Correlation of genes (*HFE*, *TF*, *TFR2)* expressions and iron deficiency anemia in children

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Abstract

Iron deficiency anemia (IDA) is a prevalent nutritional condition, especially impacting children globally. It arises when there is inadequate iron to satisfy the body's requirements, resulting in diminished hemoglobin levels and compromised oxygen delivery. Iron deficiency anemia in children may lead to developmental delays, cognitive impairments, and heightened vulnerability to infections, making it a critical public health issue that needs greater study. The transferrin gene (*TF*) encodes the protein transferrin, which is responsible for iron transport in the bloodstream. The transferrin receptor 2 gene (*TFR2*) encodes a receptor that binds transferrin, facilitating cellular iron uptake. Additionally, the hemochromatosis iron regulator gene (*HFE*) is involved in regulating iron homeostasis by influencing the body's iron receptors.

The research included 50 children with iron deficiency anemia and healthy control group of 30 children for comparison analysis, measuring the expression levels of certain genes. The methods included collecting of blood samples from male and female children aged (eleven months to nine years), all diagnosed with iron deficiency anemia and free from chronic conditions. The diagnosis of iron deficiency anemia was verified with a complete blood count (CBC) and an iron assay for each sample.

Gene expression studies for all samples demonstrated a statistically significant elevation in the expression of the *TF*, *TFR2*, and *HFE* genes relative to the control group. Additionally, we noted a favorable link between the gene expression levels of *HFE* and *TF*, as well as between *HFE* and *TFR2*. These results provide additional data on the involvement of these genes in the regulation of iron levels inside the body. The increased expression of *TF*, *TFR2*, and *HFE* in children with iron deficiency anemia indicates an adaptive mechanism to counteract iron deficit. Consequently, the elevated expression of these genes may serve as a marker for iron deficiency in the body.

Introduction

Iron deficiency is the most common nutrient deficiency in the world and is a public health problem. The World Health Organization (WHO) reported in 2008 that 24.8% of the global population is anemic, with iron deficiency (ID) accounting for half of the anemia cases. In underdeveloped nations, between 40%-50% of children under the age of five get iron deficiency anemia (Özdemir, 2015). Iron deficiency is characterized by insufficient iron in the body that does not inhibit hemoglobin formation, but iron deficiency anemia (IDA) is characterized by a reduction in hemoglobin levels resulting from iron deficiency (Powers, 2022).Transferrin (TF) is

a major iron transport protein in the bloodstream, contributing to the maintenance of cellular iron homeostasis by modulating cellular iron uptake. Each transferrin molecule can carry one or two iron ions prior to interacting with a transferrin receptor on the cell membrane. The transferrin–iron complex is internalized by endocytosis, releasing iron ions for metabolic processes. The transferrin receptor is returned to the cell surface, while the transferrin molecule is liberated to re-enter the plasma transferrin pool. The transferrin receptor in serum mostly originates from maturing red blood cells. Cells may control their iron absorption by adjusting the expression of the transferrin receptor on their surface and by storing excess iron as ferritin (Skikne *et al*., 1990). Transferrin receptor-2 (TFR2) is a type II transmembrane glycoprotein that is similar to TFR1 (Kawabata *et al*., 1999) and can bind diferric-transferrin (holo-TF), but with a lower affinity than TFR1. In iron homeostasis TFR2 appears to have regulatory rather than transport functions, since TFR2 mutations in humans(Camaschella *et al*., 2000) and TFR2 inactivation in mice(Wallace et al., 2005; Roetto *et al*., 2010) . TFR2 is expressed mainly in the liver (Fleming et al., 2000) and is encoded by the *TFR2* gene located on chromosome 7q22 (Silvestri *et al*., 2014). Research on cultured hepatoma cell lines indicates that TFR2, when associated with holo-TF, may concurrently interact with the hemochromatosis iron regulator (HFE). The HFE- and TF-binding sites on TFR1 overlap, but those on TFR2 are distinct (Johnson and Enns, 2004; Alessio *et al*., 2012). The HFE-TFR2 complex is hypothesized to function as a sensor for circulating iron levels (Chen J, Enns, 2012).

Materials and methods

Samples

This research comprised 80 children aged between 11 months and 9 years. Fifty children with iron deficiency anemia and thirty children without iron deficiency anemia, serving as the control group, were proven to have iron deficiency anemia using complete blood count (CBC) tests and iron assays for each sample.

