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The Effect of the Diet and Other Genetic Factors on a Unique β-Thalassemia Genotype IVS1.1 [G>A] / IVS2.1 [G>A] Discovered in Kirkuk City

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Abstract:
Background: Thalassemias are inherited hemoglobin disorders characterized by reduced or absent globin chain synthesis, resulting in a variety of clinical phenotypes ranging from severe chronic anemia requiring lifelong transfusion and iron chelating therapy to asymptomatic individuals.

Methods: The study was done on a family consisting of four individuals, a minor father and a minor mother (carriers), major two daughters. During sampling, one of the girls has facial deformities and a typical thalassemic face with changes on the skull (younger daughter B). The older daughter(A) has no facial deformities and has a normal skull like a healthy non-thalassemic individual.
The study aimed to detect if the two daughters have the same mutations and the reason for the differences in facial bone deformities between them.
The results were obtained by reverse hybridization using B- globin strip assay MED and pedigree analysis of the family.

Results: The two thalassemia major daughters have a unique genotype never seen before in another study IVS1.1 [G>A]/ IVS2.1 [G>A]. Each mutation inherited from the minor mother IVS2.1 [G>A] and minor father IVS1.1[G>A]. The combination of the two mutations is seen first in this family never seen before in another study. This new combination in the two intervening sequences 1,2 on the B-globin gene sequence causes a complete absence of a beta chain. That means the two daughters are thalassemia major [B0/B0].

Conclusion: The genotype IVS1.1[G>A]/IVS2.1[G>A] (B0/B0) is unique and first seen in Kirkuk city. The phenotypes between the two daughters were affected by the diet and the mutation in the MCIR gene.

Introduction:

The genetic abnormality of the α- and β-globin genes, which causes thalassemia, leads to persistent anemia and inefficient erythropoiesis. While non-transfusion-dependent thalassemia patients enhance duodenal absorption of dietary iron to hasten erythropoiesis, transfusion-dependent thalassemia patients need red cell transfusions to keep their blood hemoglobin levels within the normal range. Iron overload, oxidative stress, organ dysfunction, and other complications are brought on by these changes (1).

To achieve a negative iron balance and alleviate the problems brought on by an excess of iron, effective iron chelators are required. Patients with thalassemia may also be given medications like hydroxyurea, N-acetylcysteine, ascorbic acid, vitamin E, and glutathione to prevent oxidative cell and tissue damage and improve quality of life (2).

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It's interesting to note that certain vegetables, cereals, and functional natural goods, like mango, tea, caffeine, and curcumin, are beneficial for their health-promoting capabilities by enhancing the body's natural antioxidant defense system. Natural products have a wide range of pharmacological effects, but they are safer when administered the conventional way (3).

Pathophysiology of B-thalassemia Major

A. Consequence of Defective or Absent β-chain

Anemia: It is produced by the following mechanisms:

1. **Diminished synthesis of HbA**:

Microcytic, hypochromic, "underhemo-globinized" RBCs with subnormal oxygen transport capacity result from a deficiency in HbA production (4, 5).

A significant drop in HbA limits the blood's ability to carry oxygen. HbF and HbA2, two non-β-containing hemoglobins, are up. HbF has a greater oxygen affinity than HbA. As a result, the already inadequate oxygen transport to tissues is exacerbated (6).

2. **Ineffective erythropoiesis**:

It refers to a situation in which the bone marrow tries to make cells but cannot release viable cells into the bloodstream. The absence (β0) or impaired β-chains (β+) synthesis is related to β-thalassemia major. The imbalance in α and β-globin production causes red cells and their precursors to live shorter lives. Because α -chain synthesis is typical in developing normoblasts and red cells, these unstable free α-chains collect, precipitate, and form intracellular, insoluble inclusions. Normoblasts with such inclusions do not mature and experience intramedullary apoptosis in the marrow, resulting in inefficient erythropoiesis (4,7).

The ineffective erythropoiesis is caused by the precipitated α-globin chains and damaged cellular components activating apoptotic pathways (6).

