How to Cite:
Jabbar, E. E., & Mahdi, W. T. (2022). The hepcidin gene polymorphism in patients with β -thalassemia. *International Journal of Health Sciences*, *6*(S1), 11594–11601.
https://doi.org/10.53730/ijhs.v6nS1.7845

The hepcidin gene polymorphism in patients with β -thalassemia

Esraa Emad Jabbar
Al-Qadisiyah University/College of Science/Department of Biology
Corresponding author email: Asraaimad0@gmail.com

Prof. Dr. Wejdan Thamer Mahdi
Al-Qadisiyah University/College of Science/Department of Biology

***Abstract*---**This study was conducted to study the hepcidin gene polymorphism and its effect in patients with β-thalassemia. Blood samples were collected from patients visiting (for the Center for Genetic Blood Diseases - Thalassemia) in Al-Diwaniyah for ages (15-35) for males. The results were as follows: The present study enrolled 50 patients with Thalasemia and 40 apparently healthy subjects. The mean age of patients was 39.31 ± 12.89 and that of control subjects was 28.74 ± 10.77 years and there was no significant difference between patients and control subjects in mean age (P = 0.277). Again, there was no significant difference in the frequency distribution of patients and control subjects according to age (P = 0.0125). Patients' group included < 20 years, n (%) in patients 17 (34.00 %), and in control 7 (20.00 %), whereas, 20-25 years, n (%) in patients 6 (12.00%) and in control 7 (20.00 %), whereas, 30-35 years, n (%) in patients 8 (16.00%) and in control 3 (8.57 %).

***Keywords*---**hepcidin gene, polymorphism, β-thalassemia.

Introduction

Thalassemia is a blood disorder caused by a genetic mutation that leads to impaired production of the hemoglobin chain. Thalassemia patients usually have complications such as anemia, issues related to blood transfusion, hepatic or cardiac involvement, and psychosocial effects (Sardar et al., 2021). Mutations in the -globin gene are the most prevalent cause of genetic diseases in humans, with 350 -thalassemia mutations reported to date (De Sanctis et al.,2017).

Its problems have a negative influence on afflicted persons' quality of life, including negative consequences on their general health, school performance,

mental health status, and physical, social, and psychological elements of their existence. (Ansari et al.,2014; Nashwan et al.,2018).

Metabolism of iron

Iron demand, iron storage, erythropoiesis, hypoxia, and inflammation all influence hepcidin, the master iron regulator.It is predominantly generated in the liver then released into the circulation. (Nicolas et al., 2001; Muckenthaler et al., 2017). Hepcidin identifies the membrane-bound protein ferroportin, which is the sole iron exporter, once it is in circulation. The hepcidin-ferroportin combination is absorbed and destroyed by lysosomes when hepcidin binds to ferroportin, blocking iron transfer from the intracellular to the extracellular environment. (Muckenthaler et al., 2017; Nemeth et al., 2004).Because ferroportin is present in enterocytes, parenchymal hepatic cells, and macrophages, the amount of hepcidin in circulation, as well as ferroportin on extracellular membranes, regulates dietary iron absorption in the duodenum, liver iron storage, and macrophage iron recycling (Muckenthaler et al .,2017)

It's not unexpected that organisms developed sophisticated systems for iron uptake, recycling, and effective tissue distribution because iron is relatively limited in forms that may be utilised for biological activity. Although iron is necessary, too much of it can cause the creation of extremely harmful reactive oxygen species (ROS), which can harm DNA, proteins, and lipid membranes, resulting in organ failure.(Arezes &Nemeth, 2015; Papanikolaou & Pantopoulos, 2005; Oikonomidou et al., 2016)

Physiological consequences to Overabundance of iron

When serum iron levels rise above normal, iron binds to the unsaturated sites of transferrin molecules (about two-thirds of their capacity is vacant) in an attempt to reduce the deleterious effects of free iron. More free iron will be created in plasma in the form of non-transferrin-bound iron (NTBI) if iron levels surpass theiron binding capability. NTBI is highly harmful and readily absorbed by all tissue cellular membranes. As a result of this unregulated uptake, free iron will accumulate inside the cell. The liver, heart, and endocrine system are the most commonly afflicted organs by such accumulations. This labile iron (NTBI) is primarily responsible for iron toxicity and the resulting ROS(Kruszewski,2004).

