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Levels of EPOR Protein and Gene Expression of *SLC11A2, SLC40A1* in Children with Iron Deficiency Anemia

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Abstract
Iron has many different roles in the body. About 65 to 80 percent of the body's iron is in the blood in the form of hemoglobin. Hemoglobin is a protein in red blood cells that transports oxygen to tissues in the body. If iron is lacking in the diet, iron reserves in the body are used. Once this supply is depleted the formation of hemoglobin is affected. This means the red blood cells cannot carry oxygen needed by the cells. When this happens, iron deficiency occurs and anemia results. The Solute Carrier Family 11 Member 2 (*SLC11A2)* and Solute Carrier Family 40Member 1 (*SLC40A1)*gene plays a crucial role in iron metabolism and homeostasis in the body, The erythropoietin receptor (EPOR) gene plays a crucial role in erythropoiesis and iron metabolism, with its protein product playing a central role in erythroid cell survival and proliferation.
The study included 50 children with iron deficiency anemia and healthy control group of 30 children for comparison analysis and 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.
The studies of gene expression of *SLC11A2* and *SLC40A1* in IDA children and healthy children observed significant increase of gene (*SLC11A2)* expression and non-significant decrease of gene *(SLC40A1*). Study showed significant negative correlation between *SLC11A2* and *SLC40A1*gene expression. Observed increase in EPOR level in IDA children when compared with healthy children but this increasing was non-significant. The concluding *SLC11A2* gene expression elevates in iron deficiency anemia to increase intestinal iron absorption, while *SLC40A1* gene expression diminishes in correlation with the severity of iron deficiency to keep cellular iron levels. Also we conclude that the EPOR level rises in response to iron insufficiency, with the increase being proportionate to the extent of iron reduction to prevent the formation of deformed red blood cells.

Keywords: EPOR protein, SLC11A2, SLC40A1, Iron deficiency anemia.

INTRODUCTION

Iron plays a crucial role through various life stages of human. Iron homeostasis is primarily regulated by iron absorption which is mediated via Solute Carrier Family 11 Member 2(SLC11A2), (Qiu *et al.*,2025) Solute Carrier Family 11 Member 2 is a mammalian iron transporter encoded by the *SLC11A2* gene. SLC11A2 has a vital role in iron homeostasis by mediating iron uptake in the intestine and kidneys and by recovering iron from recycling endosomes after transferrin endocytosis. (Romero-Cortadellas *et al.,* 2022) and iron export protein Solute Carrier Family 40 Member 1 SLC40A1 (Qiu *et al.*, 2025) The *SLC40A1* gene plays a crucial role in iron metabolism and homeostasis in the body, It encodes the only known mammalian iron exporter protein, which is essential for maintaining both cellular and systemic iron balance (Sangokoya *et al.*, 2013). The erythropoietin receptor (EPOR) gene plays a crucial role in erythropoiesis and iron metabolism. EPOR signaling is essential for erythroblast survival and proliferation, and it also influences red blood cell size and

cell cycle progression (Hidalgo *et al.*, 2021). The EPOR gene encodes a membrane receptor that interacts with erythropoietin (EPO) to induce the proliferation and differentiation of erythroid progenitors (Yoshimura and Misawa, 1998). The EPOR gene is crucial for erythropoiesis, with its protein product playing a central role in erythroid cell survival and proliferation(Yoon *et al.*, 2006).

MATERIALS AND METHODS

Study population

This research comprised 80 children (50 with IDA and 30 as healthy control) with age (11 months -9 years). Using complete blood count (CBC) tests and iron assay for each sample.

Human Erythropoietin receptor evaluation

Human Erythropoietin receptor (EPOR) ELISA Kit is to assay EPOR levels in Human serum, plasma and culture media. This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an Antibody specific to EPOR. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific Antibody. Then a Horseradish Peroxidase (HRP)-conjugated Antibody specific for EPOR is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain EPOR and HRP conjugated EPOR Antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of EPOR.

Molecular analysis

Total RNA Extraction

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:

1. A volume of 250µL of blood was combined with 750µL of trizol® LS Reagent in an Eppendorf tube.
2. Homogenized the mixture using a vortex.
3. 200 µL of chloroform was added to the mixture and shaken violently for 15 seconds.
4. The mixture was subjected to incubation on ice for 5 minutes.
5. The mixture underwent centrifugation at 12,000 rpm for 10 minutes at 4°C. 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.
6. The aqueous phase was transferred to a fresh 1.5 ml tube, and an equivalent amount of isopropyl alcohol was added.
7. The mixture was inverted for 10 seconds and incubated at -20 °C for 10 minutes.
8. The mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C, after which the supernatant was carefully collected.
9. 80% ethanol was added and thoroughly mixed by inversion or vortexing.
10. Centrifuged at 12,000 rpm for 5 minutes at 4°C, then meticulously decanted the supernatant.
11. Dried the pellet with hot air.
12. RNA was solubilized in RNase-free water and incubated for 10 minutes at 60°C.
13. Preserve in a frozen state until required.

