Mitochondrial ferritin expression in erythroid cells from patients with alpha-thalassaemia

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ABSTRACT

Background: Patients with thalassaemia who received regular transfusions had increased iron accumulation, leading to iron overload, which was associated with oxidative stress. Mitochondrial ferritin, encoded by the FTMT gene is an iron-storage protein in the mitochondria. The aim of this work was to investigate the expression levels of FTMT in the reticulocytes of patients with alpha-thalassaemia who were regularly transfused and rarely transfused compared with healthy controls and to evaluate the relationships of the levels of FTMT mRNA with malondialdehyde (MDA) and ferritin in these patients.

Methods: The levels of FTMT mRNA in the reticulocytes of patients (30 regularly transfused and 30 rarely transfused) and 30 healthy individuals were assessed by quantitative reverse transcription-polymerase chain reaction. The levels of ferritin and MDA were analysed by ELISA and by a thiobarbituric acid reactive substance assay, respectively.

Results: The levels of FTMT mRNA, ferritin and MDA in both groups of patients were significantly increased compared with those in the healthy controls. In addition, the levels of FTMT mRNA, ferritin and MDA in the regularly transfused patients were significantly higher than those in the rarely transfused patients. Furthermore, the relative expression levels of FTMT in patients correlated with those of MDA and ferritin.

Conclusion: These results suggest that the elevation of expression levels of FTMT in the reticulocytes of patients with alpha-thalassaemia may be associated with iron loading and oxidative stress.

KEYWORDS

Mitochondrial ferritin; thalassaemia; iron overload; oxidative stress

Introduction

Alpha-thalassaemias are a group of genetic blood diseases characterized by an alteration in alpha-globin production. Clinical manifestations in alpha-thalassaemia patients vary from mild to severe anaemia depending on the type of genetic abnormality. Anaemia in transfusion-dependent thalassaemia (TDTs) is alleviated by a blood transfusion. However, multiple blood transfusions lead to iron overload, and thus, the patient will suffer from the complications of having an iron overload status [1,2]. Patients with TDTs require regular transfusions to survive, but patients with mild clinical symptom or non-transfusion-dependent thalassaemia (NTDT), who seldom require blood transfusions, may have excess iron mainly due to the increased iron absorption from the gastrointestinal tract and iron deposition in the body with age [2,3]. Most alpha-thalassaemia patients and those with alpha-beta-thalassaemia co-inheritance are considered to be thalassaemia intermedia and are non-transfusion-dependent thalassaemia [2]. However, moderate-severe anaemia is seen in some types, especially in non-deletional haemoglobin (Hb) H disease, i.e. Hb H with Hb Constant Spring (CS) and Hb H with Hb Pakse [2]. Non-deletional Hb H patients may require occasional or frequent transfusions [2]. Some non-transfusion-dependent thalassaemia patients also requires regular transfusions to correct their growth failure, bone changes and marked hepatosplenomegaly [2].

The pathophysiology of iron overload is that the increased iron, which exceeds the capacity of iron-binding protein to bind it, is present in the free form or non-transferrin-bound iron (NTBI) form, which can give rise to hydroxyl free radicals through the Fenton reaction [4]. Hydroxyl free radicals contribute a high degree of oxidation in the cell and these reactive oxygen species can oxidize the cell components such as fats, proteins, RNA, and DNA, leading to cell and tissue damage [5,6]. Determination of malondialdehyde (MDA) has been suggested as a tool to assess the level of lipid peroxidation that occurs in cells experiencing oxidative stress [7].

Mitochondrial ferritin, encoded by the FTMT gene, is a form of ferritin located inside the mitochondria [8]. It has a key role in iron trafficking in the mitochondria and is important for cellular activities, including respiration, production of reactive oxygen species, and

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regulation of apoptotic pathways [9]. It has a structure and function similar to those of cytosolic H-ferritin [9], possesses ferroxidase activity, is involved in Fe(II) oxidation, and stores iron, such that it protects the mitochondria from iron-mediated oxidative damage, presumably via sequestration of potentially harmful excess free iron [10,11]. Mitochondrial ferritin has a very restricted tissue expression. High levels have been found in the testis, but only very low levels have been found in iron storage organs [12]. However, its level was increased dramatically in erythroblasts of patients with sideroblastic anaemia and has been suggested to protect organelles from oxidative damage [13]. Increased synthesis of this type of ferritin does not involve translational control, as FTMT mRNA lacks an iron response element. Haem is specifically synthesized within the mitochondrial matrix by the ferrochelatase enzyme, which catalyzes the insertion of ferrous iron into protoporphyrin IX. Thus, mitochondrial ferritin has the potential to be an important regulator of local iron trafficking and defends against the possible interaction between free iron and reactive oxygen species, which are both abundant in the mitochondria [12]. It has been reported that cell overexpressing the FTMT gene are more resistant to oxidative damage induced by hydrogen peroxide and to the apoptotic signal induced by TNF-α in the presence of actinomycin A, suggesting that the protein withholds iron and prevents it from entering dangerous redox cycles [12].

