

Reduced hepcidin expression enhances iron overload in patients with HbE/ β -thalassemia: A comparative cross-sectional study

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Abstract. Iron homeostasis is regulated by hepcidin (HEPC) that controls the dietary iron absorption and iron recycling. HEPC deficiency contributes to iron overload in β -thalassemia patients. The present study aimed to investigate the correlation between HEPC concentration and serum iron status among hemoglobin E (HbE)/ β -thalassemia patients and their parents (HbE trait and β -thalassemia trait) compared with healthy controls. This study is a comparative cross-sectional study in which iron profile and HEPC level were examined in 65 HbE/ β -thalassemia patients (pretransfusion) and 65 parents at the Hospital Sultanah Nur Zahirah and in 130 students as healthy controls from Universiti Sultan Zainal Abidin, Terengganu, Malaysia. Furthermore, six samples from each group (HbE/ β -thalassemia patients, parents and healthy controls) were randomly selected for gene expression analysis of HEPC and ferroportin1 (FPN1) using reverse transcription quantitative PCR. The results demonstrated that serum HEPC level were significantly decreased in HbE/ β -thalassemia patients and their parents ($P < 0.001$) compared with healthy controls. In addition, the gene expression analysis showed a dramatically downregulated HEPC in HbE/ β -thalassemia patients and their parents ($P = 0.001$) compared with healthy controls. However, there was a marked upregulation of FPN1 in HbE/ β -thalassemia patients and their parents ($P = 0.001$) compared with healthy controls. Iron profiling results revealed a significantly increased serum ferritin in HbE/ β -thalassemia patients and their parents compared with healthy controls ($P < 0.001$). In summary, the present study demonstrated that HEPC expression level and

serum level were significantly decreased in HbE/ β -thalassemia patients and their parents, which was combined with a marked increased FPN1 expression level and serum ferritin level compared with healthy volunteers. These findings supported the hypothesis that downregulated HEPC could lose its function as a negative regulator of FPN1, resulting in iron overload in HbE/ β -thalassemia patients. Subsequently, assessing HEPC and FPN1 gene expression may be a useful tool to determine the risk of iron toxicity in patients with HbE/ β -thalassemia and their parents, and could therefore be considered as a therapeutic target in the management of iron burden in these patients.

Introduction

Thalassemia is an inherited disease associated with reduced synthesis of one or more globin chains and resulting in a defective hemoglobin (Hb) production and earlier damage to red blood cells (1,2). Hemoglobin E (HbE)/ β -thalassemia is the most common severe thalassemia in South and Southeast Asian countries (3). Clinical burden of HbE/ β -thalassemia varies markedly, and ranges from mild or asymptomatic anemia (β -thalassemia trait) to severe anemia (HbE/ β -thalassemia major), which requires blood transfusions (4). Microcytic hypochromic anemia and iron overload represent the most frequent complications and major challenges in managing thalassemia (5). Iron is essential to numerous biological processes, such as cellular respiration and oxygen transport (6). Iron homeostasis must be maintained to avoid iron overload and iron deficiency, which can have severe consequences (7). Cardiomyopathy, which is a common cause of death, can be caused by cardiac iron overload in patients with transfusion-dependent thalassemia (8,9). Patients with non-transfusion-dependent thalassemia are also at high risk for iron overload and its consequences, such as liver cirrhosis (10). Severe thalassemia patients require blood transfusion to improve anemia via suppression of erythropoiesis (11,12). Because HbE/ β -thalassemia is associated with increased serum iron, regular evaluation of iron level in these patients is therefore required (13,14).

Hepcidin (HEPC) is a hormone that is produced by the liver and released into the circulation (15,16). HEPC interacts with ferroportin1 (FPN1), a HEPC-receptor and an iron exporter

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protein, which is situated on the plasma membrane of iron exporting cells, such as duodenal enterocytes, hepatocytes, spleen cells (17) and erythropoietic cells (18). FPN1 exports iron from cells into the blood (19,20). FPN1/HEPC complex inhibits the ability of these cells to export iron (21). Therefore, higher HEPC expression will reduce iron export from storage cells into the blood via suppression of FPN1 (18). HEPC production is regulated by erythropoiesis (22). Increased erythropoiesis reduces HEPC production and increases iron level due to the high release of iron from storage cells and enteric absorption for Hb synthesis (23).