Molecular Analysis

Total RNA Extraction

1. Total RNA from all samples was extracted using the TRIzol® LS Reagent in accordance with the manufacturer's procedure (Trizol LS Reagent) as detailed below:

2. A volume of 250µL of blood was combined with 750µL of trizol® LS Reagent in an Eppendorf tube.
3. Homogenized the mixture using a vortex.
4. 200 µL of chloroform was added to the mixture and shaken violently for 15 seconds.
5. The mixture was subjected to incubation on ice for 5 minutes.
6. The mixture underwent centrifugation at 12,000 rpm for 10 minutes at 4℃. Upon centrifugation, the mixture segregates into a lower organic phase, an interphase, and a colorless upper aqueous phase. RNA is confined only to the aqueous phase.
7. The aqueous phase was transferred to a fresh 1.5 ml tube, and an equivalent amount of isopropyl alcohol was added.
8. The mixture was inverted for 10 seconds and incubated at -20 °C for 10 minutes.
9. The mixture was centrifuged at 12,000 rpm for 10 minutes at 4℃, after which the supernatant was carefully collected.
10. 9.80% ethanol was added and thoroughly mixed by inversion or vortexing.
11. Centrifuged at 12,000 rpm for 5 minutes at 4℃, then meticulously decanted the supernatant.
12. Dried the pellet with hot air.
13. RNA was solubilized in RNase-free water and incubated for 10 minutes at 60℃.
14. Preserve in a frozen state until required.

Assessment of RNA Purity and Concentration

The NanoVue Nanodrop spectrophotometer (England) was used to measure the concentration and purity of extracted RNA for determining the quality of samples for subsequent RT-qPCR analysis. The RNA content of the samples varied from 73 to 147 ng/µl, and the absorbance was assessed at two specific wavelengths (260 and 280 nm) to evaluate RNA purity. An A260/A280 ratio of around 2.0 indicated the RNA sample's purity.

Quantitative Real-Time PCR

The expression levels of the HFE, TF, and TFR2 genes were estimated by Quantitative Real-Time PCR (qRT-PCR). TransStart® Top Green qPCR Super Mix (SYBR Green) was used to confirm the expression of the target gene. Primer sequences for these genes were produced by Alpha DNA Ltd (Canada) and kept in a lyophilized state at -20°C. The mRNA levels of the

endogenous control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene were used as an internal control to standardize the mRNA levels of the target genes.

Complementary DNA (cDNA) Synthesis of mRNA

Total RNA was reverse transcribed into complementary DNA (cDNA) by using a cDNA kit from Addbio Company, Korea. The process was done in a reaction volume of 25µl following the manufacturer's guidelines.

Primer Design

The primer design procedure was conducted using Primer3 web version 4.1.0 (available at http://primer3.ut.ee) for the HFE, TF, TFR2, and GAPDH genes, and then verified by the University Code of Student Conduct (UCSC) programs. Synthesis and lyophilization were conducted by Alpha DNA Ltd. (Canada).

Primer Preparation

For each test in this investigation, the required primers were produced as follows: Following the dissolution of the lyophilized sample in nuclease-free water according to the manufacturer's guidelines, a stock solution with a concentration of 100µM was made and preserved at -20°C. By diluting 10µL of each primer stock solution in 90µL of nuclease-free water, a working solution with a concentration of 10µM was produced and stored at -20°C until used.

Statistical analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. T-test was used to significant compare between means. Chi-square test was used to significantly compare between percentage (0.05 and 0.01 probability) and estimate of correlation coefficient between variables in this study (Al-Gharban and Dhahir, 2015).

Result

HFE, TF and TFR2 gene expression in patients with IDA and healthy control

The expression of the *HFE*, *TFR2*, and *TF* genes in patients with iron deficiency anemia (IDA) was compared to that of healthy controls, and the results are presented in Table 1. The mean *HFE* gene expression in patients with IDA was 5.33 ± 1.72, while in healthy controls it was 1.0. Similarly, the mean *TFR2* gene expression was 8.62 ± 2.73 in IDA patients and 1.0 in healthy

controls. The mean *TF* gene expression was 4.04 ± 1.2 in patients with IDA and 1.0 in healthy controls. The increase in mean gene expression of *HFE*, *TFR2*, and *TF* was highly significant in patients with IDA compared to healthy controls (P<0.001, P<0.001).

(Table 1): *HFE*, *TFR2*, and *TF* Gene Expression in IDA children and Healthy Controls

Gene/Marker	Group	n	Mean ± SD	Range	p-value
HFE	IDA Patients	50	5.33 ± 1.72	0.79 – 12.37	< 0.001
	Healthy Controls	30	1.0	1.0-1.0	
TFR2	IDA Patients	50	8.62 ± 2.73	1.0	< 0.001
	Healthy Controls	30	0.99 – 17.45	1.0-1.0	
TF	IDA Patients	50	4.04 ± 1.2	1.0	< 0.001
	Healthy Controls	30	1.68 – 6.54	1.0-1.0	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; HS: Highly significant at P ≤ 0.001.

Correlation between genes expression

The results indicate a significant positive correlation between *HFE* and *TFR2* gene expression (r = 0.435, p = 0.001), as well as between *HFE* and *TF* gene expression (r = 0.313, p = 0.001).