Free iron and hemichromes accumulate in the α-chains, generating reactive oxygen species (ROS). ROS degrades hemoglobin, membrane proteins, and lipids, reducing membrane stability (8).

3. **Extravascular hemolysis:**

α-chain inclusions are also found in RBCs discharged from the bone marrow. α-chain inclusions damage red cell membranes. Extravascular hemolysis is caused by free α-chain inclusion-bearing red cells produced from defective progenitors (normoblasts) that escape intramedullary apoptosis are destroyed in the spleen and contribute to hemolytic anemia (4, 9).

4. **Synthesis of fetal hemoglobin (HbF):**

Although synthesis of y-chains ceases in babies after 6 months, it may continue in thalassemia. They combine with α-chains to produce a significantly higher level of HbF (α2 y2), ranging from 20 to 90 percent (4,10).

Significant anemia is caused by a combination of low HbA, high HbF, ineffective erythropoiesis, and prolonged hemolysis. The body tries to compensate by increasing the rate of erythropoiesis (see Figure (1)) (6).

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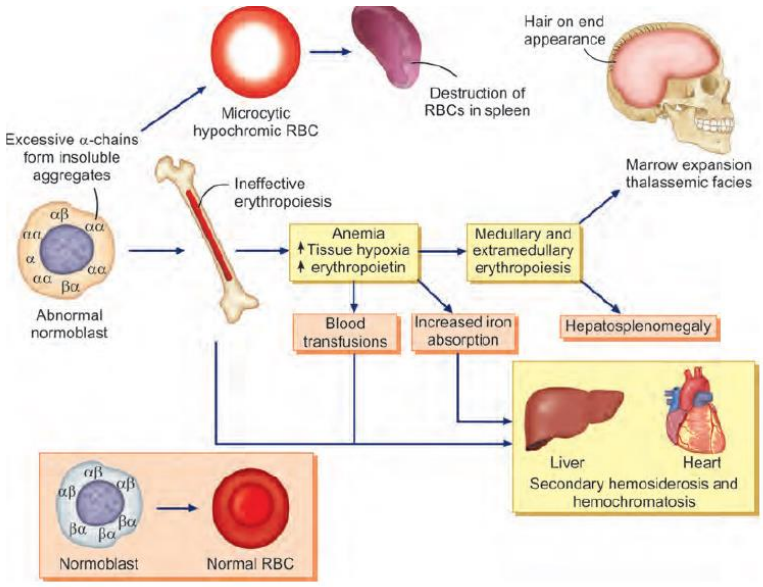
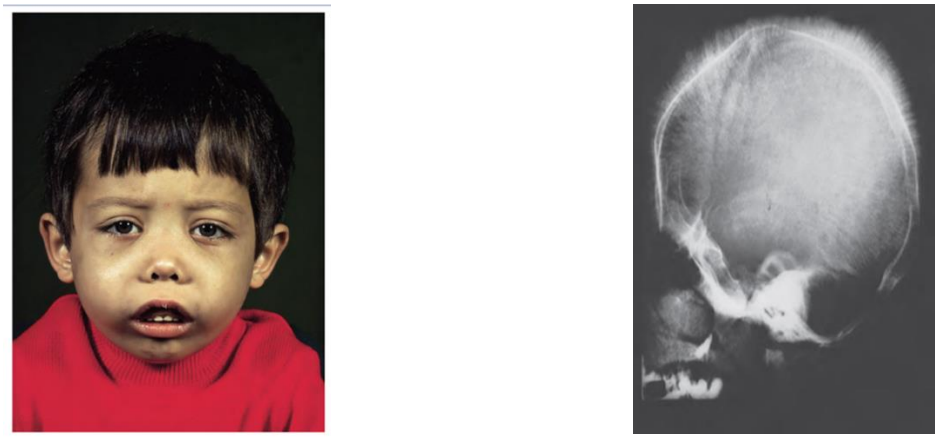


Figure (1): Pathogenesis of β-thalassemia major and its consequences (Nayak and Raj, 2017).