The present study is a comparative cross-sectional study, in which a total of 260 participants, including 65 HbE/ β -thalassemia patients (pretransfusion), 65 HbE trait and β -thalassemia trait (patients' parents) and 130 healthy controls were involved for the evaluations of iron profile, HEPC gene expression and serum HEPC level. The aim of the present study was to investigate the association between HEPC expression and serum levels and iron overload among HbE/ β -thalassemia patients.

Materials and methods

Samples recruitment and ethics. The present study is a comparative cross-sectional study that included a total of 260 blood samples ($n=260$) collected from 65 HbE/ β -thalassemia patients and 65 parents at the Hematology Department of Hospital Sultanah Nur Zahirah, Terengganu. A total of 130 blood samples were collected from healthy postgraduate students at the Universiti Sultan Zainal Abidin (UniSZA), Gong Badak Campus, in Terengganu, Malaysia, who were included as the controls. All blood samples were collected between August 2019 and July 2020. The study was approved by UniSZA Human Research Ethics Committee [(approval no. UniSZA.C/2/UHREC/628-2 J1d.2) (73)] and the Medical Research and Ethics Committee [(approval no. KKM/NIHSEC/P19-1143) (11)]. The procedures were in accordance with the ethical standards and each participant to this study was given a participant information sheet (PIS) and an informed consent form (ICF) prior to blood samples collection. Both PIS and ICF were written in Bahasa Melayu and English languages to allow better understanding from all participants. Briefly, 5 ml of blood were collected from each participant by a trained nurse staff. The inclusion criteria were as follows: i) Transfusion dependent HbE/ β -thalassemia patients (pretransfusion) and their parents (HbE trait and β -thalassemia trait); ii) healthy participants with normal hematological blood profile and hemoglobin analysis; and iii) transfusion dependent HbE/ β -thalassemia patients who required lifelong regular blood transfusion. The exclusion criteria were as follows: i) Patients or parents with iron deficiency anemia (IDA) or alpha thalassemia trait (DNA analysis for alpha globin gene was performed); and ii) healthy participants with low hemoglobin and red cell indices, abnormal hemoglobin variants.

Laboratory investigations. Capillary electrophoresis and the VARIANT™ II β -Thalassemia Short Program Reorder Pack (Bio-Rad Laboratories, Inc.), which is a fully automated high-performance liquid chromatography (HPLC) system, were used to confirm the patient's diagnosis and ensure that

healthy controls were thalassemia-free. Complete blood counts [red blood cells (RBCs), Hb, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW)] were analyzed using SYSMEX XN-1000 automated Hematology Analyzer (Sysmex America, Inc.). Iron profile [serum iron, total iron binding capacity (TIBC) and unsaturated iron binding capacity (UIBC)] was determined using UniCel® DxI 600 Access® Immunoassay (Beckman Coulter Inc.) and serum ferritin level was estimated using UniCel® DxI 800 (B) Access® Immunoassay (Beckman Coulter Inc.). Serum HEPC level was determined using Human HEPC ELISA Kit (cat. no. E-EL-H0077; Elabscience Biotechnology Co., Ltd.) according to the manufacturer's protocol.

RNA extraction and cDNA synthesis. Total RNA was extracted from the peripheral blood samples of all participants using QIAamp RNA Blood Mini Kit (Qiagen GmbH) according to the manufacturer's protocol. RNA purity and concentration were evaluated via a NanoPhotometer® NP80 (Implen GmbH).

Reverse transcription quantitative (RT-q)PCR. GoTaq 2-Step RT-qPCR (Promega Corporation) was used to synthesize cDNA from RNA samples (100 ng) according to the manufacturer's protocol. All PCR amplifications were conducted using 2 μ l of cDNA mixed with 20 μ l of GoTaq PCR master mix, following a 2-step amplification protocol as follows: A starting denaturing step for 2 min at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C, and annealing and extension for 1 min at 60°C by using 1-Step RT-qPCR Systems (Applied Biosystems). Data were analyzed by 1-Step One Software v2.3 (Applied Biosystems). Beta actin (β -actin) was used as the reference gene. The relative expression levels of HPEPC and FPN1 were normalized to endogenous control and were expressed as $2^{-\Delta\Delta C_q}$ (24). The sequences of the primers used are listed in Table I. All experiments were performed in triplicates.