(Table 2): Correlation Analysis of *HFE*, *TFR2*, and *TF* Gene Expression in IDA children

		HFE	TFR2	TF
HFE	r	1	0.435*	0.313*
	p		0.001	0.001

Discussion

Our findings indicated that transferrin gene expression levels in children with iron deficiency anemia are statistically elevated compared to those in healthy children (Figure 1). This means that Transferrin levels rise in response to iron insufficiency in an attempt to capture iron and fill the deficiency, our results agree with (Bermejo and García-López, 2009) show High transferrin signifies low iron, which means there is less iron bound to transferrin, allowing for a high circulation of non-bound iron transferrin in the body, revealing a possible iron deficiency anemia. The liver increases the production of transferrin as a form of homeostasis to enable transferrin to bind to iron and transport it to the cells. Also The findings of our work agree with those of Idzerda *et al*. (1986), which demonstrated that rats subjected to a low-iron diet served as a model

for examining the regulate of transferrin gene expression in response to iron deficit. Transferrin mRNA was quantified in several organs from both normal and iron-deficient rats. The analysis revealed that the transferrin mRNA level in the liver of normal rats was around 6,500 molecules per cell, but in iron-deficient rats, it was 2.4 times more.

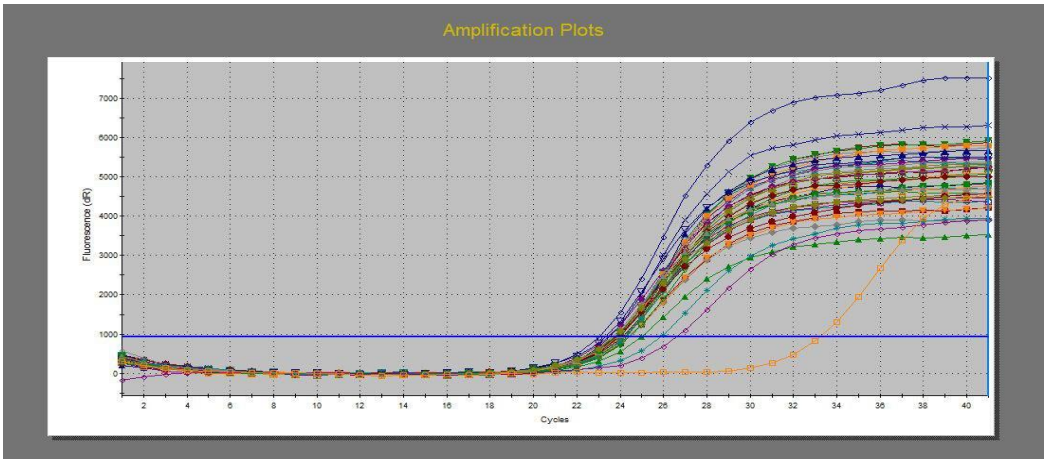

Figure (1): The Real Time PCR amplification plots of TF gene expression in IDA children

Our work demonstrates an increased expression of the *TFR2* gene in children with iron deficient anemia (Figure 2), and this finding is similar with the prior research conducted by Ferguson *et al*., 1992. A research was conducted to evaluate the serum transferrin receptor's capacity to differentiate between iron-deficiency anemia and anemia of chronic inflammation, as well as to detect iron deficiency in patients with liver illness. The average transferrin receptor level in 17 normal controls was 5.36 ± 0.82 mg/L, but in 17 patients with iron-deficiency anemia, it was 13.91 ± 4.63 mg/L ($p < 0.001$). The average serum receptor level was normal in all 20 patients with acute infection, including five with acute hepatitis, and was also normal in 8 of 10 anemic individuals with chronic liver disease. Receptor levels were within the normal range for all but 4 of the 41 individuals diagnosed with anemia of chronic illness. The serum transferrin receptor level is unaffected by these illnesses, making it a dependable laboratory indicator of iron deficiency anemia. Our findings align with those of Skikne *et al*. (1990), who demonstrated that cells may control their iron absorption by regulating the expression of the transferrin receptor on their surface. Consequently, serum transferrin receptor levels indicate the extent of erythropoiesis and the need for iron. As the availability of iron to the tissues diminishes, the expression of transferrin receptors rises.