B. Consequences of Ineffective Erythropoiesis

1. **Changes in bone marrow:** The kidney produces erythropoietin (EPO) in response to severe hemolytic anemia. EPO affects the bone marrow, causing erythroid hyperplasia (11).
2. **Changes in bone**: On the outside, the increasing erythropoietic marrow erodes the bony cortex and induces new bone production. The skull vault, maxilla, and facial bones are particularly affected. Due to fresh bone growth, an X-ray of the skull reveals a characteristic hair-on-end ("crew-cut") appearance. With a large forehead (also termed frontal bossing), cheekbones, and upper jaw, distinctive facies known as thalassemic (Chipmunk) facies develops. Because of trabeculations, X-rays of the metatarsals, metacarpals, and phalanges reveal a mosaic pattern (4, 12). See figure (2)



A B

Figure (2) Bone deformities in thalassemia patients. A. The facial appearance of a child with β-Thalassemia major. The skull is bossed with prominent frontal and partial bones; the maxilla is enlarged. B. Skull x-ray in β-Thalassemia major. There is a 'hair-on-end appearance resulting from the expansion of the bone marrow into cortical bone (Nayak and Raj, 2017).

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3. **Extramedullary hematopoiesis**: Extramedullary hematopoietic foci develop in the liver and spleen as the bone marrow cannot compensate for the anemia, resulting in hepatosplenomegaly (ineffective erythropoiesis) (4,13).
4. **Cachexia**: Severe anemia produces hypoxia in most tissues, and the active erythroid progenitors compete with these tissues for critical nutrients. If left untreated, this can progress to cachexia (4,14).

C. Iron Overload and its Consequences

Causes of iron overload:

1. Increased dietary iron absorption from the duodenum: Hepcidin, produced in the liver, is the main regulator of iron homeostasis. Hepcidin is a hormone that regulates iron uptake and storage. Hepcidin levels in the blood are suppressed by ineffective erythropoiesis. Iron absorption is higher when hepcidin levels are low (4,8). Hepcidin production is inhibited by increased erythropoietic activity, which encourages the absorption of more iron in the gut, resulting in iron toxicity (6).
2. Hemolysis.
3. Repeated transfusion (which is the usual mode of treatment).

Consequences: Hemosiderosis and secondary hemochromatosis are caused by increasing iron excess. Excessive iron deposition causes organ parenchyma damage, particularly in the heart (a leading cause of death), liver, and pancreas (15).

Materials and Methods:

Patients

During randomly sampling of 50 patients for detecting the most frequent mutation that causes beta–thalassemia in Kirkuk city in Azadi hospital thalassemia center, A unique genotype IVS1.1(G>A)/IVS2.1G>A) appeared in one patient that suffer from thalassemia major. She is the second child of a family consisting of four individuals during the interview with the patient family, we noticed that the older daughter suffers from thalassemia major without any facial deformities. the older daughter was ginger (refers to the person with red hair and pale skin).

The facial differences between the two daughters open the door to make a further investigation into the members of this family. Pedigree analysis was done interpretation of the B-globin strip assay results for all the family, and further interviews with all the members of the family.

2 ml of peripheral blood was aspirated from all the family members. There was no kinship between the parents.

Methodology

Pedigree analysis

Pedigree diagrams are used in human genetics to show how a characteristic, anomaly, or disease is passed down through the generations. A square or symbol represents a male, while a circle or symbol represents a female. A horizontal line (marriage line) connects a

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male symbol with a female symbol, indicating mating; offspring symbols are joined in a row beneath the mated pair, indicating sibship. A vertical line connects the offspring symbols to the marriage line, which appears from left to right in the order of birth. A solid or blackened symbol indicates the character's possession, whereas an open or clear symbol indicates the character's absence (16).