Statistical analysis. All statistical analyses were performed using SPSS software (version 20; IBM Corp.). Kruskal Wallis was applied for data comparison and $P < 0.05$ was considered to indicate a statistically significant difference. Multiple Mann-Whitney Test with Bonferroni correction was applied for 2-group comparisons and the level of significance was set at 0.017.

Results

Demographic data. The study included 65 transfusion-dependent HbE/ β -thalassemia patients, 65 parents and 130 healthy controls. All groups comprised 100 men and 160 women. The majority of HbE/ β -thalassemia patients were aged between 11 and 32 years, while parents were aged between 39 and 56 years. Furthermore, the median age of healthy controls was between 18 and 32 years (Table II).

Increased HbE and HbF in HbE/ β -thalassemia patients. To confirm the diagnosis of HbE/ β -thalassemia in patients, their parents and thalassemia-free controls, HPLC analysis

Table I. Sequences of the primers used for reverse transcription quantitative analysis.

Gene name	Forward primer	Reverse primer	Accession no.
Hepcidin	5'-TTTCCCACAACAGACGGGAC-3'	5'-AGCTGGCCCTGGCTCC-3'	NM_032541
Ferroportin1	5'-TTGCCGGAGTCATTGCTGCTA-3'	5'-TGGAGTTCTGTACACCATT-3'	NM_016917
β -actin	5'-GAGCGCGGCTACAGCTT-3'	5'-TCCTTAATGTACGCACGATTT-3'	NM_007393

Table II. Demographic data of HbE/ β -thalassemia patients, parents and healthy controls.

Characteristics	Healthy controls (n=130)	Parents (n=65)	HbE/ β -thalassemia patients (n=65)
Age range, years	18-32	39-56	11-32
Sex			
Male, n (%)	31 (23.8%)	26 (40.0%)	43 (66.2%)
Female, n (%)	99 (76.25)	39 (60.0%)	22 (33.8%)

HbE/ β -thalassemia, hemoglobin E/ β -thalassemia.

Table III. Comparison between medians of hemoglobin (Hb) variants in HbE/ β -thalassemia patients, parents and healthy controls.

Studied variables	Healthy controls (n=130) Median (Iq)	Parents (n=65), median (Iq)	HbE/ β -thalassemia patients (n=65), median (Iq)	X ² -statistic ^a (df)	P-value ^b	Reference range
HbA, %	96.20 (4.03)	81.80 ^c (20.60)	2.30 (2.35)	203.9 (2)	0.001	>90.0
HbA ₂ , %	2.80 (0.50)	4.80 (0.50)	4.80 (2.15)	175.8 (2)	0.001	2.0-3.3
HbF, %	0.30 (0.11)	0.80 ^c (0.65)	47.2 (11.60)	181.8 (2)	0.001	<1.0
HbE, %	0.00	0.20 ^c (1.10)	44.6 (10.58)	89.00 (2)	0.000	-

^aKruskal Wallis test was applied. ^bHbA in HbE/ β -thalassemia patients vs. Healthy controls (P<0.001); HbA in HbE/ β -thalassemia vs. parents (P=0.001); HbA in Parents vs. Healthy controls (P=0.056); HbF in HbE/ β -thalassemia patients vs. Healthy controls (P<0.001); HbF in HbE/ β -thalassemia vs. parents (P=0.001); HbF in Parents vs. Healthy controls (P=0.006); HbE in HbE/ β -thalassemia patients vs. Healthy controls (P<0.001); HbE in HbE/ β -thalassemia vs. parents (P<0.001); HbE in Parents vs. Healthy controls (P=0.001). ^cMajority of parents are β -thalassemia and only 8 samples are HbE traits. Hb, hemoglobin; HbE/ β -thalassemia, hemoglobin E/ β -thalassemia.

and capillary electrophoresis was performed for all blood samples from all participants prior to further laboratory analysis. The results demonstrated a significant decrease in HbA in HbE/ β -thalassemia patients compared with parents (P=0.001) and healthy controls (P<0.001; Table III). However, there was no significant difference in HbA of parents compared to healthy controls (P=0.056). Furthermore, there was a significant increase in HbF (P=0.001) and HbE (P<0.001) in HbE/ β -thalassemia patients compared with their parents and healthy controls. The results also showed a significant increased HbF in parents compared to healthy controls (P=0.006).

