The novel SLC40A1 (T419I) variant results in a loss-of-function phenotype and may provide insights into the mechanism of large granular lymphocytic leukemia and pure red cell aplasia

Hongfei Wu, Xiang Ren, Meili Ge*, Peiyuan Dong, Shichong Wang, Huiming Yi, Xingxin Li, Jiali Huo, Xuan Zheng, Mengying Gao, Jinbo Huang, Jing Zhang, Min Wang, Peng Jin, Neng Nie, Yingqi Shao, Yizhou Zheng*

State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China

Abstract

Variants in the solute carrier family 40 member 1 (SLC40A1) gene are the molecular basis of ferroportin disease, which is an autosomal dominant hereditary hemochromatosis. Here, we present a patient with pure red cell aplasia (PRCA) and large granular lymphocytic leukemia (LGLL) associated with an extremely high levels of serum ferritin and iron overload syndrome. Whole exon sequencing revealed a novel heterozygous variant in SLC40A1 (p.T419I), which was found in his daughter as well. A series of functional studies in vitro of the T419I variant in ferroportin were conducted and the results revealed a reduced capacity of iron export from cells without changes in protein localization and its sensitivity to hepcidin. Intracellular iron storage in mutated cells was significantly higher than that of wild-type. These findings suggest that the novel variant p.T419I can cause the classical form of ferroportin disease and an elevated intracellular iron level indicates a potential novel pathogenic mechanism underlying PRCA and LGLL.

Keywords: Ferroportin disease, Iron overload, Large granular lymphocytic leukemia, Pure red cell aplasia, SLC40A1 variant

* *Address correspondence: Dr Meili Ge, MD, PhD and Dr Yizhou Zheng, MD, PhD, Diagnostic and Therapeutic Center for Anemic Diseases, State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, China. E-mail address: gemeili503@126.com (M. Ge), zheng_yizhou@hotmail.com (Y. Zheng).*

Funding: This work was supported by grants from the National Natural Science Foundation of China under grant numbers 81770119 & 81700120.

CRediT authorship contribution statement: H.W.: conceptualization; methodology; investigation; data curation; writing - original draft.

X.R.: methodology; investigation; data curation; writing - original draft.

P.D., S.W., H.Y., X.L., J.H., X.Z., M.G, J.H., Y.S., J.Z., M.W., P.J., and N.N.: methodology; data curation; writing – review & editing.

M.G. and Y.Z.: conceptualization; project administration; funding acquisition; resources; supervision.

All authors gave final approval to the manuscript and consent to publish the data.

The authors declare no conflicts of interest.

The funding source had no involvement in the study design, collection, analysis, or interpretation of the data, writing of the report, and in the decision to submit the article for publication.

The data generated or analyzed in this study are all available from the corresponding author upon request.

Blood Science, (2022) 4, 29-37

Received September 28, 2021; Accepted November 21, 2021.

http://dx.doi.org/10.1097/BS9.0000000000000099

1. INTRODUCTION

Hemochromatosis type 4A (OMIM 606069), also called ferroportin disease (FD), is caused by loss-of-function variants in the gene encoding the solute carrier family 40 member 1 (SLC40A1), which is also known as ferroportin 1 (FPN1). It is reported in many ethnic groups and is considered to be the second most common type of hereditary iron overload after *HFE*-related hemochromatosis.[1]

FPN1, the solely known cellular iron export protein in mammals, is expressed in all types of cells that transport iron into blood, including macrophages, enterocytes, hepatocytes, erythroid cells, lymphocytes, etc.[2,3] It is post-translationally regulated by hepcidin, a peptide hormone synthesized by the liver in response to iron, inflammation, and a variety of stressors.[4,5] Hepcidin binds to the FPN1 cavity and induces the internalization and degradation of FPN1 in lysosomes, leading to a decreased iron efflux into the blood.[6–8] The cellular and systemic iron homeostasis are finely controlled by the hepcidin–ferroportin axis to maintain adequate iron concentration, meet body needs, and avoid toxicity.[9]

Variants in the SLC40A1 gene have two types of pathogenic effects on FPN1 protein function: gain-of-function vs loss-of-function, both of which exert their disease phenotype in an autosomal dominant fashion. Unlike the variants of gain of function (such as Q248H,[10] N144D,[11] and C326S[12]), which do not injure the expression or iron export capability but make FPN1 resistant to its inhibitor hepcidin, loss-of-function variants (such as A77D,[13] G490D,[14] and L129P[15]) impair the iron-export capability of FPN1, causing a normal or low level of plasma iron