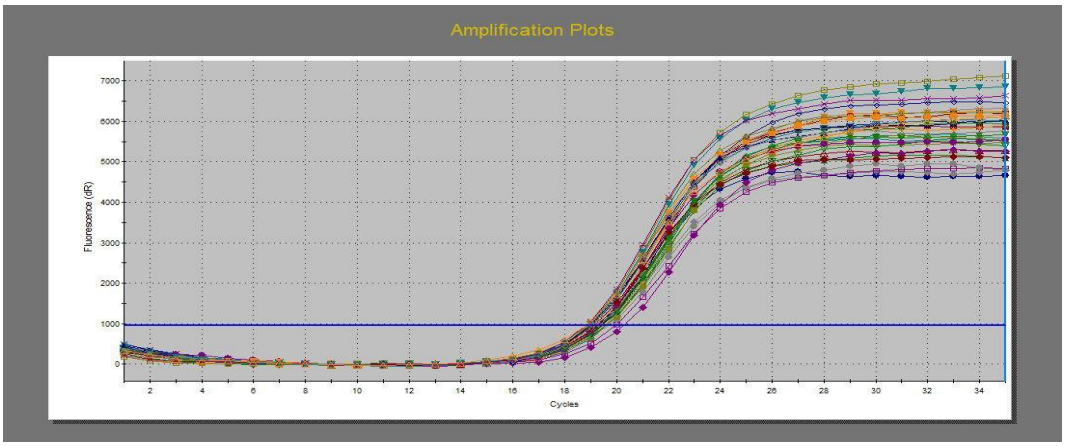

Figure (2): The Real Time PCR amplification plots of TFR2 gene expression in IDA children

Our findings indicate a substantial elevation in *HFE* gene expression in children with iron deficiency anemia compared to healthy children (Figure 3), corroborating a study by Beiranv *et al*. (2015), which reported that *HFE* gene expression levels in patients with iron deficiency anemia were significantly higher than in other groups.

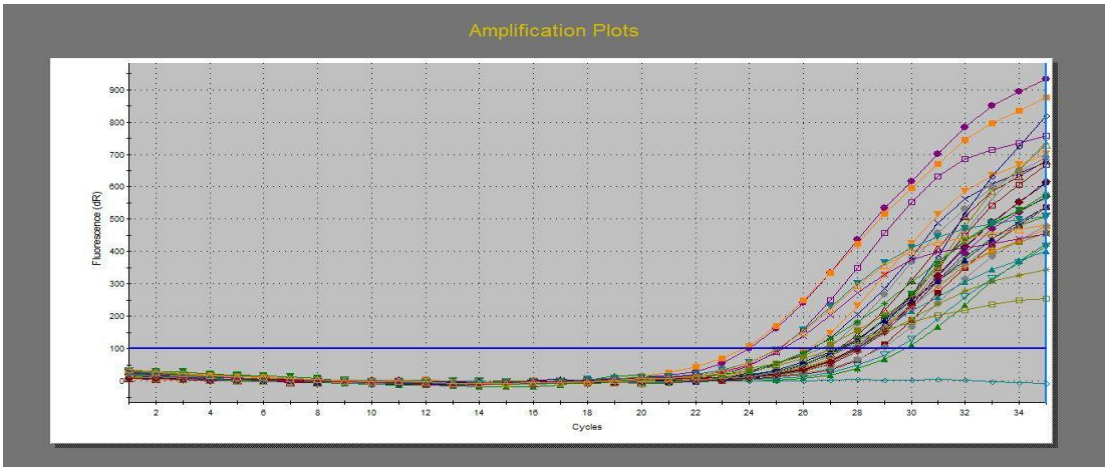

Figure (3): The Real Time PCR amplification plots of HFE gene expression in IDA children

A research by Goswami and Andrews (2006) demonstrates that TFR2 competes with TFR1 for binding to HFE, regardless of Fe2-TF levels. The greatest competition for the HFE-TFR1 interaction was seen in the presence of both Fe2-TF and elevated TFR2 expression. In hepatocytes, elevated serum Fe2-TF enhances TFR2 expression (Robb and Wessling-Resnick, 2004; Johnson and Enns, 2004) while concurrently decreasing TFR1 (Hubert *et al*., 1993). These modifications would preferentially promote the development of an HFE-TFR2 complex rather

than an HFE-TFR1 complex. Consequently, an iron status sensing function for HFE and the HFE-TFR2 interaction arises (Goswami and Andrews, 2006). This allows us to deduce that an elevation in TFR2 correlates with an increase in HFE, indicating a positive relationship between TF and HFE, which aligns with our findings.

Our findings indicate a favorable association between the expression of *TFR2* and the *HFE* gene. This occurs because TfR2 detects fluctuations in blood-iron concentrations via its interaction with holo-TF. Although structurally similar to the iron-endocytosis protein TfR1, it has a diminished affinity for TF, possesses an alternative binding site for HFE, and is subject to distinct trafficking and regulatory mechanisms. The distinctions, along with the tissue expression profile of TfR2, suggest that the role of TfR1 is to bind and internalize iron for cellular use, while the role of TfR2 is to detect blood iron concentrations. TfR2 regulates body iron absorption in accordance with blood iron concentrations (Worthen and Enns, 2014).

We conclude that the elevated expression of the genes TF, TFR2, and HFE serves as an indicator of iron deficiency anemia in the body.

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