Reverse hybridization

Strip-based reverse hybridization Using biotinylated primers, multiplex PCR amplification was performed on the extracted DNA. The amplified B-globin products are then hybridized preferentially to a test strip containing the wild-type and mutant oligonucleotide probes fixed as parallel lines. Following that, the color of the bound biotinylated sequences is developed (17.18).

Results and discussion :

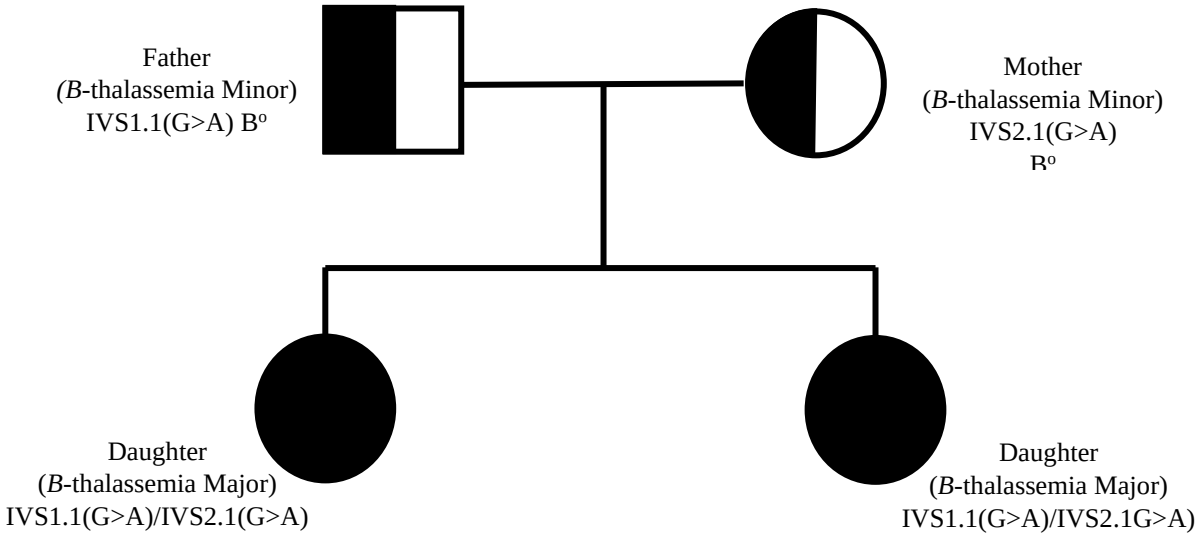

Figure (3): Pedigree analysis of the famly members (Recessive Pedigree).

