Transforming growth factor 15 increased in severe aplastic anemia patients

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ABSTRACT

Objectives: The patients with severe aplastic anemia (SAA) usually rely on red cell transfusion which lead to secondary iron overload. Transforming growth differentiation factor-15 (GDF-15) plays an important role in erythropoiesis and iron regulation. In this study, we investigated the level of GDF-15 and other indexes of iron metabolism in SAA patients to explore the correlation with GDF-15 and iron overload in SAA.

Methods: The levels of serum GDF-15, hepcidin (Hepc), and erythropoietin (EPO) were determined by ELISA. The levels of serum iron (SI), ferritin, TIBC, and transferrin saturation (TS) were measured by an auto analyzer. Iron staining of bone marrow cells was used for testing extracellular and intracellular iron.

Results: The GDF-15 level in the experimental group was higher than that of the control group and normal control group (all p < 0.05). The Hepc level in the experimental group and case-control group were both higher than that of healthy controls (all p < 0.05). The Hepc level was significantly lower in the experimental group patients who had excessive GDF-15 (r = -0.766, p = 0.000). There was a positive correlation between the level of GDF15 and EPO in the experimental group (r = 0.68, p < 0.000). The level of GDF15 in SAA patients was positively correlated with SI levels (r = 0.537, p = 0.008), TS levels (r = 0.466, p = 0.025), and sideroblasts (%) (r = 0.463, p = 0.026). Moreover, there was a positive correlation between GDF-15 level and blood transfusion-dependent time (r = 0.739, p = 0.000).

Discussion: Our data indicated that GDF-15 plays an important role in iron metabolism in SAA. GDF-15 might be a novel target for SAA therapy.

Introduction

Severe aplastic anemia (SAA) is a rare disease characterized by severe pancytopenia and bone marrow failure, which is associated with high mortality rates. Many studies on the pathogenesis of SAA had been concentrated on immune mechanisms. Up to now, SAA has been recognized as an autoimmune disease with bone marrow failure mediated by hyperfunctional T lymphocytes [1,2]. Moreover, patients with SAA were susceptible to secondary iron overload due to iron utilization disorder and transfusion dependence [3]. Iron metabolism disorders, especially iron overload, can damage bone marrow hematopoietic function and seriously unfavorable to the prognosis of SAA patients. In recent years, the risk of bone marrow failure with iron overload has been paid more and more attention, and the related researches also gradually become a hot spot.

Iron exists in the form of hemoglobin and myoglobin, which is vital for the delivery and storage of oxygen. Conversely, serum non-transferrin bound iron can overload and damage cell membrane permeability in the liver, heart, pancreas, brain, and joint parenchymal cells in pathological state, contributing to oxidant-mediated cell injury [4]. Iron overload is mainly manifested in the increased concentration of free serum iron (SI) and bound SI. It is generally believed that iron overload can be diagnosed as the standard of serum ferritin (SF) concentration >300 ng/mL and transferrin saturation (TS) >45% [5,6]. A growing number of studies have indicated that iron overload can cause damage to cardiovascular, endocrine, liver, kidney, or nervous system leading to many chronic diseases.

Growth differentiation factor-15 (GDF-15), a member of the transforming growth factor-β superfamily, is the primary regulator of systemic iron homeostasis and iron availability for erythropoiesis [7]. Recent studies confirmed that serum GDF-15 level of genetic hemolytic anemia disorders, such as severe beta thalassemia and congenital dyserythropoietic anemia (CDA), increased significantly, which is related to hepcidin (Hepc) produced by erythroblast [8–10]. GDF-15 inhibits the expression of Hepc in liver cells through BMP-SMAD signaling pathway and regulates Hepc-ferroportin axis, ultimately promoting intestinal iron absorption and iron release from mononuclear phagocytic system [11]. Therefore, GDF-15 might involve in the progress of the iron overload.
But there are few reports about the serum GDF-15 level in SAA patients.

In our study, the transfusion-independent SAA patients during the bone marrow recovery period after immunosuppressive therapy (IST) were selected as the research object. The levels of GDF-15 were detected to explore the role of GDF-15 in SAA patients with iron overload.