Assessment of RNA Purity and Concentration

The 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 *SLC11A2* and *SLC40A1*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 25 µl 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 theSLC11A2,SLC40A1 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. The t-test was used to significant compare between means (Gharban, 2024). Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimation of correlation coefficient between variables in this study was done.

Results

Levels of Iron and erythropoietin receptor (EPOR) in IDA children and healthy control subject

The comparison of iron levels between patients with iron deficiency anemia (IDA) and healthy control participants has been conducted and findings shown in Table 1. The mean iron levels were 5.57±1.92 in patients with iron deficiency anemia (IDA) and 12.95±2.80 in the healthy control group, indicating a highly significant decrease in IDA children compared to healthy controls (P≤0.001). Additionally, the comparison of erythropoietin receptor (EPOR) levels between IDA children and healthy controls was conducted and results presented in Table (3-1). The mean Erythropoietin receptor levels were 586.51± 111.03 in children with iron deficiency anemia (IDA) and 577.10± 126.6 in the healthy control group, indicating a non-significant increase in IDA patients compared to the healthy controls (P=0.760).

Table (1): Iron and EPOR levels in IDA children and healthy controls

Indicator	Group	No.	Mean ± SD	Range	p-value
Iron level (µmol/L)	IDA Patients	50	5.57 ± 1.92	1.80 – 10.50	≤ 0.001 † HS
	Healthy Controls	30	12.95 ± 2.80	8.00- 19.00	
EPOR level	IDA Patients	50	586.51 ± 111.03	355.13 – 882.52	0.760 † NS
	Healthy Controls	30	577.10 ± 126.6	320.21- 882.52	

n: number of cases; SD: standard deviation; †: independent samples t-test; HS: highly significant at P <0.001; NS: non- significant at P >0.05

SLC11A2 and SLC40A1 genes expressions in IDA children and healthy control

The expression of the *SLC11A2* gene has been compared between children with iron deficiency anemia (IDA) and healthy control participants and the results were demonstrated in table (3-2). The mean expression of the *SLC11A2* gene was 5.25 ± 1.2 in patients with iron deficiency anemia (IDA) and 1.0 in healthy controls. The increase in SLC11A2 gene expression in patients with IDA compared to healthy controls was highly significant (P < 0.001). Additionally, the comparison of SLC40A1 gene expression between patients with IDA and healthy controls was conducted and the results were demonstrated in table (2). The mean expression of the SLC40A1 gene was 0.98 ± 0.21 in children with iron deficiency anemia (IDA) and 1.0 in healthy controls; the mean expression in IDA patients exhibited a non-significant small drop compared to healthy controls (P= 0.727).

Table (2): *SLC11A2 and SLC40A1 genes* expressions in IDA children and healthy control

Gene	Group	No.	Mean ± SD	Range	p-value
SLC11A2	IDA Patients	50	5.25 ± 1.2	3.25 – 7.79	< 0.001 † HS
	Healthy Controls	30	1.0	1.0-1.0	
SLC40A1	IDA Patients	50	0.98 ± 0.21	0.42 – 1.57	0.727 † NS
	Healthy Controls	30	1.0	1.0-1.0	

n: number of cases; SD: standard deviation; †: independent samples *t*-test; HS: Highly significant at P ≤ 0.001t; NS: non-significant at P > 0.05.

Correlation between genes expressions

The current findings indicate a significant negative correlation between the expression of the SLC11A2 and SLC40A1 genes (r=-0.369, p=0.001).

Table (3): Correlation between *SLC11A2 and SLC40A1 genes* expressions

Gene		*SLC11A2*	*SLC40A1*
SLC11A2	R	1	-0.369*
	P		0.001
SLC40A1	R		1
	P		

DISCUSSION

Our findings indicate a significant decrease in iron levels among children with iron deficiency anemia (IDA) compared to healthy controls. This agrees with the results of Powers and O'Brien (2019), who demonstrated that laboratory findings reveal low serum iron in cases of iron deficiency anemia. Additionally, Sanad *et al.* (2011) found that serum iron levels were significantly lower in children with IDA relative to healthy controls, this discovery is consistent throughout various age groups and populations (Abdelhaleim *et al.*, 2019).The significant reduction in iron levels is a characteristic feature of iron deficiency anemia and is consistently detected when comparing IDA patients to healthy controls (Abdelhaleim *et al.*, 2019; Sanad *et al.*, 2011).