Reduced Hb concentration and red cell indices in thalassemia patients. There was a significant decrease in RBCs count, Hb concentration and red cell indices (except RDW) in HbE/ β -thalassemia patients and their parents (P=0.001)

compared with healthy controls. The results also revealed a significant decrease in Hb concentration of parents compared to healthy controls (P=0.003) (Table IV).

Increased serum ferritin in thalassemia patients. The results from iron profile revealed a significant increase in serum ferritin in HbE/ β -thalassemia patients and their parents (P<0.001) compared with healthy controls. However, serum ferritin level was >20 times higher in HbE/ β -thalassemia patients than in healthy volunteers (Table V). Serum iron and transferrin saturation were also significantly increased in HbE/ β -thalassemia patients (P=0.001) compared with parents and healthy controls. Serum iron in parents were significantly increased compared to healthy controls (P=0.016). However, TIBC and UIBC were significantly decreased in HbE/ β -thalassemia patients compared with parents and healthy controls (P=0.001; Table V and Fig. 1).

Table IV. Comparison between median haematological parameters in HbE/ β -thalassemia patients, parents and healthy controls.

Studied variables	Healthy controls (n=130), median (Iq)	Parents (n=65), median (Iq)	HbE/ β -thalassemia patients (n=65), median (Iq)	X ² -statistic ^a (df)	P-value ^b	Reference range
RBCs, $\times 10^{12}/l$	4.54 (0.51)	5.40 (0.84)	3.00 (0.710)	163.5 (2)	0.001	4.50-5.50
Hb, g/l	132.0 (15.0)	121.0 (19.00)	75.10 (22.90)	166.8 (2)	0.001	120-150
PCV, l/l	0.400 (0.05)	0.390 (0.040)	0.220 (0.04)	147.5 (2)	0.001	0.350-0.450
MCV, fl	88.0 (4.93)	64.90 (14.85)	62.30 (6.85)	189.3 (2)	0.001	83.0-101.0
MCH, pg	28.95 (1.80)	20.50 (4.10)	20.40 (4.10)	143.9 (2)	0.001	27.0-32.0
MCHC, g/l	329.5 (12.25)	319.0 (24.0)	301.0 (36.50)	125.04 (2)	0.001	315.0-345.0
RDW, % CV	13.20 (1.60)	15.70 (4.30)	24.60 (6.40)	172.7 (2)	0.001	11.6-14.8

^aKruskal Wallis test was applied. Multiple Mann-Whitney Test with Bonferroni correction was applied and level of significance was set at 0.017.

^bHb in HbE/ β -thalassemia patients vs. Healthy controls (P<0.001); Hb in HbE/ β -thalassemia vs. Parents (P=0.001); Hb in Parents vs. Healthy controls (P=0.003). Hb, hemoglobin; HbE/ β -thalassemia, hemoglobin E/ β -thalassemia; RBCs, red blood cells; PCV, packed cell volume; RDW, red cell distribution width; MCH, packed cell volume; MCV, mean cell volume; MCHC, mean corpuscular hemoglobin concentration.

Table V. Median iron profile and hepcidin levels in HbE/ β -thalassemia patients, parents and healthy controls.

