to examine the sequence data. The BLAST SEARCH tool was used to record the data, which had the same gene, C677T, between 9800 and 9930, and showed no alterations from the reference gene (NG_013351). These findings show that the participants in this research were wild type for this location, meaning they did not carry the C677T mutation.

Keywords: Beta thalassemia major, the MTHFR gene, C677T polymorphism ،DNA Sequencing.

1. INTRODUCTION

The severe anaemia condition known as thalassaemia is typified by a lack of a particular kind of hemoglobin-forming protein chain. Due to the loss or lack of formation of one or more haemoglobin polypeptide chains, either the alpha (α) or beta (β) chain, thalassaemia results in low haemoglobin levels in red blood cells (erythrocytes). Because of this deficit, haemoglobin (Hb) loses its primary role in carrying carbon dioxide (CO2) and oxygen (O2) throughout the body, causing anaemia (1).

The HBA1 and HBA2 genes, which produce the alpha chains of haemoglobin, as well as the ζ-globin gene, which is active throughout foetal development, are part of the alpha globin chain genes, which are found on chromosome 16 at 16p13.3. Located on chromosome 11 at 11p15.4, the beta globin chain genes include the basic β gene (HBB), the δ gene (HBD) that produces haemoglobin A2, the γ genes (HBG1/HBG2) that produce foetal haemoglobin (HbF), and the early

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foetal ε gene (HBE1). These genes have a chronological control over their expression; foetal genes like HBZ and HBE1 are active first, followed by adult genes like HBA and HBB after birth (2).

Mutations affecting the genes of polypeptide protein chains, such as the substitution or deletion of a gene or nucleotide in the DNA, are one of the primary causes of thalassaemia. These mutations impair haemoglobin synthesis and the development of functional red blood cells. Because these cells are hypochromic and tiny (microcytic), they are unable to carry enough oxygen to fulfil the body's demands and are thus functionally inefficient (3).

Due to the conversion of alanine to valine, the C-to-T mutation at nucleotide 677 (SNP C677T) results in a heat-labile form of the enzyme that has decreased activity. High levels of homocysteine, an antioxidant that produces free radicals through autoxidation, promote lipid peroxidation, lower nitric oxide (NO), and damage endothelial cells, which may result from this (4).

It is an enzyme that is encoded by the MTHFR gene, which is around 20,374 base pairs long, has 12 exons, and is found on chromosome 1 (more precisely, at location 1p36.3). The TATA box is not present in any of the distinct regulatory elements that control gene expression, including SP1, AP1, AP2, CAAT, and GC. Additionally, the promoter region is structurally similar to other genes involved in the metabolism of homocysteine (Hcy), including genes coding for cystathionine β-synthase (CBS), methionine synthase (MS), and methionine synthase reductase (5).

The C677T polymorphism has a high relative frequency worldwide, however, its incidence varies by ethnic group and geographic area. According to a thorough meta-analysis of population-based research, the incidence of the TT genotype was around 7.7%, while the global frequency of the T allele was estimated to be 0.24%. However, it was evident by looking at other groupings that there was a great deal of diversity in the prevalence of the T allele: 10.3% among Africans, 31.2% among North Americans, 27.8% among South Americans, 19.7% among Asians, 20.3% among Australians, and 34.1% among Europeans (6).

Europeans had a greater frequency of the T allele and the TT genotype than Africans did. There are significant differences in the T allele's prevalence throughout Asian groups. According to studies done on Asian populations, East Asian nations have a noticeably greater incidence of this particular genetic polymorphism (44.7% in China, 40.3% in the Republic of Korea, and 39.9% in Japan). On the other hand, the prevalence was lower in South Asian nations (11.4% in India, 16% in Pakistan, and 4.5% in Sri Lanka) (7). Thus, the study's objectives were to screen for the C677T polymorphism in the methylenetetrahydrofolate reductase gene in Anbar, Iraqi patients with β-thalassemia major.