It's been proposed that the LIP is a low-molecular-weight intermediate or transitory pool between extracellular iron and intracellular firmly bound iron. Jacobs (Jacobs, 1977). The Fenton and Haber-Weiss processes, which create ROS, are catalyzed by the intracellular LIP, which is redox active (Jacobs,1977). Excess ROS are cytotoxic, causing damage to important organs through interactions with biological components such as DNA, proteins, and lipids (Fibach & Rachmilewitz , 2008)

Hepcidin

In the year 2000, the hepcidin molecule ('hep' hepatic origin, 'cidin' antimicrobial action) was identified as a novel antimicrobial peptide working in part on innate

immunity, was identified in its active form from human blood ultrafiltrates and urine as a cysteine-rich peptide generated in the liver (Krause et al.,2000& Park et al.,2001).
Hepcidin is produced by adipocytes, macrophages, lymphocytes, neutrophils, pancreatic -cells, and renal cells (Kulaksiz et al., 2008). Hepcidin is a peptide hormone that helps the human body maintain iron homeostasis. transports iron from the daily food via the intestinal enterocyte apical membrane, where it is either stored as ferritin or exported into the plasma by hepcidin-ferroportin (Fpn) as an exporter .Hepcidin (hepatic bactericidal protein) is a cysteine-rich peptide, There are 25 amino acids in it, as well as four disulfide bridges. It plays an important part in the body's iron control .The formation of hepcidin is connected to the stimulation of iron in plasma and its subsequent storage. The absorption of iron from the food is hampered by this increase in iron.(Krause et al., 2000) (Ganz

2003; Kemna et al., 2008; Politou& Papanikolaou, 2004)

The role of hepcidin in thalassemia patients

Urinary and serum hepcidin levels are drastically reduced in iron-loading anemias not treated with frequent transfusions, such as -thalassemia intermedia (Kearney et al., 2007; Nemeth & GANZ, 2006; ORIGA et al., 2007). Due to the eager uptake of non-transferrin bound iron by hepatocytes, hepcidin deficiency permits excessive iron absorption and is impacted by iron overload. Other organ iron excess appears to be related to the rate of iron absorption. Rapid iron buildup, such as that observed in severe hepcidin insufficiency (juvenile hemochromatosis, thalassemia intermedia), is linked to significant iron deposition in the heart and several endocrine organs. Transfusions, rather than dietary iron absorption, are the most common cause of iron excess in -thalassemia major. Hepcidin concentrations are much greater in chronically transfused patients than in nontransfused patients, owing to both increased iron burden and the relief of inefficient erythropoiesis (Kearney et al., 2007 & ORIGA et al., 2007).

Molecular analysis
Primers

ARMS-PCR primers for Hepcidin gene polymorphisms (rs10421768 -582A > G) were designed in this study using NCBI-SNP data base and Primer1 ARMS-PCR primers design online. These primers were provided from (ScientificReseracher. Co. Ltd. Iraq) as following tables:

Table (1)
The ARMS-PCR primers with their sequence and amplicon size

Primer	Sequence (5'-3')	Product size
G allele Wildtype Primer	CTGACACTGGGAAAACACCG	205bp
A allele Mutant type Primer	CTGACACTGGGAAAACACCA	
Common Reverse Primer	CTCACTTCCCCATCGCCTAC	

Genomic DNA Extraction

Genomic DNA from blood samples were extracted by using gSYAN DNA kit extraction kit (Frozen Blood) Geneaid, and checked by using Nano drop spectrophotometer at (260/280 nm), it was ranging between 1.8- 2.2, with purity average equal 2, and DNA concentration mean was 20 ng/µl.

ARMS-PCR Method

ARMS-PCR assay was performed for detection and genotyping of Hepcidin gene polymorphisms (rs10421768 -582A > G) β-thalassemia patients and in healthy control blood samples.