Studied variables	Healthy controls (n=130), median (Iq)	Parents (n=65), median (Iq)	HbE/ β -thalassemia patients (n=65), median (Iq)	X ² -patients statistic ^a (df)	P-value ^b	Reference range
Iron, $\mu\text{mol/l}$	18.60 (8.65)	24.40 (6.70)	32.90 (10.15)	601.5 (2)	0.001	10.7-32.2
UIBC, $\mu\text{mol/l}$	45.95 (14.90)	43.10 (12.60)	22.30 (6.80)	89.29 (2)	0.001	27.8-63.6
TIBC, $\mu\text{mol/l}$	60.45 (8.72)	57.90 (12.00)	37.10 (8.40)	97.29 (2)	0.001	42.96-80.55
Transferrin saturated, %	23.50 (12.25)	25.00 (12.00)	54.80 (15.20)	77.90 (2)	0.001	15-50%
Ferritin, $\mu\text{g/l}$	46.20 (44.23)	97.50 (80.60)	1383.0 (1098.8)	1324.0 (2)	0.000	11.0-306.8
Hepcidin, ng/ml	4.823 (5.235)	0.590 (0.056)	0.0489 (0.043)	101.2 (2)	0.001	0.16-10
Hepcidin/ferritin	0.3365 (0.467)	0.033 (0.029)	0.0115 (0.009)	87.9 (2)	0.001	Up to 1

^aKruskal Wallis test was applied. Multiple Mann-Whitney Test with Bonferroni correction was applied and level of significance was set at 0.017.

^bSerum iron in HbE/ β -thalassemia patients vs. Healthy controls (P= 0.001); serum iron in HbE/ β -thalassemia vs. Parents (P=0.05); serum iron in Parents vs. Healthy controls (P=0.052); serum ferritin in HbE/ β -thalassemia patients vs. Healthy controls (P<0.001); serum ferritin in HbE/ β -thalassemia vs. parents (P=0.001); serum ferritin in Parents vs. Healthy controls (P=0.016); serum hepcidin in HbE/ β -thalassemia patients vs. Healthy controls (P<0.001); serum hepcidin in HbE/ β -thalassemia vs. Parents (P=0.208); serum hepcidin in Parents vs. Healthy controls (P<0.001). HbE/ β -thalassemia, hemoglobin E/ β -thalassemia; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity.

Reduced serum HEPC level in HbE/ β -thalassemia patients and parents. Serum HEPC level was significantly reduced in HbE/ β -thalassemia and their parents (P<0.001) compared with healthy controls (Table V and Fig. 2). However, there was no significant difference in the HEPC level between HbE/ β -thalassemia and their parents (P=0.208).

HEPC downregulation in HbE/ β -thalassemia patients and parents. Six blood samples of each group were randomly selected for gene expression analysis by RT-qPCR. The results demonstrated a significant lower HEPC gene expression in HbE/ β -thalassemia patients and their parents (P=0.001) compared with healthy controls. However, there was no significant difference in HEPC gene expression between HbE/ β -thalassemia patients and their parents (P=0.208; Fig. 3).

Upregulated FPN1 in HbE/ β -thalassemia patients and parents. The results from RT-qPCR demonstrated a significant upregulation (P=0.001) of FPN1 gene in HbE/ β -thalassemia patients and their parents (7-folds and 3-folds higher, respectively) compared with healthy volunteers (Fig. 4).

Discussion

HbE/ β -thalassemia remains one of the most common inherited blood disorders, which is characterized by an abnormal β -globin chain production. HbE/ β -thalassemia is prevalent worldwide and represents a major health problem in numerous countries, such as Southeast Asian countries like Malaysia (3,25). The complication arising from this blood disorder is not only due to ineffective erythropoiesis but also to iron overload due to increased gastrointestinal iron absorption

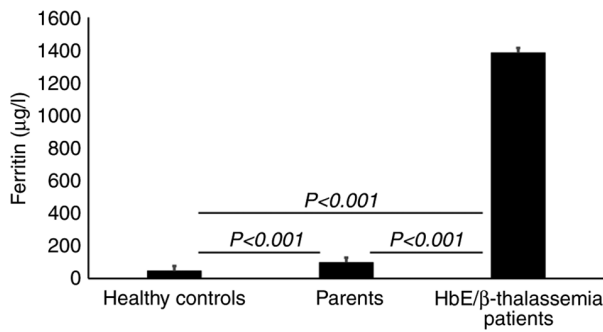


Figure 1. Serum ferritin concentrations. Results are presented as the median ferritin concentrations and show a significant increased ferritin level in HbE/β-thalassemia patients and their parents ($P<0.001$) compared with healthy controls. HbE/β-thalassemia, hemoglobin E/β-thalassemia.