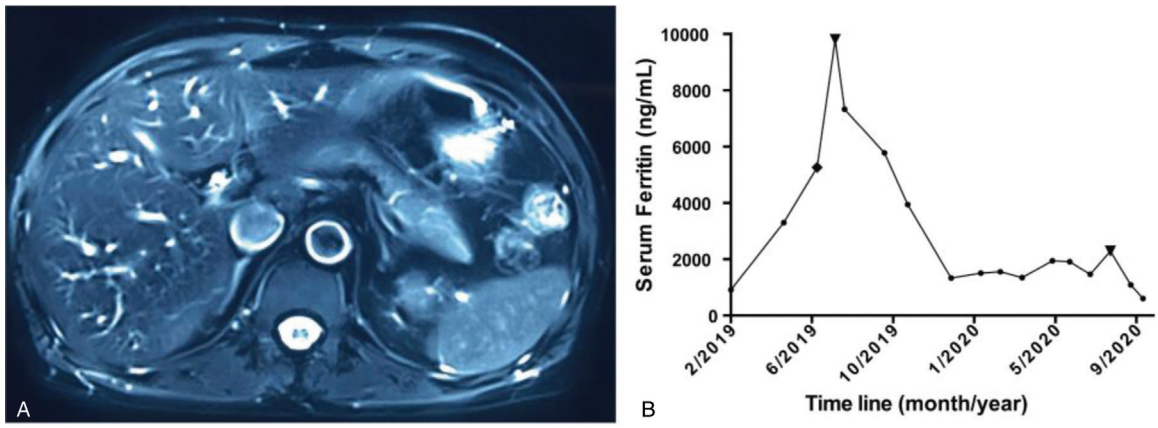

Figure 1. Clinical details of the patient. (A) MRI of the upper abdomen showed a decreased intensity of T2WI-fat-suppression signal in the spleen while approximately normal in the liver. (B) Changes of serum ferritin levels after dietary iron restriction (square) and treatment of deferasirox (triangle). MRI = magnetic resonance imaging.

and transferrin saturation despite high serum ferritin (at least in early stages of disease). Patients often have predominantly iron accumulation in mesenchymal cells/tissues such as macrophages and Kupffer cells, which are different from classical hemochromatosis and these cases do not respond well to phlebotomy.[1]

The first case of FD was clinically reported in 1999[16] and subsequently a heterozygous variant in FPN1 (A77D) was identified in the same family in 2001.[17] From then on, numerous variants of the SLC40A1 gene have been disclosed so far in patients with the classic phenotype of FD worldwide.[1] In China, it was until 2015 that the first variant in the SLC40A1 gene was identified and yet it was a phenotype of gain-of-function with classical manifestations of hereditary hemochromatosis.[18] To date, only one loss-of-function variant previously reported was identified in a healthy 37-year-old man only with hyperferritinemia and low transferrin saturation.[19]

Here, we present a Chinese patient with pure red cell aplasia (PRCA) and large granular lymphocytic leukemia (LGLL) associated with an extremely high level of serum ferritin and iron overload syndrome without previous transfusion. In in vitro studies, the novel mutant ferroportin (T419I) fails to export iron, thus a novel pathogenic mechanism in this triad is proposed by taking other studies into account.

2. METHODS

2.1. Details of patient

A 49-year-old man with a 5-year history of PRCA and LGLL in remission was referred to the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences (CAMS) & Peking Union Medical College (PUMC) for a routine follow-up. He was newly diagnosed with type 1 diabetes mellitus 3 months earlier at a local hospital. After admission, a blood test showed a high level of ferritin (3306 ng/mL), which was significantly higher than previous results (891–1495 ng/mL), meanwhile normal transferrin saturation and serum iron concentration were detected. A series of screening tests, including C-reactive protein (CRP), autoantibodies, ultrasonography, etc found no obvious inflammatory/infectious diseases; in addition, the patient had no history of blood transfusions or iron supplementation. The patient denied iron chelation therapy therefore restrictions on dietary iron intake were recommended. However, the ferritin increased to 5262 ng/mL 1 month later, and 2 months later, it increased to an extremely high level of 9800 ng/mL. Abdominal MRI showed decreased T2 signal intensity in the spleen on T2WI-fat-suppression (T2WI-FS) images and approximately normal intensity in the liver, which was consistent with the mode of iron overload in FD (Fig. 1A). Deferasirox was administered and a decrease in ferritin was achieved, however, ferritin level would increase subsequently and deferasirox was restarted upon discontinuation of deferasirox due to its side effect on kidney function (Fig. 1B).

2.2. Molecular study

Considering the unexplainable hyperferritinemia, iron overload, and newly diagnosed diabetes in the patient, hereditary hemochromatosis was suspected and a whole exon sequencing was performed at Kindstar Global gene (Beijing) Technology, Inc., P.R. China. After that, a Sanger sequencing for SLC40A1 was performed to verify the novel variant. Whole blood samples were obtained from the patient and his daughter, and all procedures were in strict accordance with the manufacturer's instructions and quality control standards.

