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Case Report

An unusual case of iron deficiency anemia is associated with extremely low level of transferrin receptor

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Abstract: A case study of a female patient, diagnosed with iron deficiency anemia, was unresponsive to oral iron treatment and only partially responsive to parenteral iron therapy, a clinical profile resembling the iron-refractory iron deficiency anemia (IRIDA) disorder. However, the patient failed to exhibit microcytic phenotype, one of the IRIDA hallmarks. Biochemical assays revealed that serum iron, hepcidin, interluekin 6, and transferrin saturation were within the normal range of references or were comparable to her non-anemic offspring. Iron contents in serum and red blood cells and hemoglobin levels were measured, which confirmed the partial improvement of anemia after parenteral iron therapy. Strikingly, serum transferrin receptor in patient was almost undetectable, reflecting the very low activity of bone-marrow erythropoiesis. Our data demonstrate that this is not a case of systemic iron deficiency, but rather cellular iron deficit due to the low level of transferrin receptor, particularly in erythroid tissue.

Keywords: Iron deficiency anemia, iron therapy, hepcidin, soluble transferrin receptor

Introduction

Iron deficiency anemia is the most common blood disorder, produced by a variety of underlying causes of either inadequate iron supply or increased iron requirements [1]. The majority of iron for erythropoiesis is provided by recovered iron from senescent erythrocytes [2] and only 1-2 mg of iron are absorbed daily from diet for an adult [3]. The dietary iron absorption is precisely regulated by the axis complex of hepcidin-ferroportin and a number of proteins may modulate the expression of hepcidin [3]. Hepcidin, a key iron systemic hormone produced in liver, inhibits both iron absorption from the intestine and iron release from macrophage stores [3].

Cellular iron uptake is mainly via the internalization of the complex iron-transferrin-transferrin receptor [3]. Ferritin iron represents an alternative for iron uptake in erythroid precursor [4]. Erythroid cells require large amount of iron daily for normal erythropoiesis. In the bone marrow developing erythroid cells express a high level of surface transferrin receptors to meet the iron needs for hemoglobin synthesis [5]. An imbalance of either the amount or recycling of transferrin and transferrin receptor may lead to cellular iron deficiency and subsequent hemoglobin deficit regardless of the serum iron level [6-9].

Blockage of systemic or cellular iron homeostasis may result in either iron overload or iron deficiency blood disorders. In addition, blood loss, hemolysis, erythropoietin, or hypoxemia can also stimulate erythropoiesis [10]. Clinically, in case of iron-deficiency anemia, storage iron declines until iron delivery to the bone marrow is insufficient for erythropoiesis. This is usually monitored with clinical indicators, beginning with low plasma ferritin, followed by decreased plasma iron and transferrin saturation and culminating with low-hemoglobin content in red blood cells [2].

Here we reported a rare anemia patient with an unusual iron refractory iron deficiency anemia (IRIDA)-like disorder, unresponsive to oral iron treatment but partially responsive to parenteral iron therapy. Several iron metabolism markers aforementioned were examined. Our data demonstrate that this is not a systemic iron deficien-

cy, but rather cellular iron deficit due to the extremely low level of transferrin receptor, particularly in erythroid tissue.

Materials and methods

Human samples

Erythrocytes from a patient and her two daughters and one son were collected for the study. All subjects have given informed consent and the study was approved by the Institutional Review Board of Nanjing University.

Clinical blood parameters

Blood routine examination was performed with blood analyzer XT-1800i (Sysmex, Shanghai, China). Urinary test was performed with urinary sediment analyzer SCANXL (Kobold, Hofheim, Hessen, Germany). Serum ferritin was measured using Advia Centaur XP chemiluminescence immunoassay analyzer (Siemens, Munich, Germany). Serum iron was measured with biochemistry analyzer AU640 (Beckman Coulter, Brea, California). O-tolidine was used for fecal occult blood tests. Serum β_2-microglobulin was determined with chemiluminescence immunoassay analyzer IMMULITE® 2000 (Siemens). Bone marrow iron levels were assessed by Prussian blue staining.