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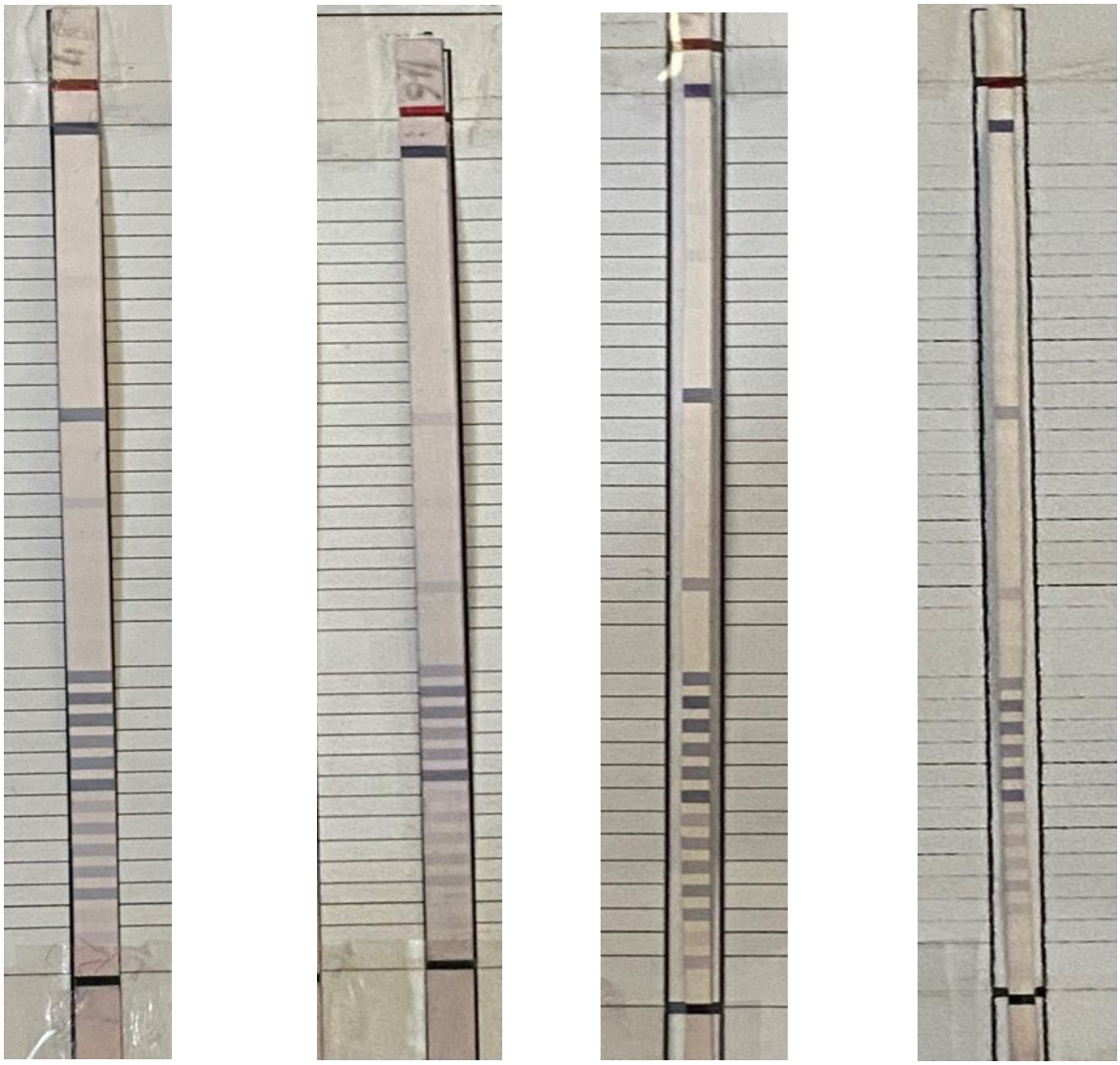

Minor IVS1.1[G>A] Minor IVS2.1[G>A] Heterozygous compound Daughters IVS1.1[G>A] + IVS2.1[G>A] Bo + Bo Thalassemia Major

Bo Father B0 mother

Figure (4): The interpretation of B-globin StripAssay for the family members

The results show that the father was minor IVS1.1[G>A] B0, the mother was IVS2.1[G>A] BO and the daughters were heterozygous compound IVS1.1 [G>A] /IVS2.1 [G>A] B0/B0. IVS2.1[G>A] One mutation, [G>A] change which occurs at the first nucleotide of intron 2 in the β-globin gene (designated IVS2-1 G>A), destroys the 5' donor splice site at the end of exon 2 and prevents correct splicing of pre mRNA.The splice junction mutation IVS 2.1 [G>A] has been detected in the Mediterranean peoples (Greece, Italians, and Tunisians) and recorded in most Arab countries, with a high frequency in north Jordan (20%) and is the most prevalent mutation in Kuwait and Iran (19,20).

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The IVS1-1 [G>A] is a mutation that stops mRNA splicing because the substitution of guanine for adenine [G>A] changes the splice junction, resulting in a non-functional mRNA that is destroyed inside the nucleus, resulting in the phenotype of B0 (the affected gene does not synthesize the B-chain) (21).

The genotype of the patients according to consanguinity status

Table (1): The relationship between consanguinity marriage and inheritance of a homozygous and heterozygous compound genotype in the patients.