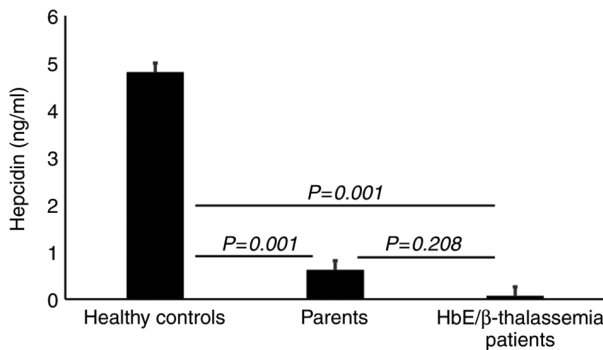


Figure 2. Serum hepcidin concentrations. Results show a significantly lower HEPC concentration in HbE/β-thalassemia patients ($P=0.001$) compared with their parents and healthy controls. Results also demonstrate a significant decrease in HEPC concentration in parents ($P=0.001$) compared with healthy controls. HbE/β-thalassemia, hemoglobin E/β-thalassemia.

and blood transfusions (26). Iron overload in β-thalassemia patients is a major cause of mortality and morbidity (27).

Iron deposition leads to a marked cellular damage and organ dysfunction (28,29). Excess iron deposition is associated with cardiac hypertrophy and dilatation (30,31). Long term iron deposition also damages thyroid, parathyroid and adrenal glands (32). HEPC is a key regulator of iron homeostasis produced by hepatocytes and regulating intestinal iron absorption (16,33). FPN1 serves a key role in regulating dietary iron absorption in the duodenum, whereas serum HEPC levels control the concentration of FPN1 on the basolateral cell surface of enterocytes (34). The interplay between the iron exporter FPN1 and HEPC is critical for iron homeostasis, inflammation and anemia (35).

In the present study, the diagnosis of HbE/β-thalassemia patients, their parents and thalassemia-free healthy controls was confirmed by capillary electrophoresis and HPLC analysis. As a novelty of the present study, gene expression of HEPC and FPN1 together with serum HEPC level were studied in HbE/β-thalassemia patients and their parents and compared with those in healthy controls. The results revealed a significant downregulation of HEPC in HbE/β-thalassemia patients and their parents ($P=0.001$) compared with healthy controls. The downregulated HEPC was associated with a significant increase in FPN1 expression level in HbE/β-thalassemia patients

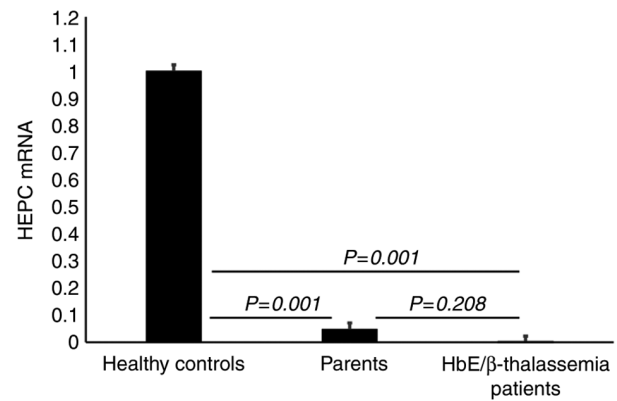


Figure 3. Gene expression analysis of HEPC. Results display a significant downregulation of HEPC gene expression in HbE/β-thalassemia patients and their parents ($P=0.001$) compared with healthy controls. However, there was no significant difference in HEPC gene expression in HbE/β-thalassemia patients ($P=0.208$) compared to their parents. HEPC, hepcidin; HbE/β-thalassemia, hemoglobin E/β-thalassemia.