This genetic study was in accordance with the ethics guidelines of the Declaration of Helsinki for Human Research. Written informed consent was obtained from the subjects for genetic analysis according to study protocols approved by the Ethics Committees of the Institute of Hematology, CAMS, and PUMC.

2.3. Generation of FPN constructs

The FPN1 constructs were produced by Genewiz Inc. (Suzhou). The wild-type FPN cDNA was synthesized by PCR using the In-fusion primers FPN sense and antisense by Sanger Sequencing, and the new variant c.1256C > T in the SLC40A1 gene was introduced into the wild-type FPN cDNA by site-directed mutagenesis in two PCR steps using the oligonucleotides FPN-mut sense and antisense. Both wild-type and mutant cDNAs were cloned into the pEGFP-N1 vector (Genewiz, Suzhou) in frame with the enhanced green fluorescent protein (EGFP) coding sequence. The sequence of wild-type and mutant FPN constructs were confirmed by sequencing.

2.4. Cell culture and transfections with FPN constructs

The human embryonic kidney cells (HEK293T, State Key Laboratory of Experimental Hematology, Tianjin, China) were

incubated at 37°C in a 5% CO_2 humidified atmosphere in Dulbecco modified Eagle medium, supplemented with glutamine 2 mM, 10% fetal calf serum, with or without penicillin-streptomycin (100 to 100 mg/mL) (Invitrogen, Life Technologies, Carlsbad, CA). Cells were grown in 6-well plates and transfected 24 hours later (70%–90% confluence) with the wild-type and mutant FPN constructs and pEGFP1-N1 vector using lipofectamine 3000 (Invitrogen, Life Technologies, Carlsbad, CA). Cell monolayers were dissociated with Gibco™ TrypLE™ Express Enzyme (Gibco, Thermofisher, Grand Island, NY), and transfection efficacy was estimated by inverted microscope (Olympus IX73) and flow cytometry on a FACSCantoII flow cytometer (Becton Dickinson, San Jose, California) 24 to 48 hours after transfection.

2.5. Live-cell fluorescence

To localize the wild-type and mutant FPN-EGFP fusion proteins, cells were grown in confocal dishes (15 mm) and transfected as described; 24 hours after transfection, Hoechst 33342 (Sigma–Aldrich) was added to stain cell nuclei for 30 min, and live-cell fluorescence images were acquired on a confocal microscope UltraVIEW VoX (PerkinElmer, UK).

2.6. Fluorescence measurement

The sensitivity of the wild-type and mutant FPN-EGFP fusion proteins to hepcidin was firstly assessed by fluorescent images to probe internalization and degradation of membrane FPN, and flow cytometry was also used to compare the change of fluorescence intensity before and after treatment of hepcidin-25 (0.36 μM) (Bachem Inc., Switzerland). After that, a dose-dependent assay was performed by measuring fluorescence in live HEK293T cells. Cells grown in confocal dishes (15 mm) or 6-well plates were transfected with the wild-type or mutant FPN constructs, and 24 hours after transfection, they were treated with one single dose of 0.36 μM or a dose gradient of 0.0, 0.18, 0.36, 0.72, or 1.08 μM synthetic hepcidin-25 for 4 hours, then cells in confocal dishes were subjected to take fluorescent images, and cells in 6-well plates were washed with PBS, harvested and re-suspended in 500 μL of PBS/well. The inhibition effect of hepcidin-25 was verified by flow cytometry on FACSCanto II and the fluorescence was measured in a Corning 96-well black plate at 488 nm excitation and 510 nm emission in a Multinode Plate Reader, Synergy H4 (Bio-Tek, VT).