2. MATERIALS AND METHODS

2.1 Samples collection

People with thalassaemia major, aged 3–52 years, from the districts and regions of Ramadi city/Anbar Governorate provided 60 samples to the Thalassaemia Specialist Centre at Ramadi General Hospital for Women and Children. In terms of healthy individuals, 30 samples were gathered between August 20, 2024, and September 14, 2024, with ages ranging from 16 to 65.

2.2 Blood collection

Blood was drawn from the veins to obtain samples. The venous blood was extracted using disposable plastic syringes, the volume being 4-6 ml. To extract DNA, 2 ml of blood was put straight into tubes filled with EDTA, an anticoagulant.

2.3 DNA Extraction

DNA was extracted from blood using a special kit manufactured by the gSYNC™ DNA Extraction Kit Geneaid Biotech Ltd (Add: #112 Sintai 5th Road, Section 1, 16th floor, Shijr District, New Taipei City, Taiwan R.O.C. 22181). PCR (polymerase chain reaction) amplification was performed using DNA samples extracted from blood using specific primers for each gene, according to the information listed in Table 1. According to the Transgene company instructions, a mixture was prepared using 2xEasyTaq® PCR SuperMix. The reaction was carried out using a thermal cycler (Eppendorf, Germany). Table 1 shows the primers and temperatures used for the studied gene.

Table 1: Primers and temperatures used for the studied gene.

Primer	Sequence (5′→3′ direction)	primer size bp	Ta°C
Genotype Analysis of MTHFR C677T			
F	5'-TGAAGGAGAAGGTGTCTGCGGGA-3'	198	62
R	5'- AGGACGGTGCGGTGAGAGTG-3'		

2.4 Amplification of *the* MTHFR C677T gene

Amplification PCR technology was used for all DNA samples obtained, and to obtain good results, several experiments were conducted to reach the optimal conditions for the reaction (optimization improvements) and subjected to PCR using 0.1 mM of the primer gene in this study, 2.5 µl of buffer 10X, 3 µl MgCl2, 3 µl dNTPs, 2 µl of DNA template, and 0.2 enzyme units Taq DNA polymerase in a 25 µl reaction mixture with deionized water (DW). Thermocycler conditions were: 94 ℃ for 5 min, followed by 40 cycles of denaturation at 94 ℃ for 30 s, annealing at 62 ℃ for 30 s, extension at 72 ℃ for 30 s, and a final extension at 72 ℃ for 7 min. several factors must be considered, including the concentration and purity of the genomic DNA used, the concentration of the primers, and the suitability of the program implemented on the PCR machine. Also, taking into account that the place and work tools are sterile.

2.5 DNA Sequencing

An ABI3730XL automated sequencer (Macrogen, Korea) was used to perform sequencing on the PCR-amplified fragments. Following alignment to the reference sequence in the GenBank database, genotypes were examined using BioEdit software. A popular software in molecular biology research is called BioEdit. It was once intended to be a Windows-only biological sequence editor. Numerous sequence alignment features are available, including split-window display, user-defined colouring, simple manual editing, and automated interface with other programs like Blast and Clustal W.

2.6 Primer sequence matching

Primers were made to find and sequence the expression of the MTHFR C677T gene. The primer sequences were created using the National Center for Biotechnology Information's (NCBI) reference sequences (rs). NCBI bioinformatics algorithms were able to match the genotype primer sequences.

3. RESULTS AND DISCUSSION

3.1 DNA concentration and purity assessment

The amount of DNA was measured using a Nanodrop spectrophotometer. Sample values ranged from 750 to 1600 ng/µL, while the A260/A280 ratio ranged from 1.7 to 1.9, indicating that the samples were clean. Using ethidium bromide dye and ultraviolet light, a single band indicating DNA for the various forms of thalassemia appeared on a 1% agarose gel after electrophoresis (Figure 1).