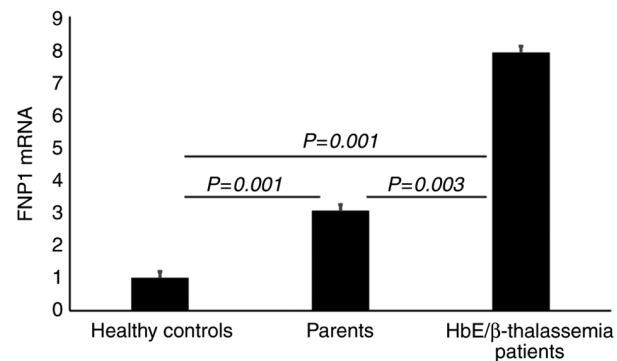


Figure 4. Gene expression analysis of FPN1. Results show a significant upregulation of FPN1 gene expression ($P=0.001$) in HbE/β thalassemia patients (>7 -fold change) and their parents (>3 -fold change) compared with healthy controls ($P=0.001$). Results also display a significant upregulation of FPN1 gene expression in HbE/β-thalassemia patients ($P=0.003$) compared with their parents. HbE/β-thalassemia, hemoglobin E/β-thalassemia.

and their parents ($P=0.001$) compared with healthy controls. These findings were similar to previous studies reporting that HEPC expression in β-thalassemia is downregulated in β-thalassemia mice (36,37). However, FPN1 is upregulated in β-thalassemia mice (38). The expression of fibroblast growth factor 23 (FGF23) was also reported as a primary regulator of hepcidin expression (39-41). Subsequently, the FGF23 expression level and its association with HEPC expression level in HbE/β-thalassemia patients will be further investigated.

Serum HEPC level, complete blood count and iron profile were also investigated in all participants from the present study. The results revealed that serum HEPC levels were significantly lower in HbE/β-thalassemia patients (pre-transfusion) and their parents ($P=0.001$) compared with healthy controls. These findings were supported by a previous study reporting that serum HEPC levels were decreased in β-thalassemia trait patients compared with healthy controls (42). However, the present findings were opposite to those from a previous study, in which serum HEPC levels were similar in β-thalassemia patients and healthy controls, which was attributed to the

blood transfusion (11). Furthermore, HEPC was shown to be upregulated by increasing the body's iron but it downregulated due to ineffective erythropoiesis (33).

The serum HEPC levels were shown to be decreased in patients with β -thalassemia major compared with healthy controls (43). Furthermore, serum iron is increased in patients with β -thalassemia major compared with β -thalassemia trait patients and healthy controls (11,12). The increase in serum ferritin in β -thalassemia patients is mainly due to the suppression of HEPC caused by ineffective erythropoiesis which then increases iron absorption (44). Consistent with these results, the findings from the present study revealed a significantly lower HEPC serum level combined with a higher ferritin serum level in HbE/ β -thalassemia patients and their parents compared with healthy volunteers. In addition, estimation of serum ferritin is a critical tool to evaluate the total body iron content (45). HEPC/ferritin ratio is therefore a marker of iron overload (42,46). Furthermore, the present study demonstrated that the HEPC/ferritin ratio was significantly decreased in HbE/ β -thalassemia patients and their parents ($P=0.001$) compared with healthy controls. These findings were similar to a previous study in which HEPC/ferritin ratio is significantly decreased in β -thalassemia patients compared with controls (47).

The results from the present study revealed a significant increase in serum iron ($P=0.001$) in HbE/ β -thalassemia patients compared with their parents and healthy controls. However, there was a significant decrease in TIBC and UIBC ($P=0.001$) in HbE/ β -thalassemia patients compared with the two other groups. These findings were in agreement with previous studies reporting that TIBC and UIBC are significantly decreased in β -thalassemia patients compared with healthy controls (48,49).

The present study reported an elevated serum level of ferritin, which is the most significant indicator for iron overload, in HbE/ β -thalassemia patients and their parents compared with healthy controls. The elevation of ferritin was attributed to the enhanced iron absorption in gastrointestinal tract due to the significant reduction of HEPC level in these patients.