2.7. Western blotting

The iron export capacity of the wild-type and the mutant FPN proteins was analyzed by western blot immunodetection of ferritin in the proteins of cell lysates. Cells were grown in 6-well plates, transfected with the wild-type or mutant FPN constructs, 24 to 36 hours after transfection cells were treated with 100 μM ferric ammonium citrate (FAC, Absin Bioscience Inc., China) for 18 hours, and then treated with 100 μM cycloheximide (Cell Signaling Technology, USA) for 1 hour to reduce the continuous synthesis of FPN, followed by 21 hours incubation with 0.72 μM synthetic hepcidin-25 (Bachem Inc., Switzerland) and/or an iron mobilizer guanosine-5-diphosphate (GDP, 10 μM) (MedChemExpress), which is an inhibitor of hepcidin. Then, cells were washed and re-suspended with PBS, centrifuged at 400*g* at 4°C for 5 min. Cell pellets were homogenized and lysed in 200 μL of RIPA buffer containing 1× protease inhibitor cocktail (Thermoscientific, USA), followed by sonication for 30 seconds and ten times in ice-water (Bioruptor Plus, Diagenode, Belgium). Protein concentrations were determined in the supernatant of 14,000*g* at 4°C for 15 min, using the Pierce™ BCA Protein Assay Kit (Thermoscientific, USA). A total of 20 to 30 μg of protein were electrophoresed on a 10% SDS-PAGE and transferred onto 0.20 mm Immobilon-PSQ PVDF membrane (Millipore). Blots were blocked in blocking buffer (5% BSA) for 2 hours at room temperature and incubated with the primary antibodies: rabbit anti-human FPN1 (1:500) (Novus Biologicals, UK; or Sigma–Aldrich), rabbit anti-human ferritin (1:1000) (Cell Signaling Technology) overnight at 4°C, and with a monoclonal antibody rabbit anti-GAPDH (1:1000) (Cell Signaling Technology) for 1 hour at room temperature, as a loading control. After extensive washing with TBST, blots were incubated with a working solution of SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermoscientific, USA) for 5 min and then exposed the blots to ChemiDoc Imaging System (Bio-Rad) to acquire signal images.

2.8. Statistics

All experiments were carried out four to five times. The intensity of the chemiluminescent signal in the wild-type and mutant groups were compared using a 2-sided Student *t* test, and the analysis was performed using the statistical package SPSS 17.0 (SPSS Inc., Chicago). A two-sided *P* value less than .05 (*) was considered statistically significant.

3. RESULTS

3.1. Identification of the novel FPN (T419I) variant

WES data (Fig. 2A) on the patient and his daughter revealed that a novel heterozygous allelic variant in the exon 7 of SLC40A1 gene in patient II-1, the c.1256C > T transition resulted in the substitution of threonine with isoleucine at position 419 (p.T419I) (Fig. 2B, 2C). Structural models of wild-type and mutant FPN were generated using SWISS-MODEL (https://swissmodel.expasy.org/), showing an extracellular loop change (Fig. 2D). This new variant was not found in 40 Chinese healthy controls. The 23-year-old daughter (III-1) was also heterozygous for the novel variant but with normal serum ferritin (21.41 ng/mL). Other relatives were not tested but it should be noted that the father died from heart failure of uncertain cause, implicating iron overload cardiomyopathy as one possible etiology.

3.2. Cellular localization of wild-type and mutant FPN (T419I)

Live-cell fluorescence images acquired on a confocal microscope showed that mutant EGFP-tagged FPN (T419I) properly localized at the plasma membrane in a manner comparable to the wild-type (Fig. 3A, 3B), indicating that the mutant FPN could reach the cell membrane where it would be able to exert its action as an iron exporter. No obvious difference in fluorescence intensity on the cell surface was observed.

3.3. Sensitivity of wild-type and mutant FPN (T419I) to hepcidin

Fluorescent images taken by confocal microscope showed that hepcidin-25 could trigger the internalization and degradation of both wild-type and mutant FPN, which was further confirmed by flow cytometry (Fig. 4A, B, C). In other words, this novel mutant FPN (T419I) was as sensitive as wild-type FPN to hepcidin at a concentration of 0.36 μM.

Fluorescence measurement in live cells after treating with an increasing dose of hepcidin-25 showed decreased fluorescence with the increment of hepcidin concentration but not in a manner of total dose-dependence (Fig. 4D).