It is of interest to determine the expression pattern of this gene in regularly transfused and rarely transfused alpha-thalassaemia patients. Thus, the purpose of this work was to assess the expression levels of FTMT mRNA in reticulocytes of alpha-thalassaemia patients and to evaluate its relationship with the levels of MDA and ferritin.

Materials and methods

This research has been approved by the Khon Kaen University Ethics Committee for Human Research, Khon Kaen, Thailand (HE581470). Informed consent was obtained from all subjects.

Subjects

Subjects included regularly transfused (RegT) (n = 30) and rarely transfused (RarT) (n = 30) α-thalassaemia patients attending the Srinagarind Hospital, Khon Kaen University, Thailand and healthy individuals (n = 30) who met the following criteria: Hb ≥ 13 g/dl for males, ≥ 12 g/dl for females; MCV > 80 fl; Hb typing A2A; Hb A2 < 4% and plasma ferritin > 15 μg/L) [14]. Some RegT and RarT patients were treated with iron chelation when their successive ferritin levels reached ≥ 1,000 μg/L or ≥ 800 μg/L, respectively according to the guidelines recommended by the Thalassaemia International Federation [1,2].

Sample preparation

Approximately 7 ml of fresh venous blood was collected. Fresh whole blood was collected in a tube containing EDTA and used for plasma and cells preparation. Clotted blood was also collected and centrifuged for serum separation. The reticulocyte fraction was prepared according to Suebpeng et al. [15]. Serum and plasma were stored at approximately −80°C until use. Total RNA was extracted from the reticulocyte fraction by using Trisure® reagent (Bioline Ltd., Australia). The RNA concentration and purity were determined spectrophotometrically at 260 nm and 280 nm, using a Nano Vue™ Spectrophotometer.

Determination of the expression of FTMT

The expression levels of FTMT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed by using quantitative reverse transcription real-time polymerase chain reaction (RT–PCR). Approximately 100 ng of RNA was used with the SensifAST™ SYBR No-ROX One-Step kit (Bioline Inc., Taunton, MA, USA). The primer sequences for FTMT were TATTCTTTCAC-CAGTCCCGG (sense) and AGAGCGTGCAATTCAGCAAC (antisense) [13], and for GAPDH were

| Hb typing                  | Reg T patients | RarT patients |
|----------------------------|----------------|---------------|
| EABart’s                   | 12             | 0             |
| (HbE and some HbA and some HbBart’s) | 1              | 0             |
| A2A/Bart’s H               | 5              | 5             |
| (HbA2 and some HbA and some HbBart’s and some HbH) |              |               |
| CSEABart’s                 | 9              | 7             |
| (Hb Constant Spring and some HbA and some HbBart’s) | 0              | 0             |
| CSA2ABart’s H              | 0              | 12            |
| (Hb Constant Spring and some HbA and some HbBart’s) |              |               |
| CSA2AABart’s               | 3              | 0             |
| (Hb Constant Spring and some HbA2 and some HbA and some HbBart’s) | 5              | 5             |
| CSA2AH                     |                |               |
| (Hb Constant Spring and some HbA2 and some HbA and some HbBart’s) | 0              | 6             |
| A2AH                       |                |               |
| (Hb Constant Spring and some HbA2 and some HbA and some HbBart’s) | 0              | 12            |
| Total                      | 30             | 30            |
GAAGGTGAAGGTCGGAGTC (sense) and GAAGATGGT-GATGG (antisense). The reaction was performed by using the Illumina® Eco™ Real-time PCR system.

The RT–PCR conditions were: reverse transcription for 10 min at 42°C; the PCR reaction for 95°C for 10 min, 40 cycles of 94°C for 15 sec, 58°C for 15 sec and 72°C for 15 sec. The Ct value was used to calculate the expression level of FTMT normalized to that of GAPDH and relative to healthy individuals by using the equation $2^{-\Delta\Delta Ct}$ [16].

**Laboratory measurements**

Whole blood was used for haemoglobin and MCV determinations by using an automated haematology analyser (KX-21; Sysmex Corp., Kobe, Japan). Plasma ferritin and serum MDA were analysed by enzyme linked immunosorbent assay (ELISA) using a commercial kit (RayBiotech, Inc., USA) and a thiobarbituric acid-reactive substance assay [17], respectively.

**Statistical analysis**

The data are reported as the means ± standard deviations (SD). Statistical analyses were performed with the SPSS statistical package (version 19; SPSS Inc., Chicago, IL, USA). The Kruskal–Wallis test was used for comparisons among RegT patients, RarT patients and healthy individuals. The Mann–Whitney U-test was used to compare the two patient groups and each patient group with the healthy individual group. The Spearman rho test was used to calculate the correlation and $p < 0.05$ was considered statistically significant.