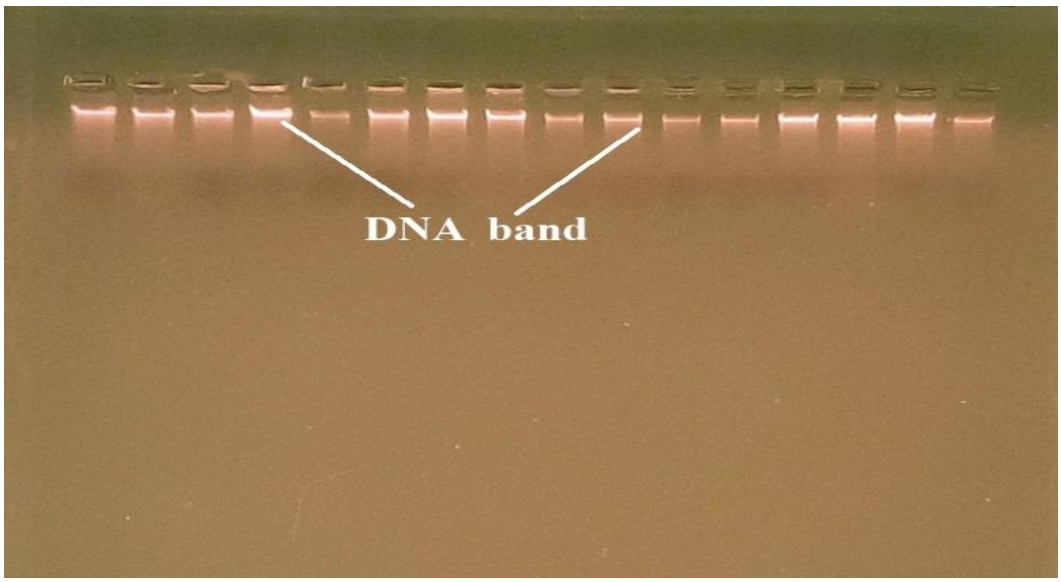


Figure (1): Electrophoresis of the DNA extraction on a 1% agarose gel at 70% voltage for 40 minutes.

3.2 Identification of the MTHFR C677T gene polymorphism.

Using specialised primers specific to the MTHFR C677T gene and DNA extracted from blood samples of the thalassaemia patients under study as a template for constructing the desired gene, which was 198 bp in size, the polymerase chain reaction (PCR) was conducted under the previously mentioned conditions in the methods, and the results showed that the desired DNA fragment was obtained in all 55 samples. After that, the PCR products were electrophoresed for an hour on a 1.2% agarose gel. Several temperatures were utilised in the process, but when the reactions were performed several times to obtain the best PCR result, it was discovered that 62°C was the optimal temperature for gene synthesis Figure (2).