Code no.	Genotypes of the patients	Kind of the Genotype	Consanguinity Marriage		Total Genotype frequency
			Yes	No	
1.	IVS 1.110 [G>A] / IVS 1.110 [G>A]	Homozygous	5(12.5)%	-	5(12.5)%
2.	Codon 8/9 [+G] / Codon 8/9 [+G]	Homozygous	3(7.5)%	1(2.5)%	4(10)%
3.	Codon 8 [-AA] / Codon 8 [-AA]	Homozygous	1(2.5)%	-	1(2.5)%
4.	IVS 1.6 [T>C] / IVS 1.6 [T>C]	Homozygous	1(2.5)%	-	1(2.5)%
5.	Codon 39 [C>T] / Codon 39 [C>T]	Homozygous	-	1(2.5)%	1(2.5)%
6.	IVS 2.1 [G>A] / IVS 2.1 [G>A]	Homozygous	2(5)%	-	2(5)%
7.	Codon 8 [-AA] / IVS 2.1 [G>A]	Heterozygous Compound	8(20)%	1(2.5)%	9(22)%
8.	Codon 8 [-AA] / IVS 1.5 [G>C]	Heterozygous Compound	4(10)%	-	4(10)%
9.	IVS 1.110 [G>A] / Codon 44 [-C]	Heterozygous Compound	2(5)%	-	2(5)%
10.	Codon 5 [-CT] / Codon 6 [A>T]Hbs	Heterozygous Compound	1(2.5)%	1(2.5)%	2(5)%
11.	IVS 1.1 [G>A] / IVS 2.1 [G>A]	Heterozygous Compound	-	1(2.5)%	1(2.5)%
12.	Codon 8/9 [+G] / IVS 2.1 [G>A]	Heterozygous Compound	1(2.5)%	-	1(2.5)%
13.	IVS1.5 [G>C] / IVS 2.1 [G>A]	Heterozygous Compound	1(2.5)%	-	1(2.5)%
14.	IVS 1.116 [T>G] / IVS 1.116 [T>G]	Homozygous	1(2.5)%	-	1(2.5)%
15.	Codon 8 [-AA] / IVS 1.1 [G>A]	Heterozygous Compound	1(2.5)%	-	1(2.5)%
16.	Codon 8 [-AA] / IVS 1.6 [T>C]	Heterozygous Compound	1(2.5)%	-	1(2.5)%
17.	IVS 1.110 [G>A] / Uncharacterized	Heterozygous Compound	1(2.5)%	-	1(2.5)%
18.	IVS 1.5 [G>C] / IVS 1.116 [T>G]	Heterozygous Compound	-	1(2.5)%	1(2.5)%
19.	Codon 8/9 [+G] / Uncharacterized	Heterozygous Compound	1(2.5)%	-	1(2.5)%
		7 Homozygous Genotype (17.5)% 33 Heterozygous Compound Genotype (82.5)%	34(85)%	6(15)%	40(100)%
		40(100)%			

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According to the primary results that obtained from the 40 unrelated patients . the heterozygous compound state is most common between the patients . The consanguinity marriage between patients family is about 32 (80) % of the cases and foreign marriage is about 8 (20) % of the patients. Consanguinity plays a key role in limiting the disease's gene pool within the community, and thus in society as a whole (22). Tribalism and consanguineous marriages are widespread in areas of the world where B thalassemia (B-thal) mutations are common, and they enhance the risk of homozygosity for this and other recessive disorders (23). The genetic pool of beta-thalassemia is very rich in Kirkuk city because of the variety in ethnicity in the Kirkuk community. Therefore, the heterozygous compound state of genotypes is most common than the homozygous state. As a result of consanguinity, mutations cluster within the community, increasing the probability of a thalassemic child being born. In our family case (no 11 in table 1) the children are born from non-consanguineous couple .foreign marriage between the couple leads to a new combination in two daughters the IVS1.1 [G>A] /IVS2.1 [G>A].