Our data indicate that the EPOR level exhibited a non-significant rise in children with IDA compared to healthy controls (Figures 1, 2). Nai *et al.* (2015) demonstrate in conditions of iron deficit there is usually an elevation in erythropoietin (EPO) levels and heightened sensitivity to EPO signaling demonstrates that in mice deficient in TFR2 inside the bone marrow which simulates characteristics of iron deficit there is an elevated expression of EPO-target genes, including the Epor gene that encodes the erythropoietin receptor.

Silvestri *et al.* (2014) demonstrate Transferrin receptor 2 (TFR2) plays a role for regulating the erythropoietin receptor (EPOR) response under iron-deficient circumstances in anemia. This means EPOR gene expression tends to increase in iron deficiency conditions, likely as part of a compensatory mechanism to enhance erythropoiesis in response to reduced iron availability but expression of EPOR is regulated in iron deficiency conditions by TFR2, which regulates the expression of EPOR in response to the amount of available iron to prevent the accumulation of immature red blood cells.
Transferrin receptor 2 (TFR2) acts as a partner of EPOR, stabilizing the receptor on the cell surface (Silvestri *et al.*, 2014). This interaction is particularly important in iron-restricted conditions. TFR2 is essential for an appropriate erythropoietic response in iron-deficient anemia, as mice lacking TFR2 in the hematopoietic compartment exhibit an accumulation of immature erythroblasts and a reduction in mature erythroid cells during anemia (Rishi *et al.*, 2016). Our research revealed an increase in the gene expression of the *SLC11A2* gene in children with iron deficiency anemia (IDA) compared to the control group, which agrees with the findings of Surekha *et al.* (2022). Two hundred pregnant women were recruited and categorized into anemic and non-anemic groups. Following delivery, the placental expression of *SLC11A2* was examined by mRNA analysis, revealing that mRNA expression levels of the *SLC11A2* gene were elevated in anemic women compared to non-anemic moms. Also we agree with Leong *et al.* (2003), who investigated *SLC11A2* gene expression in iron-deficient rat pups, iron-deficient rat pups with iron supplementation, and control subjects. *SLC11A2* exhibited a fourfold increase in the low-Fe group *vs* to the control group. Conversely, *SLC11A2* gene expression was down regulated with iron supplementation. Morgan and Oates (2002) also identified *SLC11A2* increased expression was seen in iron-deficient rats, whereas decreased expression was noted in iron-loaded rats compared to controls. Gros *et al.* (1999) demonstrated that *SLC11A2* mRNA is expressed in the villous enterocytes of the duodenum and is up regulated during iron deficit and down regulated after iron loading.

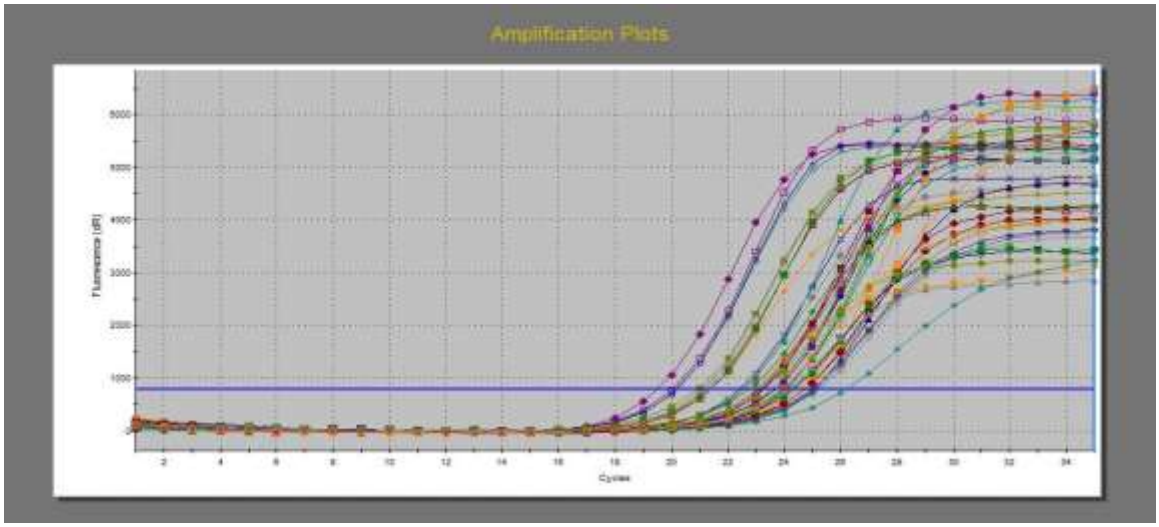

Figure (1): The Real Time PCR amplification plots of *SLC11A2* gene expression in IDA children