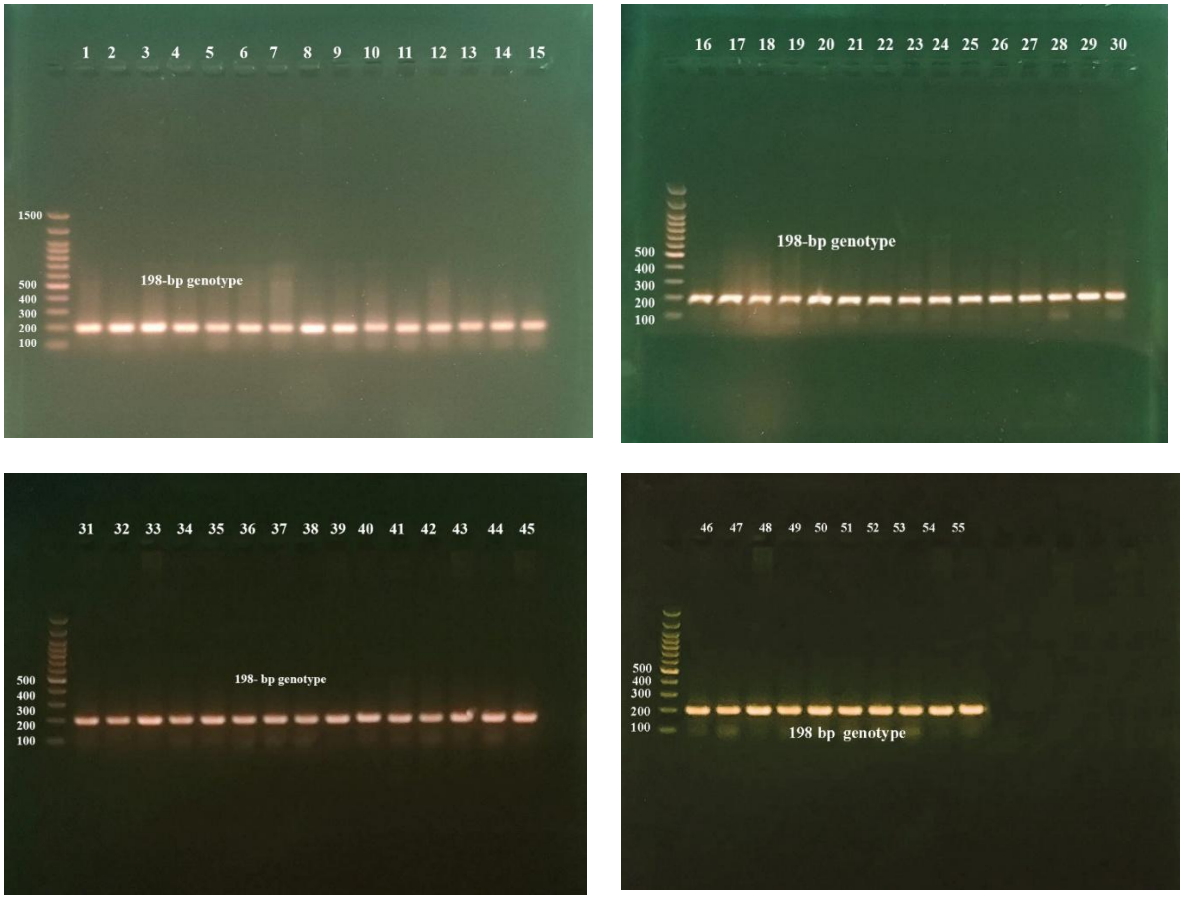


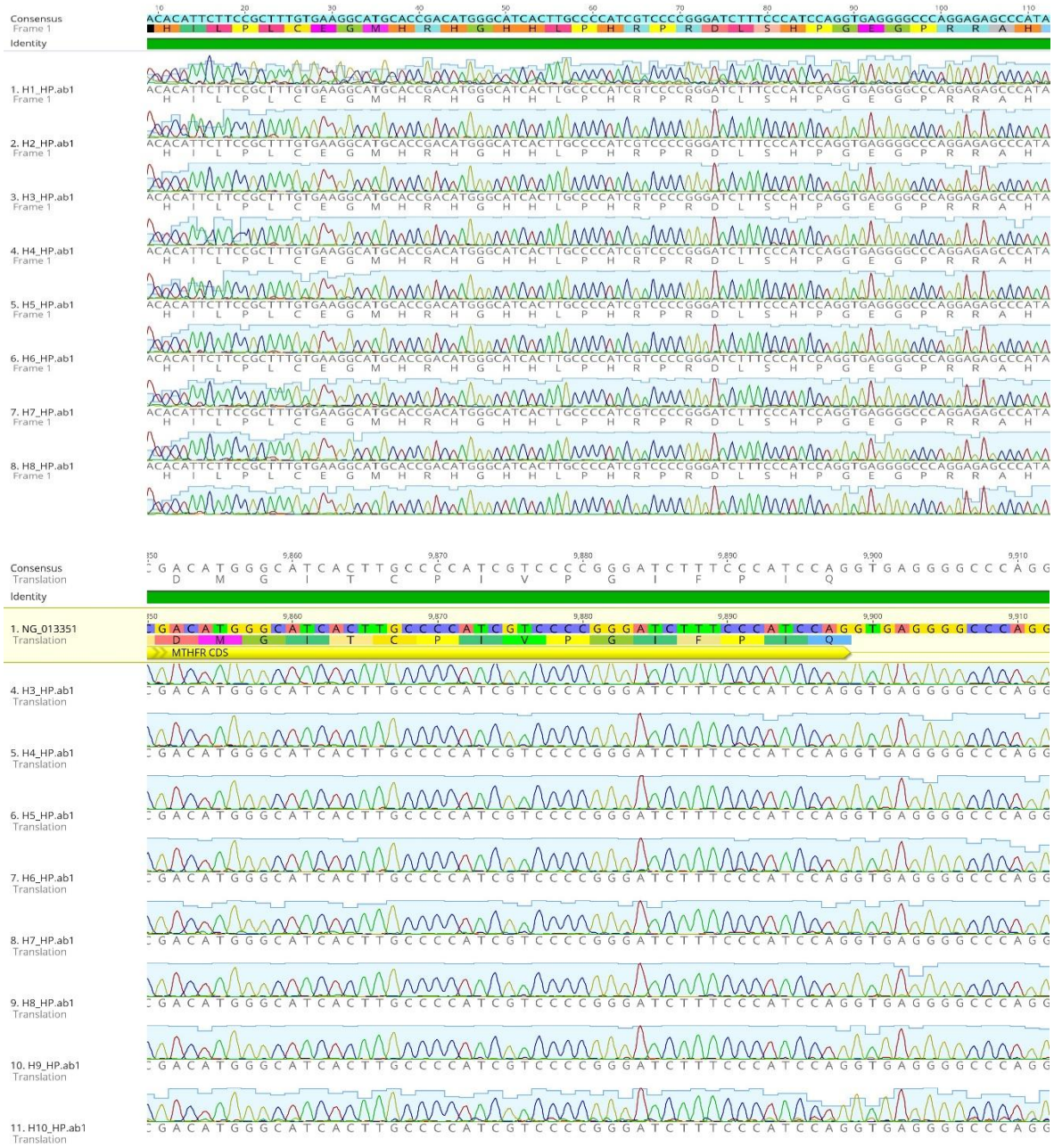
Figure 2: Electrophoresis of the PCR products of the C677T gene on a 1.2% agarose gel at 70% voltage for 60 minutes. The results showed that this gene measured 198 bp.

3.3 Results of Alignment for the genetic sequencing of gene C677T

The alignment process was performed on the data we received for the DNA sequencing for gene C677T according to the procedures that were previously explained, where the number of samples that were sent was 25 samples, 20 of which were for people with thalassaemia (from 1 to 20) and the other 5 for the control group (from 21 to 25). The results were as shown in Figure (3), where the results of the genetic sequence after the alignment are that the percentage of congruence is 100%, meaning that there is no mutation or difference in the C677T gene between patients with thalassaemia and the control group.

3.4 Gene sequencing for C677T gene polymorphism

To identify and sequence the DNA of the MTHFR C677T gene, we sent 25 samples of the products of the previous reaction extracted from blood samples of individuals with thalassaemia and the control group. This was done after sending the products of DNA replication by polymerase chain reaction technology to Macrogen Company in Korea. After determining the DNA sequence using the sequencing technology, the sequence's data was examined using the CHROMAS program and compared to data from the National Centre for Biotechnology Information (NCBI), which was recorded using the BLAST SEARCH tool and pertains to the same gene C677T as seen in Figure (3).