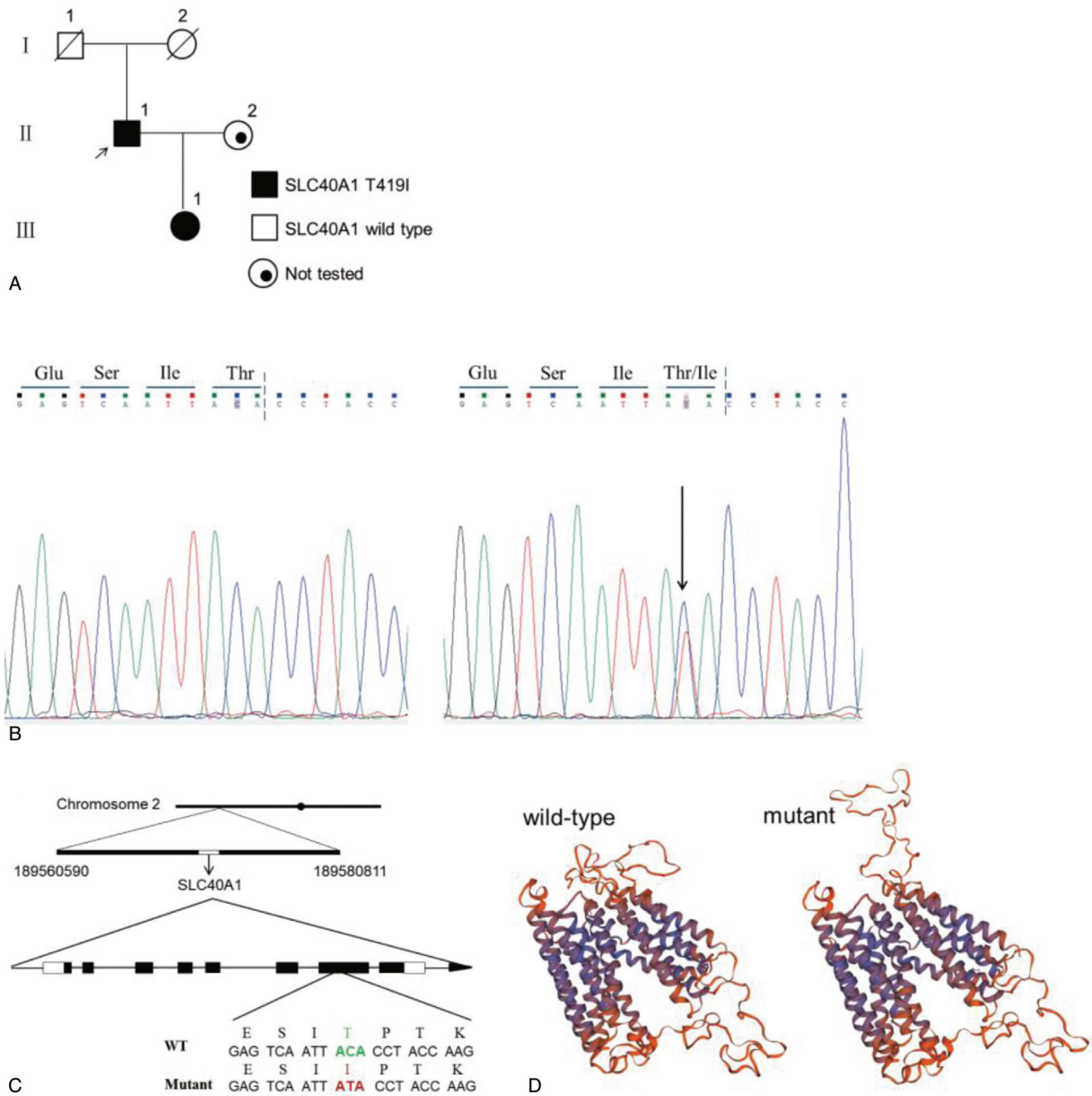

Figure 2. Novel FPN (T419I) variant. (A) Pedigree of the family. Arrow indicates index case. The same SLC40A1 variant was identified in the index case and his daughter. (B) Electropherogram of partial sequence of the exon 7 of SLC40A1 gene heterozygous sequence of the proband and his daughter; the c.1256C > T transition is indicated with an arrow. (C) The site and codon change of this novel variant. (D) Predicted changes in mutant protein structure. FPN = ferroportin, SLC40A1 = solute carrier family 40 member 1.

3.4. The iron export capacity of mutant FPN (T419I) was impaired

The results of western blotting for ferroportin showed a decreased expression level of mutant FPN-EGFP than that of wild-type (Fig. 5A). The results of western blotting for ferritin, as indicative of intracellular iron concentration, were compatible with a reduced iron export capacity of the mutant FPN (T419I) when compared to that of wild-type FPN transfected cells incubated with ferric ammonium citrate (FAC) (Fig. 5B). The relative intensities of ferritin bands were as an average of 1.35-, 1.30- and 1.25-fold higher, respectively in FPN (T419I) compared to wild-type in non-treated ($P < .05$), hepcidin- ($P > .05$) or guanosine-5-diphosphate (GDP) ($P > .05$) treated cells (Fig. 5B). The sensitivity of FPN to hepcidin was equal between wild-type and mutant FPN (T419I) transfected cells, the relative intensities of ferritin bands were an average of 1.28- and 1.23-fold higher when compared to those of nonhepcidin-treated cells (Fig. 5B, $P > .05$). The signal intensities of ferritin bands in proteins from wild-type FPN and mutant FPN (T419I) transfected cells non-treated with FAC were very weak (lanes not