The diet effect on the phenotype of the genotype IVS1.1G>A /IVS2.1G>A

Although chelation therapy is most common to iron overload in thalassemia patients, nutrition and some antioxidants play a role in decease iron overload through decreased hemolysis caused by the ROS in the RBC`S membrane. Figure (5) shows the formation of ROS (24,25, 26,27).

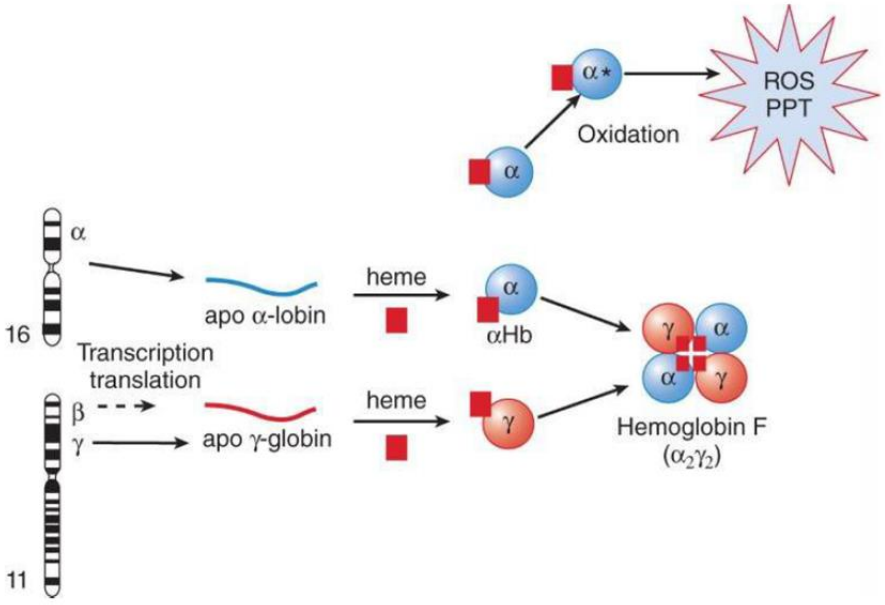

Figure (5): Assembly of hemoglobin subunits and hemoglobin tetramer in β-thalassemia. PPT, hemoglobin precipitate; ROS, reactive oxygen species (Bunn and Sankaran,2017) [28].

The effect of ginger syndrome on the bones

Daughter (A) was ginger syndrome (refers to persons with red hair and pale skin), which results from a mutation in a gene called MC1R, which codes for the melanocortin 1 receptor (29). The pigment found in red hair that makes it red is called pheomelanin (30). These

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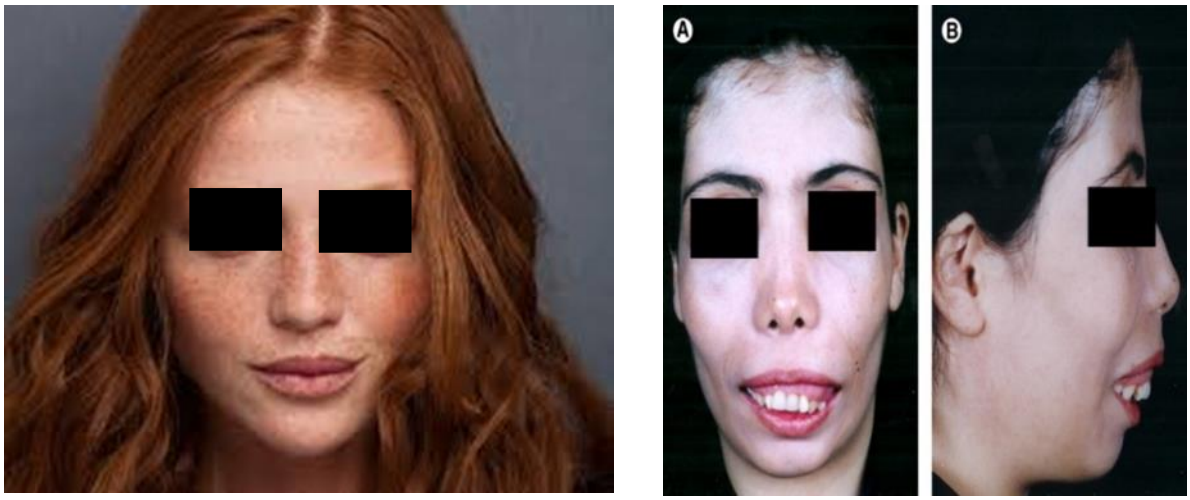
individuals can produce vitamin D, which is crucial for bone health in low light (they are better at making vitamin D) conditions than people with darker skin and hair (31).
During sampling interviews with family members (especially the father), they insist that the main cause of the differences in bone abnormalities (facial) in daughter B results from an unhealthy diet that she is dependent on, eating a lot of fast food and drink cocak instead of drinking tea to avoid iron accumulations or iron overload . In contrast, daughter A(older) keeps on a healthy diet including a lot of vegetables and meat-free and regularly drinks tea after every meal to avoid iron overload (2).
These findings lead to the conclusion that why she did not have bone deformities in comparison with her sister with darker skin and hair in addition to her healthy diet (1), and even though they have the same mutations in the same alleles. See figure (6).