Hemoglobin visualization assay

Hemoglobin levels were assessed semiquantitatively by native polyacrylamide gel electrophoresis (PAGE) separation and blotting followed by chromophore-enhanced visualization. Briefly, erythrocyte pellets were lysed for 10 min on ice in lysis buffer (1 mol/L Tris-HCl pH 7.4, 4 mol/L NaCl, 1% NP-40, protease inhibitor cocktail tablets (Roche) and centrifuged for 10 min at 20,000× g. For each sample, 0.5 μg of total protein from the supernatant were mixed with an equal volume of sample buffer (pH 7.5, 100 mmol/L Tris-HCl, 15% glycerol, 0.05% bromphenol blue) and subjected to electrophoresis at 125 V using a 4%-20% gradient Tris-glycine gel till the dye front traveled halfway to the bottom of the gel. Proteins in the gel were electroblotted onto a 0.2 μm pore-size nitrocellulose membrane (PALL, New York). Hemoglobin visualization was enhanced by incubating the membrane in a freshly made and filtered solution containing 50 mmol/L Tris (pH 7.5), 50 mmol/L imidazole, 0.5 mg/mL diaminobenzidine, and 30 mmol/L H_2O_2 for 20 min in dark. The membrane was washed and digitized with Image J (NIH).

Ferrozine iron assay

Iron content was measured using a colorimetric ferrozine-based assay with some modifications [11]. Briefly, 22 μl concentrated HCl (11.6 mol/L) was added to 100 μl serum or erythrocyte lysate (~500 μg). The mixed sample was heated at 95°C for 20 min, then centrifuged at 20,000× g for 10 min. Supernatant was transferred very gently into fresh tubes. Ascorbate was added to reduce the Fe (III) into Fe (II). Ferrozine and saturate ammonium acetate (NH_4Ac) were sequentially added to each tube and the absorbance was measured at 570 nm (BioTek ELx800, Shanghai, China) within 30 min.

Transferrin saturation

Transferrin saturation was measured as described previously [12].

Western blotting and ELISA analysis

Proteins were resolved in SDS-PAGE gels and transferred onto nitrocellulose membranes (PALL). Immunoblotting and ELISA were performed as described previously [13]. Primary antibodies goat anti-b globin (Santa Cruz Biotech. Inc.), mouse anti-transferrin receptor (Invitrogen), mouse anti-GAPDH (Abgent) were used for immunoblotting. The levels of hepcidin, soluble transferrin receptor, and interluekin-6 were analyzed with ELISA kits (Elabscience, Wuhan, China).

Results

Case history

In June 2012, we received an 81-year old female patient complaining chest tightness, fatigue, pallor and shortness of breath after physical activities. She was diagnosed with anemia with high reticulocyte count (**Figure 1A**, **1B** and **Table 1**). Vitamin B_{12} and folic acid supplements were prescribed and found to be ineffective. Iron therapy was then conducted. The patient was found unresponsive to oral iron therapy and weakly responded to the parenteral iron administration. As a result, hemoglobin content in the blood increased from 60-70 g/L

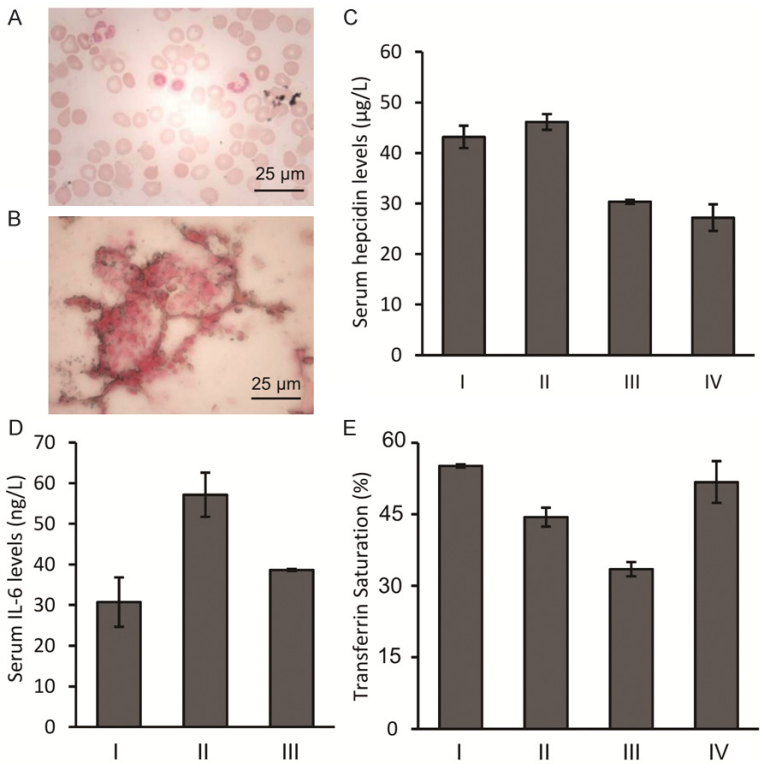

Figure 1. The patient was diagnosed with iron deficiency anemia without systemic disturbance of iron homeostasis. A. Peripheral blood film from the patient. B. Iron stain of bone marrow aspirate, which establishes the absence of stainable iron. C. Serum hepcidin levels. D. Serum IL-6 levels. E. Transferrin saturation of the patient, compared to her non-anemic children. I: Blood from the patient; II: Blood from her first daughter; III: Blood from her second daughter; IV: Blood from the patient's son.

to 86-99 g/L (reference: 113-151 g/L) upon parenteral iron administration. However, the increase was not sustained.