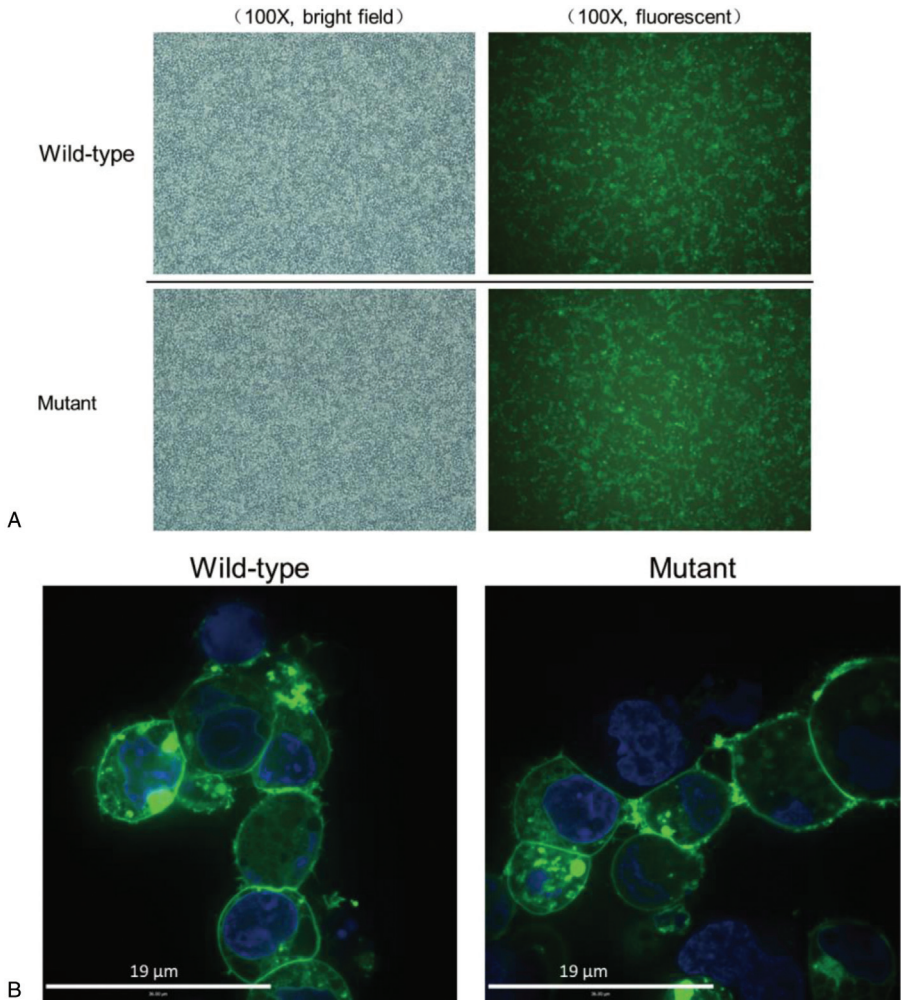

Figure 3. Cellular localization of wild-type and mutant FPN (T419I). (A) Transfection efficacy estimated by inverted microscope. (B) Live-cell fluorescence images of HEK293T expressing wild-type and mutant FPN-EGFP fusion proteins, both were localized at the cell surface. FPN = ferroportin.

shown). Unexpectedly, GDP, a hepcidin inhibitor, did not reverse the effect of hepcidin on FPN either in the wild-type or mutant group (Fig. 5A, 5B).

4. DISCUSSION

The ferroportin disease is an inherited iron overload disorder with an autosomal dominant inheritance mode but an incomplete penetrance, and the clinical presentation appears heterogeneous between patients and within families even in the case of the same genetic variant, suggesting that genetic and/or environmental factors may affect disease phenotype.[1,20] Specifically, the clinical manifestations of the disease can be modified by age, sex, and some pathological or acquired factors, such as inflammatory diseases, iron supplementation, or excessive alcohol consumption, which probably influence the expression of hepcidin.[21,22]

For the patient described in this study, an extremely high level of serum ferritin not associated with increased serum iron and its saturation indicated an iron overload different from classical hereditary hemochromatosis such as HFE hemochromatosis or acquired iron overload. And normal levels of inflammatory indicators such as CRP and IL-6 excluded the interference of potential inflammatory/infectious diseases. A decreased T2WI-FS signal intensity in the spleen confirmed a predominantly mesenchymal iron accumulation, which was typical for FD. Taking unexplained hyperferritinemia in the absence of blood transfusion, iron supplementation, and food- or drug-related factors, along with newly diagnosed diabetes and iron deposition in the spleen, the cause of iron overload in this patient was reasonably attributed to loss-of-function variant in FPN (T419I). Although the genetic background of the father of the proband was not accessible, the history of heart failure of unknown etiology indicated a possibility of iron overload cardiomyopathy. With regards to the daughter, the risk of iron accumulation was theoretically lower than that of his father due to her young age and menstrual blood loss, which was supported by her normal ferritin level. Considering the dominant mode of heritage, screening for the SLC40A1 gene is advisable since both siblings and offspring of FD patients have a 50% chance of being susceptible, although it is incomplete penetrance.