ARMS-PCR master mix preparation

ARMS-PCR master mix was prepared by using (**GoTaq® G2 Green Master Mix kit**) and this master mix done two reactions for each samples according to company instructions as following tables:

1- Wild type G allele ARMS PCR reaction Mix:

ARMS PCR Master mix	Volume
DNA template	5µl
Wild type Forward primers (10pmol)	2µl
Common Reverse Primer (10pmol)	2µl
G2 Green Master Mix	12.5µl
PCR water	3.5µl
Total volume	25µl

2-Mutant type A allele ARMS PCR reaction Mix:

ARMS PCR Master mix	Volume
DNA template	5µl
Mutant type Forward primers (10pmol)	2µl
Common Reverse Primer (10pmol)	2µl
G2 Green Master Mix	12.5µl
PCR water	3.5µl
Total volume	25µl

After that, these PCR master mix component that mentioned in table above were transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (BioRad. USA).

Detection of Genes Polymorphisms

Detection of (rs10421768 -582A > G) Polymorphism

The distribution of ARMS-PCR for Hepcidin gene polymorphisms (rs10421768 -582A > G) was detected by ARMS-PCR technique. At this locus there are three genotypes; AG, GG and AA. The wild type homozygote genotype were showed only G allele amplification at …… bp product size. the (AA) mutant type homozygote were showed in G allele only, whereas the (G/A) heterozygote were showed in both G and A allele. The presence of A or G allele were observed at 205bp product size. figure (1).

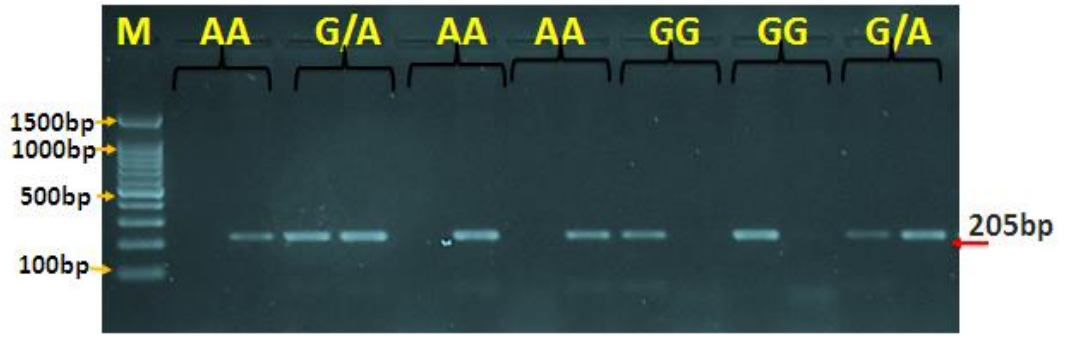

Figure 1: Agarose gel electrophoresis image that showed the ARMS-PCR product analysis of Hepcidin gene polymorphisms (rs10421768 -582 A > G). Where M: marker (1500-100bp). The (GG) wild type homozygote were showed in G allele only, the (AA) mutant type homozygote were showed in C allele only, whereas the (G/A) heterozygote were showed in both G and A allele. The presence of A or G allele were observed at 205bp product siz

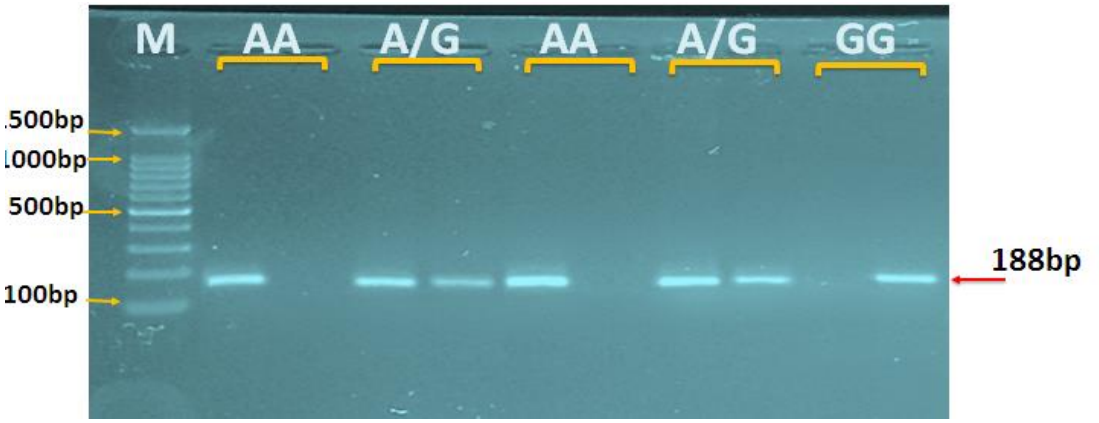

Figure 2: Agarose gel electrophoresis image that showed the ARMS-PCR product analysis of rs16944 (A/G) gene polymorphism. Where M: marker (1500-100bp).