Hemolysis was deemed to be negative because the levels of jaundice, serum bilirubin, serum lactate dehydrogenase, and urinary urobilinogen are within the range of reference. Bleeding was also excluded because repeated fecal occult blood tests showed negative. Only one test conducted on Nov. 19, 2013 was only slightly positive (+), which was due to the multiple colonic polyps.

Systemic iron metabolism parameters are not abnormal

To address the resistance to oral iron supplementation, serum hepcidin, interleukin 6 (IL-6), and transferrin saturation were evaluated. For comparison, blood samples from patient's non-anemic offspring, two daughters (sample II, III) and one son (sample IV) were collected, tested, and compared with the patient's sample (sample I). The results showed that serum hepcidin, interluekin 6, and transferrin saturation of the patient were within the normal range of the references or were comparable to her non-anemic offspring (**Figure 1C-E**).

High serum iron level after intravenous (IV) iron supplementation weakly improved the hemoglobin synthesis

Since no parameters indicated the disturbance of systemic iron metabolism, we determine improvements of the anemia symptom after IV iron treatment. A sample (sample I") from the patient was taken two months post IV iron administration, and compared with that taken two days after the IV iron therapy (sample I). Iron contents of both serum and blood cells from the patient and her children were measured. Serum iron levels of the patient (sample I and I") were markedly higher than those of her children (**Figure 2A**). Conversely, the iron content in red blood cells was much lower in the patient than in her children (**Figure 2B**), indicating anemia in the patient. The subtle decrease in serum and increase in red blood cells of iron content correlated well with the slightly increased levels of hemoglobin in patient cells after IV iron treatment (**Figure 2A-C**), although all the changes were quite small. These results confirmed the data from clinical blood test that IV iron supplementation only partially improved the hemoglobin synthesis in the patient within two months, indicating that intravenous iron was not absorbed by erythroblasts for heme biogenesis.

Low expression of transferrin receptor limited iron uptake into erythroblasts for hemoglobin synthesis

Serum transferrin receptor (sTfR) level reflects total body TfR concentration and is considered

Table 1. Clinical data of the patient

Analyte	Results	References
Hb	61	113-151 g/L
Hct	21	33.5-45%
RDW	18.7	0-14%
MCV	86.1	82-95 fl
MCH	25	27-31 pg
MCHC	290	320-360 g/L
RET	5.66	0.5-1.5%
Serum iron	11.9	6.6-28.3 mmol/L
Serum ferritin	11.3	10-291 mg/L
Transferrin	40.0	36.6-65.0 mmol/L
bone marrow iron stain	Not detectable	
Serum beta2-MG	3399	609-2164 ng/ml
Fecal occult blood	(-)	
Urine occult blood	(-)	
Serum bilirubinurobilinogen	9.9	5-20.5 mmol/L
Serum lactate dehydrogenase	188	109-245 U/L

Hb: hemeglobin, Hct: Hematocrit, RDW: Red Cell Distribution Width, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RET: reticulocytes, beta2-MG: beta2-microglobulin, RBC: red blood cells, (-): negative or within the normal range.