In this study, we report a new association of FD with LGLL and PRCA. To the best of our knowledge, this is the first case of

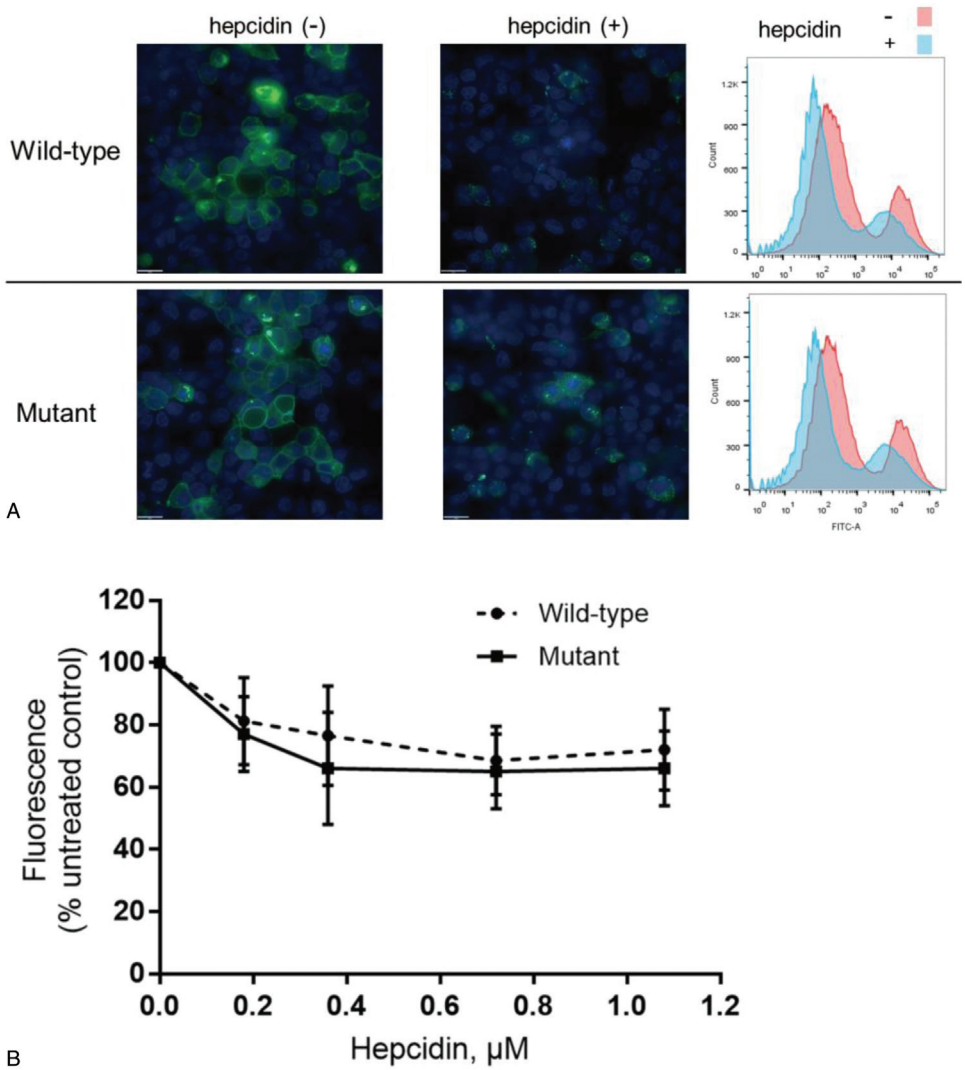

Figure 4. Sensitivity of wild-type and mutant FPN (T419I) to hepcidin. (A) Fluorescence images before adding hepcidin. (B) Fluorescence images after adding hepcidin (0.36 μM). (C) Flow cytometry images showed decreased mean fluorescence intensity after treating with hepcidin both in wild-type and mutant groups. (D) Fluorescence measurement of transfected cells non-treated (100%) and treated with increasing doses of hepcidin. Experiments were repeated independently four times, data are mean ± standard deviation. FPN = ferroportin.

dominant FD reported in the Chinese population. PRCA is known to be frequently accompanied with T-LGLL in East Asian populations and the underlying mechanism is proposed to be T-cell-mediated or autoantibody-dependent.[23] However, the detailed pathophysiology still remains to be clarified. In some previously reported cases, LGLL and PRCA are alone or simultaneously associated with rheumatoid arthritis,[24] type 1 and 2 autoimmune polyglandular syndrome,[25,26] and hemochromatosis,[27] all of which are partially or completely similar with symptoms of iron overload. All of these indicated that these diseases may not just a fortuitous but a pathological association.