Matrix: % Identity Decimal Places: 3 Style: Numbers Only

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H11_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	100%	100%	100%
H12_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	100%	100%
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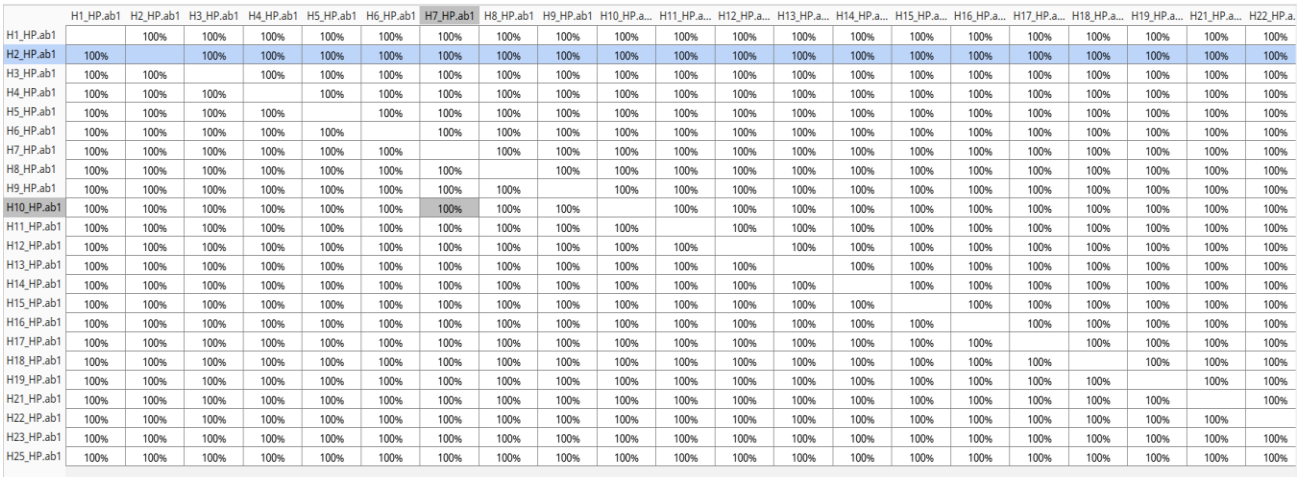
H16_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%
H17_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%
H18_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		100%	100%
H19_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		100%
H21_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	
H22_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
H23_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
H25_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%



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H1_HP.ab1		100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
H2_HP.ab1	100%		100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
H3_HP.ab1	100%	100%		100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
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H23_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
H25_HP.ab1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Figure 3: Gene sequencing for the C677T gene polymorphism

Based on phenotype, family history, and pertinent laboratory screening test findings, thalassemia/hemoglobinopathy is first suspected in anaemic individuals. The diagnosis is established by molecular genetic validation by identifying pathogenic mutations (8). Previously, molecular genetic diagnosis was complicated by the genetic heterogeneity of the disease and the mutations that were missed by conventional sequence analyses. A more accurate molecular diagnosis of hereditary haemolytic anaemia and a better comprehension of the genetic/genomic mechanisms of the disease have been made possible recently, however, by the introduction of novel molecular genetic technologies such as dosage mutation tests, which detect large deletion/duplication mutations, and multiple gene panel tests, which are performed by massively parallel sequencing (9).

The MTHFR C677T gene polymorphism is highly prevalent in beta-thalassemia intermedia. According to Rai and Kumar, (10), Mediterranean nations have a greater frequency of the homozygous mutant genotype TT than other Northern European nations. Conversely, Sipahi et al. (11) and Nagel and Muniz (12) could not discover any connection between such a mutation and beta-thalassaemia major. A notable heterogeneity might be the cause of this discrepancy. The T677 MTHFR allele's frequency across the main ethnic groupings.

The connection between oxidative alterations and the MTHR C677T genetic variant in individuals with beta-thalassaemia major. Iron excess is the primary cause of oxidative stress in beta-thalassemia patients (13). Oxidative damage is caused by producing strong reactive oxidant species and free radicals, which iron catalyses (14). As a result, assessing and maintaining antioxidant defence may help shield β-thalassemia patients from more severe disease consequences (15).

The current study's findings indicate that there was no significant correlation between the risk of developing β-thalassemia major and the genotype and allele frequencies of the MTHFR C677T gene polymorphism. The results of this, however, were based on mutation (single-nucleotide polymorphism), and notable heterogeneity was also found. As a result, the current study's findings should be evaluated and require more carefully planned research. In individuals with β-thalassemia major, no statistically significant association existed between a greater prevalence of thrombophilia and the mutation in the MTHFR C677T gene (16). Abd-Elmawla et al. (17), on the other hand, discovered that the polymorphism of the MTHFR C677T gene was statistically significantly linked to β-thalassemia.