**Results**

The characteristics of α-thalassaemia patients (RegT and RarT) and healthy controls are shown in Table 1. The mean concentrations of serum MDA in RegT patients, RarT patients and healthy controls were 2.4 μmole/L, 1.57 μmole/L and 1.18 μmole/L, respectively (Figure 1(a)). In addition, both patient groups had significantly higher MDA concentrations than the controls ($p < 0.001$). The average plasma ferritin levels of RegT patients, RarT patients and normal controls were 897 μg/L, 251 μg/L and 32 μg/L, respectively (Figure 1(b)). The levels of plasma ferritin in RegT patients and RarT patients were significantly increased compared with that of healthy controls ($p < 0.001$). In addition, there were significant differences in MDA and ferritin in RegT patients and RarT patients

![Figure 1](image-url)
The average expression level of FTMT (normalized to GAPDH relative to the healthy controls) of patients in the RegT group was significantly higher than that of patients in the RarT group (2.7-folds and 1.7-folds, respectively) \((p < 0.001)\) (Figure 1(c)). When the same experiment was performed with RNA from reticulocytes of 30 \(\beta\)-thalassaemia-HbE patients with regular transfusions, similar result was found. The average expression level of FTMT of \(\beta\)-thalassaemia-HbE patients was 3.1 folds increased as compared to healthy control \((p < 0.001)\). In addition, positive correlations were found between relative FTMT expression and log serum MDA \((r = 0.740; p < 0.001)\) (Figure 2(a)) and log ferritin \((r = 0.665; p < 0.001)\) (Figure 2(b)) as well as between MDA and ferritin \((r = 0.833; p < 0.001)\) (Figure 2(c)).

**Discussion**

Although most RarT patients with thalassaemia did not receive transfusion (20 out of 30 patients) over the past year, they still had ferritin levels (36–610 \(\mu\)g/L) higher than those of healthy controls, possibly due to increased absorption of dietary iron from the gastrointestinal tract, which arises from the increased erythropoietic activity that occurs with anaemia. Iron entering the body from both sources results in increased iron accumulation in patients, leading to high iron loading over time. The levels of ferritin in RegT patients were significantly higher than those in RarT patients (Figure 1(b)) suggesting that the amount of iron entering the body from transfused red blood cells is much higher than that from intestinal absorption over times due to ineffective erythropoiesis in RarT patients (RarT patients’ age ranged from 8 to 22 years) as the body does not have a mechanism to excrete iron. In addition, some RegT patients might develop iron overload, as indicated by the high ferritin level. The levels of ferritin are considered to be the primary index of iron overload status when a liver iron concentration measurement is unavailable [3].

This report determined the expression levels of FTMT gene, which encodes mitochondrial ferritin in reticulocytes. The erythroblast is a unique cell that is responsible for haemoglobin synthesis. Iron has to enter the cell via the transferrin-transferrin receptor system. Later, iron enters the mitochondria and is incorporated into haem. Mitochondrial ferritin is a novel type of ferritin that specifically targets the mitochondria. The FTMT mRNA does not have the IRE consensus sequence [8], and thus is not influenced by the IRE/IRP regulatory system. FTMT is expressed in cells with high metabolic activity and oxygen consumption [8,12]. This protein was observed to accumulate iron in the mitochondria of erythroblasts of patients with sideroblastic anaemia [13]. In addition, the mitochondrial environment appears to be more important than the cytosolic environment for iron incorporation into ferritin [10]. In normal physiology, most iron trafficked into the mitochondria of an erythroblast is incorporated into haemoglobin [18]. Our finding showed that the expression levels of FTMT in reticulocytes were the highest in RegT patients, followed by RarT patients and healthy controls (Figure 1(c)). These findings are consistent with the report from Cazzola et al. which showed that high-level FTMT expression was associated with iron-loaded erythroblasts in patients with sideroblastic anaemia; this association was not observed in normal erythroblasts [13] when the iron supply to the erythroid marrow exceeded the amount required for

![Figure 2](image-url)
haemoglobin synthesis. Corsi et al. [10] performed a transfection experiment in HeLa cells with the mouse FTMT gene and observed iron depletion in the cytosol. In addition, overexpression of human FTMT led to increased transferrin receptor levels and decreased cytosolic ferritin. Moreover, Nie et al. [11] demonstrated that FTMT significantly affected intracellular iron metabolism. FTMT overexpression resulted in an increase in cellular iron absorption, but a reduction in cytosolic ferritin, indicating that the iron influx was preferentially transferred from the cytoplasm into the mitochondria and incorporated into mitochondrial ferritin rather than localized in the cytosol [11].

The levels of MDA in the blood of RegT and RarT patients in our study were significantly higher than those of normal controls (Figure 1a). In addition, the levels of ferritin correlated with MDA in patients (Figure 2). These findings are supported by the fact that increased iron, which exceeds the capacity of iron-binding protein to bind it, is present in the free form, which catalyzes the Fenton reaction giving rise to hydroxyl free radicals [19]. Hydroxyl free radicals contribute a high degree of oxidation in the cell, which results in the increased levels of MDA. It is suggested that high iron loading may be one of the factors that accounts for the oxidative stress and that the expression of FTMT in RegT and RarT patients is iron loading-dependent and correlates with oxidative stress. This report supports the protective role of mitochondrial ferritin in cells, which involves the control of reactive oxygen species formation through the regulation of mitochondrial iron availability.

In conclusion, our finding demonstrates the elevation of the expression levels of FTMT in reticulocytes of regular transfused and rarely transfused patients is associated with iron storage and oxidative stress.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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