Hemoglobin A (HbA) is the most dominant type of hemoglobin found in healthy adults and is made up of two α - and two β -globin chains (50,51). The normal synthesis of β -globin chains was found to be reduced in HbE/ β -thalassemia patients (2,52). Similarly, the results from the present study showed lower HbA levels in HbE/ β -thalassemia patients compared to the reference values. A decreased synthesis of HbA causes anemia in HbE/ β -thalassemia patients due to the lack of oxygen transportation to various parts of the body (53,54). The low level of HbA would eventually lead to needed blood transfusion in severe cases of HbE/ β -thalassemia patients in order to meet the body's oxygen demand (4). The main characteristics of HbE/ β -thalassemia patients are higher blood concentrations in HbA₂, HbF and HbE (38). Due to the nature of HbE/ β -thalassemia, the production of δ -globin chain increases with a reduction in β -globin chains, leading to a raised levels of HbA₂ (25). In a normal healthy adult, the β -globin gene is expressed at low level during the early fetal life, followed by HbF switch to HbA within three to six months, as γ -globin chain is

replaced by β -globin chain (55). However, the conversion of HbF to HbA is delayed in HbE/ β -thalassemia patients due to prolonged expression of the γ -globin chain since the β -globin gene is impaired (25). Similarly, the findings from the present study demonstrated a significant increase in HbF level ($P=0.001$) in HbE/ β -thalassemia patients as one of the dominant hemoglobin variants instead of HbA. Furthermore, the present study also reported a significant elevation of HbE level in HbE/ β -thalassemia patients, which was not detected in healthy controls. HbE is the abnormal hemoglobin variant that is caused by point mutation in the β chain, where glutamic acid is replaced by lysine (52). The dominant hemoglobins in HbE/ β -thalassemia patients were HbE and HbF compared with the dominant HbA found in healthy volunteers (56).

A previous study reported an impairment in normal β chain production in HbE/ β -thalassemia patients (52). Similarly, the results from the present study showed a severe decrease in HbA level in HbE/ β -thalassemia patients compared with a mild reduction of HbA level in the parents or normal HbA level in healthy controls. The results also demonstrated that the decrease in HbA level was associated with a significant decreased in RBCs, Hb, PCV, MCV, MCH and MCHC levels ($P<0.001$) in HbE/ β -thalassemia patients compared to parents and healthy controls. Similarly, the hemoglobin level ranged from 3 to 11 g/dl depending on the phenotypes and severity of HbE/ β -thalassemia patients (52). A lower hemoglobin level indicates a hypochromic state in HbE/ β -thalassemia patients, where erythrocytes appear paler than normal (57). The anemic nature of HbE/ β -thalassemia patients are characterized by low PCV, MCV and MCH levels (52).

Taken together, the findings from the present study reported a significant upregulation of FPN1 gene ($P=0.001$) associated with a significant downregulated of HEPC gene in patients with HbE/ β -thalassemia and their parents ($P=0.001$) compared with healthy volunteers. The downregulation of HEPC was associated with a significantly lower serum HEPC level and a significant increase in serum ferritin level in HbE/ β -thalassemia patients and their parents. These findings suggested that the suppression of HEPC loses its negative regulatory function on FPN1, which resulted in increased iron and ferritin levels in HbE/ β -thalassemia patients and their parents due to increased iron absorption.

In conclusion, this study demonstrated a significant upregulation of FPN1 associated with a significant downregulation of HEPC in HbE/ β -thalassemia patients, which may contribute to an elevated iron burden in HbE/ β -thalassemia patients. The findings from the present study suggested that evaluation of HEPC and FPN1 expression levels may be considered as useful indicators of iron toxicity risks in patients with HbE/ β -thalassemia and β -thalassemia, and both genes may subsequently be used as future therapeutic targets in these patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HANAJ and WRWT designed and conceptualized the study. Data collection and experiment implementation were performed by HKMS. Data were analysed by HANAJ, WRWT, II, MFJ, ASAW and HKMS. The manuscript was drafted by HANAJ and HKMS. HANAJ, WRWT, II, MFJ, ASAW and HKMS revised and reviewed the manuscript. Supervision was provided by HANAJ and WRWT. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by UniSZA Human Research Ethics Committee (UHREC) [approval no. UniSZA.C/2/UHREC/628-2 J1d.2 (73)] and the Medical Research and Ethics Committee [approval no. KKM/NIHSEC/P19-1143 (11)]. All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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