According to prior research, the T allele (mutant allele) frequencies of the MTHFR C677T polymorphism were 21% in healthy individuals and 21.5% in β-Thalassemia major. Between β-thalassemia major patients and controls, the prevalence of MTHFR C677T mutations was somewhat higher but not statistically different (18). There was no significant correlation found between the genotype and allele frequencies of the MTHFR C677T gene polymorphism and β-thalassemia (16).

The enzyme MTHFR is reliant on flavin adenine dinucleotide (FAD). An essential coenzyme in metabolic activities, FAD is involved in the body's redox reactions and electron transport chain. The metabolism of folate and Hcy, which are both reliant on folic acid and other B vitamins, is another important function of it (19).

The reduction of 5,10-methylenetetrahydrofolate (5,10-THF) to 5-methyltetrahydrofolate (5-methyl-THF) connected to Nicotinamide adenine dinucleotide phosphate NADP is catalysed by this enzyme. Methionine synthase (MS) catalyses the process that converts Hcy to methionine (Met) by using the latter molecule as a methyl group donor. A cofactor in this process is vitamin B12. According to Mentch and Locasale (20), methionine is subsequently transformed into S-adenosylmethionine (SAM), an essential methyl group donor for a variety of bodily activities, such as the methylation of DNA and RNA, histones, phospholipids, choline, sphingomyelin, acetylcholine, and other neurotransmitters.

Perhaps the most significant mutation in the body's pathway for folate and homocysteine metabolism is the C677T mutation of the enzyme methylenetetrahydrofolate reductase (MTHFR). To reduce homocysteine to methionine, this enzyme must convert 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the physiologic form of folate. Diminished enzyme function is the effect of the C677T mutation, especially in doubly expressed (TT) persons, in which enzyme activity can drop as low as 70% (21).

Deficiency in the production of the beta chain of haemoglobin causes the fatal inherited condition β-thalassemia major. It leads to severe anaemia from early childhood, for which repeated blood transfusions are required for life. This makes the body iron overloaded and leads to damage to the liver, heart, and endocrine system (22).

The intricate interaction of C677T mutation and β-thalassemia major starts here. Patients with thalassaemia, first of all, are quite familiar with experiencing higher levels of oxidative stress as a result of iron deposition and persistent destruction of red blood cells, thereby promoting the production of free radicals. The C677T mutation results in reduced MTHFR enzyme activity, which enhances oxidative stress and causes the blood to hold onto homocysteine, a neurotoxin. These deposits can enhance the risk of heart disease or stroke by accelerating tissue damage, particularly within the brain and heart (23).

Thalassaemia patients are frequently prescribed folic acid supplements to replace the cells that are lost due to chronic anaemia. However, unless supplements are administered in physiologically active forms like methylfolate, the C677T mutation can undermine the efficacy of folate metabolism, and the body will be unable to take full advantage of them. This functional deficiency of folate can lead to further bone marrow issues or worsen the lack of production of blood cells (24).

The C677T mutation is found to be more common in thalassaemia patients than in the normal population, according to certain molecular studies in countries like Iraq where the condition is relatively common. This suggests there may be a shared genetic role or genetic overlap that may increase the severity of the disease or its complications. It should be noted that the C677T mutation is one of the factors responsible for the multifactorial pathogenesis of the patient's clinical condition and not for inducing thalassaemia itself (25).

4. CONCLUSION

In general, thalassaemia major patients with the C677T mutation are at a higher risk of manifestations of the disease, especially metabolic disturbances, oxidative stress, and a high risk of cardiovascular disease. To determine the occurrence of this mutation and alter treatment protocols, including the use of active folate, and avert the consequences of raised homocysteine, it is advantageous to conduct genetic testing in thalassaemia patients. Patients with beta-thalassemia major should get regular antioxidant treatment and iron chelation to increase their antioxidant levels.

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