The presence of A or G allele were observed at 281bp product size. the (AA) wild type homozygote were showed in A allele only, the (GG) mutant type homozygote were showed in G allele only, whereas the (A/G) heterozygote were showed in both A and G allele.

Result

Despite chelating therapy, iron overload is still one of the main complications of thalassemia. Our findings and others emphasize the role of hepcidin -582A > G polymorphism as a key component of iron homeostasis in these patients.(Zarghamian et al.,2020). Hepcidin is a 25-amino acid peptide, derived from cleavage of an 84 amino acid pro-peptide produced predominantly by hepatocytes. This molecule, encoded by the hepcidin antimicrobial peptide (HAMP) gene shows structural and functional properties consistent with a role in innate immunity. Moreover, as demonstrated in mice and humans, hepcidin is a major regulator of iron metabolism, and acts by binding to ferroportin and controlling its concentration and trafficking. In this study we investigated the influence that mutations in HAMP and/or hemocromatosis (HFE) genes might exert on iron metabolism in a group of poly-transfused thalassemic patients in preparation for bone marrow transplantation. Our results showed that the presence of the c.-582 A>G polymorphism (rs10421768) placed in HAMP promoter (HAMP-P) might play a role in iron metabolism, perhaps varying the transcriptional activation that occurs through E-boxes located within the promoter.(Andreani et al., 2009).

The G allele is meaningfully associated with TB disease. Significant differences were seen in the levels of serum iron and hepcidin but not ferritin between the -582A>G polymorphism genotypes. There was significant reverse correlation between hepcidin and iron and A high association was found between serum hepcidin levels and the HAMP -582A> G variants in patients with TB. These observations indicate a hypothetical role of this polymorphism in iron metabolism. Hepcidin could perhaps be an option for the treatment of TB (Javaheri-Kermani et al., 2014).

Since regular iron chelation treatment is likely able to override possible differences. Obviously several determinants may contribute to the clinical condition of iron overload in beta-thalassemia patients, including blood transfusions, chelation regimen and also iron absorption secondary to erythropoietic expansion. However, after considering several variables, the Authors speculate that these patients might theoretically have lower hepcidin levels than the corresponding patients without the polymorphic change .Although the paper does not report measurements of serum or urinary hepcidin of the patients studied, it indirectly suggests that the presence of the “G” nucleotide substitution makes these patients more prone to iron loading.

References

1. Andreani, M., Radio, F. C., Testi, M., De Bernardo, C., Troiano, M., Majore, S., ... & Grammatico, P. (2009). Association of hepcidin promoter c.-582 A> G variant and iron overload in thalassemia major. haematologica, 94(9), 1293.
2. Ansari, S. H., Baghersalimi, A., Azarkeivan, A., Nojomi, M., & Rad, A. H. (2014). Quality of life in patients with thalassemia major. Iranian journal of pediatric hematology and oncology
3. Arezes, J., & Nemeth, E. (2015). Hepcidin and iron disorders: new biology and clinical approaches. *International journal of laboratory hematology*, *37*, 92-98.