Our findings indicated that the gene expression level of *SLC40A1* diminished in children with IDA but the decrease was not significant compared to healthy children, since Sangokoya *et al.* (2013) (stated that *SLC40A1* expression is down regulated in an iron-dependent manner by binding of iron regulatory protein (IRP) to the IRE in the 5-prime UTR of the SLC40A1transcript. Using a reporter gene assay, they confirmed that *SLC40A1*expression decreased during iron depletion and increased significantly during iron supplementation in human HepG2 hepatocytes), Our findings agree with this, although the decrease is not

statistically significant; this may indicate that the reduction in *SLC40A1* gene expression correlates with low iron levels.

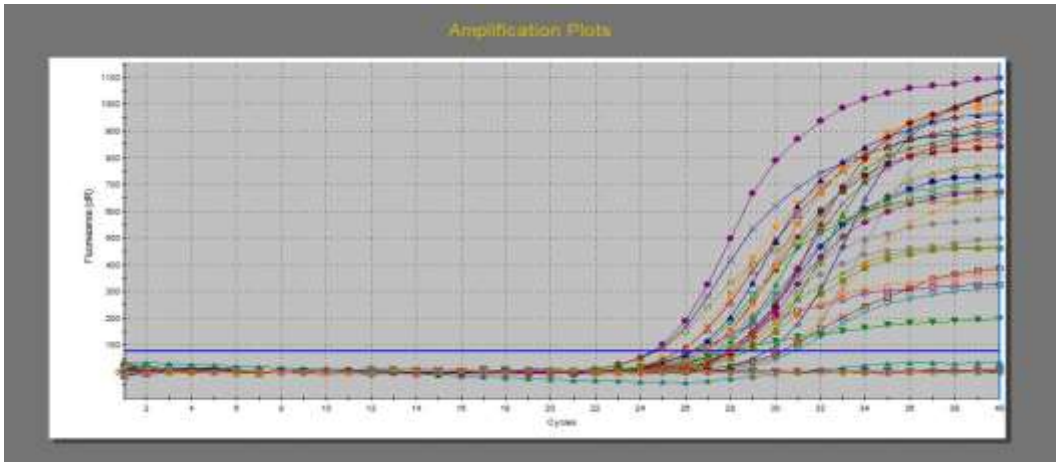

Figure (2): The Real Time PCR amplification plots of *SLC40A1* gene expression in IDA children

Our findings indicate a significant negative correlation between the gene expression of *SLC11A2* and *SLC40A1*, *SLC11A2* gene expression increases in iron deficiency due to several regulatory mechanisms: Hypoxia-inducible factor (HIF) signaling plays a critical role in upregulating *SLC11A2* expression during iron deficiency. Acute iron deficiency induces HIF signaling in the duodenum, leading to increased expression of *SLC11A2* and enhanced iron uptake. HIF-2α directly targets *SLC11A2* as a gene, demonstrating that intestinal HIF signaling is crucial for regulating systemic iron homeostasis (Shah *et al.*, 2009). The iron-responsive element (IRE) in the 3' UTR of *SLC11A2* mRNA is involved in regulating its expression in response to iron levels. Additionally, an upstream 5' exon (exon 1A) of the *SLC11A2* gene participates in iron-dependent regulation. These two regulatory regions - the 5' promoter/exon 1A region and the IRE-containing terminal exon-work together in a tissue-specific manner to control *SLC11A2* expression (Hubert and Hentze, 2002); whereas, *SLC40A1* encoded by the *SLC40A1* gene, plays a crucial role in maintaining cellular iron balance during iron deficiency through multiple regulatory mechanisms: During iron deprivation, *SLC40A1* translation is repressed by iron regulatory proteins (IRPs) binding to its 5' UTR, reducing iron export to preserve cellular iron (Sangokoya *et al.*, 2013). Additionally, miR-485-3p, induced during iron deficiency, represses SLC40A1expression by targeting its 3' UTR, further preserving cellular iron (Sangokoya *et al.*, 2013). These post-transcriptional mechanisms help cells retain iron under deficient conditions (Taylor *et al.*, 2011). *SLC40A1* gene maintains cellular iron during deficiency through a complex interplay of transcriptional, post-transcriptional, and post-translational mechanisms. These include IRP-mediated translational repression, miRNA-mediated repression, HIF-2α-dependent transcriptional activation, and increased protein stability, This multi-layered regulation ensures fine-tuned control (Delaby *et al.*, 2008).

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