Materials and methods

Study subjects

Thirty-eight new diagnosed SAA patients were selected in the Hematology Department of the General Hospital, Tianjin Medical University (Tianjin, China) between June 2012 and January 2015 according to the International AA Study Group Criteria. The disease was considered severe if at least two of the following parameters were met: Neutrophil count <0.5 × 10^9/L, platelet count <20 × 10^9/L, and reticulocyte count <20 × 10^9/L with hypocellular bone marrow. Very severe aplastic anemia was diagnosed in cases of SAA <20 × 10^9/L with hypocellular bone marrow. Very severe aplastic anemia was diagnosed in cases of SAA patients with a neutrophil count <0.2 × 10^9/L. Patients were excluded if they had congenital AA or other autoimmune diseases. Patients inducted into the group and normal controls were screened for paroxysmal nocturnal hemoglobinuria by flow cytometry using anti-CD55 and anti-CD59 antibodies, and no PNH clones had been found.

All SAA patients had received IST (anti-thymocyte globulin, ATG; anti-lymphocyte globulin, ALG; cyclosporine, CSA; glucocorticoid) and hematopoietic stimulating factors (granulocyte colony-stimulating factor, G-CSF; recombinant human erythropoietin, EPO; recombinant human thrombopoietin, TPO; and/or combination IL-11). After 6-month treatment, all 38 patients achieved remission and bone marrow hematopoietic recovery. Among them, 23/38 patients who still relied on red blood cell transfusion were selected as the experimental group, including 13 males and 10 females with a median age of 35 (range, 22–49 years). The 15/38 patients who did not need red blood cell transfusion for 1 month were selected as the control group, including 8 males and 7 females with a median age of 35 (range, 22–49 years). The 15/38 patients who did not need red blood cell transfusion for 1 month were selected as the control group, including 8 males and 7 females with a median age of 35 (range, 22–49 years). The 15/38 patients who did not need red blood cell transfusion for 1 month were selected as the control group, including 8 males and 7 females with a median age of 35 (range, 22–49 years). The 15/38 patients who did not need red blood cell transfusion for 1 month were selected as the control group, including 8 males and 7 females with a median age of 35 (range, 22–49 years). The 15/38 patients who did not need red blood cell transfusion for 1 month were selected as the control group, including 8 males and 7 females with a median age of 35 (range, 22–49 years). This study was approved by the Ethics Committee of Tianjin Medical University. Informed written consent was obtained from all patients in accordance with the Declaration of Helsinki.

Reagent and instrument

The GDF-15, Hepc, and EPO ELISA kits were purchased from R&D (U.S.A.). The SF, SI, and total iron binding capacity (TIBC) kit were purchased from Nanjing Jian-Cheng Bioengineering Institute (China). Iron metabolism indices were detected by ADVIA®2400 Chemistry System (Siemens, Nürnberg, German).

Detection of serum growth differentiation factor-15, hepcidin, and EPO level

Five milliliter EDTA anticoagulated peripheral blood samples from SAA patients and normal controls were collected and centrifuged 20 minutes at a speed of 1500 r/min. Serum samples were stored at −20°C until final analysis. The GDF-15, Hepc, and EPO level were tested by commercial ELISA kits, according to the manufacturer’s instructions (ELISA kits; R&D Systems, Minneapolis, MN, U.S.A.) and at last the samples were read with microtiter plate reader (Tadjhiz Afzar Teb, Iran) at a wavelength of 450 nm.

Detection of serum iron, serum ferritin, total iron binding capacity, and transferrin saturation

For the measurement of SI, SF, and TIBC, serum samples were processed according to the manufacturer’s instructions and measured by an auto analyzer (Siemens). TS = SI/TIBC × 100%.