A) Daughter A (older) B) Daughter B (younger)

Figure(6): A comparison between two daughters' facial appearance

We concluded that the genotype IVS1.1G>A/IVS2.1G>A influenced by other genetic factors like red hair and environmental factors like diet, consuming antioxidants, and lifestyle.
In another study done by (Al-momen et al.,2020) [2] Patients with thalassemia intermedia receiving standard medical care, such as blood transfusions and deferasirox oral iron chelation therapy, have significantly higher levels of iron chelation and slightly better hemoglobin levels when consuming Green tea regularly when compared to a control group.
In a previous study done by (Molazem et al .,2016) [32]The findings of this study demonstrated that thalassemic patients' ferritin levels may be improved and that doing so would eventually lead to a reduction in the side effects of iron overload. In fact, two months after the intervention, compared to before the intervention, the educational program's implementation in the intervention group considerably reduced the blood ferritin level.
A study done by (ozdemir et al .,2014) [33] concluded that in children with β-thal, N-acetylcysteine and vitamin E may be useful in lowering serum oxidative stress and raising pre-transfusion Hb levels. Additionally, N-acetylcysteine can lessen DNA damage.
In a study done by (Haghpanah et al .,2021) [34] Patients with TDT may benefit from using vitamin E as a safe and efficient supplement to reduce oxidative stress. Additionally, it

appears that clinical hematologic improvement in TDT patients requires a longer term of using antioxidant supplements.

A study done by (El-Haggar et al .,2018) [35]suggests that patients with TM may see a reduction in iron overload by combining regular chelation therapy with the use of amlodipine or spirulina.

In a study done by (Hussien et al .,2017) [36]When compared to antioxidants, vitamin C is a more effective booster of iron absorption from the GIT and a releaser from intracellular storage. Vitamin C must be administered to TM patients with caution and constant observation. It is crucial to examine both smaller and greater doses than 200mg over longer periods and with a wider range of age groups.

A study performed by (Soeizi et al .,2017) [37] concluded that Consuming green tea had positive effects on iron status and oxidative stress in the study participants and may help manage these risk factors in those with β-thalassemia major.

In a study done by (Koonyosying et al .,2020) [38]Through appropriate subsequent iron-chelating and diuretic actions, an edible green tea-curcumin beverage may reduce the levels of serum redox iron and lipid peroxidation product, as well as blood urea nitrogen, in iron-overloaded -thalassemia patients. Additionally, the beverage did not affect any possible hematological and biochemical variables.

According to (Abotaleb et al., 2018)[39] the study suggested that the quantity of flavonoids and the pH of the surrounding environment alter their capacity to chelate iron, which has an impact on the dosage and usage guidelines. Future research should concentrate on these elements.

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