4. Bruno, F., Bonalumi, S., Camaschella, C., Ferrari, M., & Cremonesi, L. (2010). The-582A> G variant of the HAMP promoter is not associated with high serum ferritin levels in normal subjects. *Haematologica*, *95*(5), 849.
5. De Sanctis, V., Kattamis, C., Canatan, D., Soliman, A. T., Elsedfy, H., Karimi, M., ... & Angastiniotis, M. (2017). β-thalassemia distribution in the old world: an ancient disease seen from a historical standpoint. *Mediterranean journal of hematology and infectious diseases*, *9*(1).
6. Fibach, E., & Rachmilewitz, E. (2008). The role of oxidative stress in hemolytic anemia. *Current molecular medicine*, *8*(7), 609-619.
7. Ganz, T. (2003). Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*, *102*(3), 783-788.
8. Jacobs, A. (1977). Low molecular weight intracellular iron transport compounds. 50(3):433–9
9. Jacobs, A. (1977, January). An intracellular transit iron pool. In *Ciba Foundation Symposium 51-Iron Metabolism* (pp. 91-106). Chichester, UK: John Wiley & Sons, Ltd..
10. Javaheri-Kermani, M., Farazmandfar, T., Ajami, A., & Yazdani, Y. (2014). Impact of hepcidin antimicrobial peptide on iron overload in tuberculosis patients. *Scandinavian Journal of Infectious Diseases*, *46*(10), 693-696.
11. Kearney, S. L., Nemeth, E., Neufeld, E. J., Thapa, D., Ganz, T., Weinstein, D. A., & Cunningham, M. J. (2007). Urinary hepcidin in congenital chronic anemias. *Pediatric blood & cancer*, *48*(1), 57-63.
12. Kemna, E. H., Tjalsma, H., Willems, J. L., & Swinkels, D. W. (2008). Hepcidin: from discovery to differential diagnosis93 (1), pp. 90–7
13. Krause, A., Neitz, S., Mägert, H. J., Schulz, A., Forssmann, W. G., Schulz-Knappe, P., & Adermann, K. (2000). LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS letters, 480(2-3), 147-150.
14. Kruszewski, M. (2004). The role of labile iron pool in cardiovascular diseases. *Acta Biochimica Polonica*, *51*(2), 471-480.
15. Kulaksiz, H., Fein, E., Redecker, P., Stremmel, W., Adler, G., & Cetin, Y. (2008). Pancreatic b-cells express hepcidin, an iron-uptake regulatory peptide. *Journal of Endocrinology*, *197*(2), 241-250.
16. Muckenthaler, M. U., Rivella, S., Hentze, M. W., & Galy, B. (2017). A red carpet for iron metabolism. Cell, 168(3), 344-361.
17. Nashwan, A. J., Yassin, M. A., Babu, G. D. J., Nair, S. L., Libo-On, I. L., Hijazi, H. A., ... & Soliman, A. (2018). Quality of life among adolescents aged 14 to 18 years with beta-thalassemia major (TM) in Qatar. *Acta Bio Medica: Atenei Parmensis*, *89*(Suppl 2), 16.
18. Nemeth, E., & Ganz, T. (2006). Hepcidin and iron-loading anemias. *haematologica*, *91*(6), 727-732.
19. Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., ... & Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. science, 306(5704), 2090-2093.
20. Nicolas, G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, A., & Vaulont, S. (2001). Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proceedings of the National Academy of Sciences*, *98*(15), 8780-8785.

21. Oikonomidou, P. R., Casu, C., & Rivella, S. (2016). New strategies to target iron metabolism for the treatment of beta thalassemia. *Annals of the New York Academy of Sciences*, *1368*(1)
22. Origa, R., Galanello, R., Ganz, T., Giagu, N., Maccioni, L., Faa, G., & Nemeth, E. (2007). Liver iron concentrations and urinary hepcidin in β-thalassemia. *Haematologica*, *92*(5), 583-588.
23. Papanikolaou, G., & Pantopoulos, K. (2005). Iron metabolism and toxicity. *Toxicology and applied pharmacology*, *202*(2), 199-211.
24. Park, C. H., Valore, E. V., Waring, A. J., & Ganz, T. (2001). Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Journal of biological chemistry*, *276*(11), 7806-7810.
25. Politou, M., & Papanikolaou, G. (2004). Hepcidin: A key iron regulator involved in the pathogenesis of anaemia of chronic disease. *Haema*, *7*(2), 165-174.
26. Sardar, S., Ali, E. A., & Yassin, M. A. (2021). Thalassemia and Priapism: A Literature Review of a Rare Association. Cureus, 13(4).
27. Zarghamian, P., Azarkeivan, A., Arabkhazaeli, A., Mardani, A., & Shahabi, M. (2020). Hepcidin gene polymorphisms and iron overload in β-thalassemia major patients refractory to iron chelating therapy. BMC Medical Genetics, 21(1), 1-5.