Iron staining of bone marrow cells

Bone marrow samples were aspirated from SAA patients and normal controls. Bone marrow smears were stained in 10 g/L potassium ferrocyanide for 50 minutes before being washed in tap water for 10 minutes and counterstained in 0.1% aqueous safranin. The standard of judging extracellular and intracellular iron was according to the ‘The National Clinical Test Regulation of Operation’ [12]. Extracellular iron grade: 1. ‘−’: no blue iron grain; 2. ‘+’: to have a small amount of iron grain or occasional small iron beads; 3. ‘++’: a lot of iron particles, beads, and a few patches; 4. ‘+++’: as many iron bead, bead, and a few patches; 4.‘++++’: abundance of iron particles, beads, and many small pieces. Intracellular iron: the proportion of sideroblasts in 100 polychromatic erythroblasts and metarubrics were recorded. Ring sideroblast is that the nucleated erythrocyte contains more than six iron particles and the iron particle around more than two-thirds of the nuclear diameter.

Statistical analysis

All the statistical analysis was performed using SPSS 21.0 (SPSS, Inc, Chicago, IL, U.S.A.). A parametric unpaired t-test was applied and date was presented as mean ± SEM for Gaussian distribution data. Pearson correlation test was used for correlated data. A value of p < 0.05 was considered as statistically significant.
Results

Severe aplastic anemia patients had higher growth differentiation factor-15 and lower hepcidin levels compared with normal controls

The GDF-15 level in the experimental group (941.99 ± 180.51 pg/mL) was significantly higher than that of the case-control group (743.65 ± 181.80 pg/mL, p = 0.000) and healthy controls (658.18 ± 167.87, p = 0.001) (Figure 1).

The Hepc level in the experimental group (27.01 ± 8.13 ng/mL) was significantly lower than that of the case-control group (36.38 ± 9.96 ng/mL, p = 0.000). The Hepc level in the experimental group and case-control group were both higher than healthy controls (21.51 ± 4.63 ng/mL) (all p < 0.05). Hepc level was significantly lower in the experimental group patients who had excessive GDF-15 (r = −0.766, p = 0.000).

Severe aplastic anemia patients with transfuse dependence had higher EPO level related with the level of growth differentiation factor-15

The EPO level in the experimental group was higher (102.01 ± 30.62 mU/mL) than that of the case-control group (59.92 ± 16.87 mU/mL, p = 0.000) and healthy controls (6.31 ± 1.99 mU/mL, p = 0.000). Additionally, there was a positive correlation between the level of GDF-15 and EPO in the experimental group (r = 0.68, p < 0.000) (Figure 2).

Detection of iron metabolism-related indexes

The average level of SI in the experimental group (43.67 ± 23.52 μmol/L) was significantly higher than that in the case-control group (25.58 ± 12.97 μmol/L, p = 0.003) and the normal control group (19.95 ± 7.27 μmol/L, p = 0.000), while the SF in the experimental group (421(43,2136) μg/L) was significantly higher than that in case-control group (153(62,1256) μg/L) and normal control group (65(32,867) μg/L). The expression of GDF-15 in SAA patients was positively correlated with the levels of SI (r = 0.537, p = 0.008) and TS (r = 0.466, p = 0.025) (Figure 3) (Table 1).

Iron staining of bone marrow cells in severe aplastic anemia patients

The expression level of GDF-15 in SAA patients was positively correlated with the proportion of sideroblasts (%) (r = 0.463, p = 0.026) (Table 2).
The level of growth differentiation factor-15 was positively correlated with blood transfusion dependence in severe aplastic anemia patients

To confirm the effect of GDF-15 on the prognosis of SAA, we analyzed the correlation among GDF-15 level, degree of hematopoietic recovery, and curative effect in the experimental group. The results showed a significantly positive correlation between GDF-15 expression level and blood transfusion-dependent time ($r = 0.739, p = 0.000$).

**Discussion**

Numerous studies have confirmed that SAA is a hyperactive effector T-cell-mediated autoimmune disease which targets bone marrow hematopoietic cells [13]. Meanwhile, we found that a large number of SAA patients obtained a good effect following IST (ATG/ALG combining with CSA), which also confirms the conclusion that the abnormal immune mechanism played an important role in the pathogenesis of SAA. Although IST is an effective therapy for SAA, most patients still rely on blood transfusion after IST even if their bone marrow hematopoietic function has been restored.