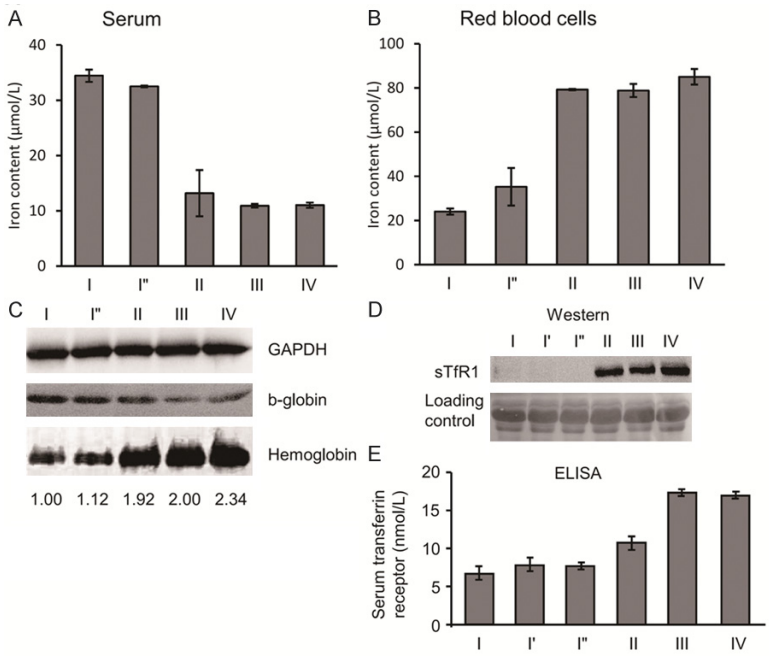

Figure 2. Intravenous iron supplementation slightly improved hemoglobin synthesis in patient within two months. A. Serum iron content of the patient and her offspring. B. Iron content in red blood cells of patient and her offspring. C. Levels of hemoglobin in the red blood cells of the patient and her offspring. Hemoglobin was stained in a native PAGE gel; b-globin and GAPDH were immunoblotted. D. Immunoblot of sTfR; E. ELISA of the sTfR. I, I', I": two days, two weeks, and two months after intravenous iron therapy. I, II, III, and IV were defined same as in **Figure 1**.

to be a sensitive, quantitative measure of tissue iron deficiency [14]. We compared the level of sTfR in the patient with those in her children. Strikingly, it was found that patient serum contained remarkably lower level of sTfR than her children (**Figure 2D**, **2E**), suggesting that patient cells, particularly erythroblasts, expressed much less TfR to take up iron for hemoglobin synthesis. For compensation, two events occurred. First, the level of globin protein is elevated compared to the healthy controls (**Figure 2C**). Secondly, the percentage of circulating reticulocytes (4.93-8.9%) was consistently higher than the reference values (1%-1.5%), indicating expanded erythropoiesis in the patient.

Discussion

Taken together, our data indicate this is not a case of systemic iron deficiency, but rather cellular iron deficit. The efficacy of intravenous iron supplementation was limited by the extremely low level of transferrin receptor for erythroblasts to take up iron. As a result, anemia was developed and refractory in the patient.

Laboratory tests show hypochromic normocytic anemia with normal serum hepcidin and iron, which is distinct from IRIDA, a disease caused by TMPRSS6 mutation with two of hallmarks, hypochromatic microcytic anemia and low transferrin saturation [15]. Though the patient showed the IRIDA-like clinical responsiveness to iron therapy, apparently this is not an IRIDA case.

During the period of treatment, the large red blood cells (100.7-115.8 fl, reference 82-95 fl) were observed with normal serum concentration of folate and vitamin B_{12}. The large cells might result from the slow shrinkage of reticulocytes over a normal red blood cell circulating lifetime. The number of reticulocytes is a good indicator of bone marrow activity because it represents recent production. Although the reticulocyte count in the patient declined from 8.9% to 4.93% after iron treatment, this was still remarkably higher than the reference interval, indicative of normal compensatory erythropoiesis and not a systemic iron deficiency anemia with insufficient hemoglobin synthesis.

A defect in transferrin/transferrin receptor cycling due to *Sec15l1* deletion produces "hemoglobin-deficit" mice characterized by a hypochromic, microcytic anemia [6-8]. This seems unlikely to be the cause of the anemia in our patient, since either microcytic anemia or abnormal serum level of transferrin would be expected. Nonetheless, our patient exhibited a close correlation between the iron content and hemoglobin concentration after iron supplementation. This suggests that heme biosynthesis is intact. The hemoglobin-deficit was very likely directly affected by the low level of transferrin receptor in erythroid tissues. One case was reported that a patient's autoreactive IgM immunoprecipitated the transferrin receptor and diminished iron uptake by erythroblasts, leading to a microcytic anemia with a high level of serum iron [16]. This autoreactive IgM mechanism could not explained our patient's anemia. Other cases with low levels of sTfR are linked with iron overload [17], chronic renal failure, and aplastic anemia as well as those post bone marrow transplantation (reviewed in [18]). Though the possible complexity of aging in the current case, the undetectable transferrin receptor is still a novel case to cause the anemia. The primary cause, either mutation of *TfR* or how TfR is drastically downregulated, remained to be determined.

From therapeutic view of point, our patient exhibited an IRIDA-like disease: no response to oral iron treatment, but narrow improvement from IV iron administration with a high level of serum iron. Membrane permeable iron-loaded chelator was reported to redistribute the iron successfully between outside and inside cells and between organelles within cells (see reviews [19, 20]). Therefore, such chelators may be an alternative therapeutic option to avoid iron overload.

In summary, our data demonstrated that this is not a case of systemic iron deficiency, but cellular iron deficit due to the extremely low level of transferrin receptor, particularly in erythroid tissue. Long-term intravenous iron supplementation should be tightly monitored to avoid iron overload-mediated damage to other organs.

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Disclosure of conflict of interest

None.

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