In SAA patients, anemia and hypoxia cannot be alleviated in a short period owing to ineffective erythropoiesis, which stimulate the secretion of EPO and increase the intestinal absorption of iron. Ineffective erythropoiesis is characterized by erythroid hyperplasia and finally gives rise to anemia and tissue iron overload due to transfusion stasis imbalance occurred in SAA patients, and high GDF-15 level, which suppresses Hepc, might play an important role in iron overload due to transfusion dependence. Therefore, GDF-15 may be the link between erythropoiesis and regulation of Hepc, and become one of the indicators of iron overload in SAA patients.

Our study measured the serum level of GDF-15 and Hepc in SAA patients and normal controls. Results demonstrated that the SAA patients with transfusion dependence had higher level of GDF-15 and lower level of Hepc compared with the case–control group and normal control group. And we found that the level of GDF-15 in SAA patients was positively correlated with the levels of SI, TS, and the proportion of sideroblasts in bone marrow, while it was negatively correlated with Hepc level. The results indicated that iron homeostasis imbalance occurred in SAA patients, and high GDF-15 level, which suppresses Hepc, might play an important role in iron overload due to transfusion dependence. Therefore, GDF-15 may be the link between erythropoiesis and regulation of Hepc, and become one of the indicators of iron overload in SAA patients.

In vitro, EPO can promote the secretion of GDF-15 by pronormoblasts. A recent study reported that the secretion of GDF-15 by erythroid progenitor cells was

Table 1. Detection of iron metabolism-related indexes in SAA patients and normal controls.

|                  | Experimental group | Case–control group | Normal control group |
|------------------|--------------------|--------------------|---------------------|
| SI (μmol/L)      | 43.67 ± 23.52      | 25.58 ± 12.97      | 19.95 ± 7.27        |
| SF (μg/L)        | 421(32,2136)       | 153(62,1253)       | 65(32,867)          |
| TS (%)           | 67.74 ± 15.78      | 40.07 ± 14.11      | 39.07 ± 9.85        |

Table 2. The extracellular iron distribution of bone marrow cells in the SAA group and control group.

|                | Extracellular iron | Intracellular iron Proportion of sideroblasts (%) |
|----------------|--------------------|---------------------------------------------------|
|                | (−) (+) (++) (+++) | (++)                                              |
| Experimental   | 1 1 10 10 1        | 74.96 ± 8.05                                      |
| group          |                    |                                                   |
| Case–control   | 2 6 55 2 0         | 52.07 ± 12.93                                      |
| group          |                    |                                                   |
| Normal control | 0 9 6 0 0          | 49.60 ± 15.60                                      |
dependent on EPO and transferrin receptor 2 [21]. In this study, we also found GDF-15 level was significantly higher in patients who had excessive EPO. It suggested that EPO might promote erythroid differentiation by regulating GDF-15 secretion.

In clinic, we found that the response time of SAA patients after IST was different. Part of the patients can be recovered in 3–6 months, while some patients need even more than 1 year to recover. SAA patients with poor response of treatment still need red blood cell infusion and usually combined with iron overload. In this study, we showed that iron overload was associated with abnormal level of GDF-15 and Hepc. Therefore, we need to further study the correlation between GDF-15, immune abnormalities, and iron metabolism in SAA patients to explore novel therapeutic targets.

In summary, iron overload can cause a series of pathological changes in many tissue and organs. In a variety of diseases, iron overload affects disease progression and clinical outcomes. In our study, we showed that transfusion-dependent SAA patients had increased GDF-15 level with more decreased Hepc level. The increased level of GDF-15 was positively correlated with EPO level, iron overload, and blood transfusion-dependent time. It indicated that GDF-15 might play an important role in ineffective erythrocytopoiesis and iron metabolism in SAA. Further researches on the pathway of GDF-15 regulated by pronormoblasts and the mechanisms of Hepc suppressed by GDF-15 might provide new targets for the treatment of SAA.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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