Recent findings determined that erythroid cells express FPN, and erythroblast-specific ablation of SLC40A1 gene (FPN deficiency) leads to mild hemolytic anemia (attributed to oxidative injury) and systemic iron deficiency, indicating a normal function of FPN is essential for erythroid cells to escape from the toxicity of excessive intracellular iron.[28] And donating iron to circulating transferrin-bound iron pool in the case of serum iron deficiency is an important role of RBCs.[29] Taking our case into consideration, a loss-of-function variant of FPN perturbs this physical role of RBCs and predisposes erythroid cells to oxidative injury due to excessive intracellular iron, which might be a pathological cause of PRCA.

On the other hand, iron has long been established as a critical mediator of T cell development and proliferation and imbalanced iron homeostasis can be detrimental to T cells. Iron deficiency may lead to decreased T-lymphocyte proliferation and impaired natural killer cell activity. And each of these defects can be reversed by iron supplementation.[30] When activated, T cells intake more iron by increasing surface expression of transferrin receptor 1.[31] Rats loaded with iron by iron-dextran injection have been reported to harbor increased $CD8^+$ T cells, although the relationship between iron overload and T lymphocytes remains controversial.[32,33] In humans, somatic gain-of-function

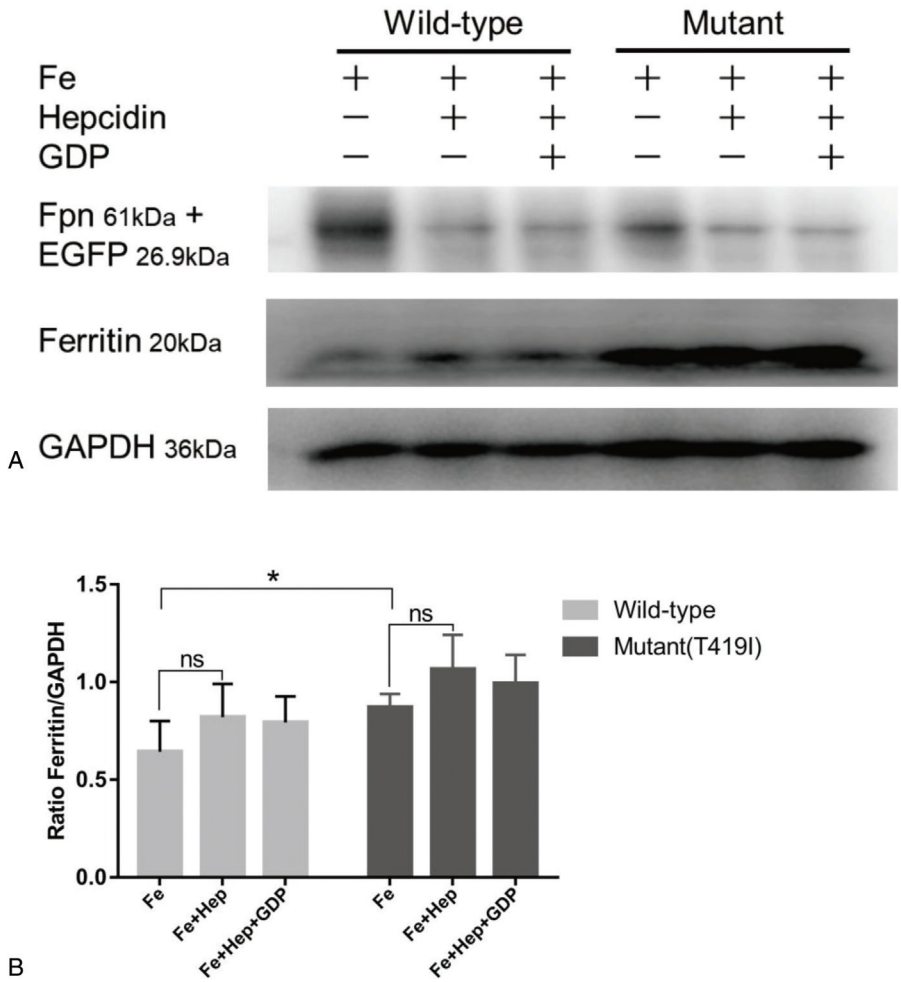

Figure 5. Iron export capacities of wild-type and mutant (T419I) FPN. (A) Western blot of cells treated with 100 μM ferric ammonium citrate, 0.03 μM hepcidin ± 10 μM GDP, wild-type FPN (lanes 1–3) and mutant FPN (T419I) (lanes 4–6). Image is representative of five independent experiments. (B) Representation of the relative intensity ratio of ferritin/GAPDH bands, data are mean ± standard deviation of five independent experiments of western blotting. Fe = ferric ammonium citrate, FPN = ferroportin, GDP = guanosine-5-diphosphate.

variants in STAT3 were associated with LGLL and PRCA, and the activated STAT3 could induce the expression of hepcidin, leading to a decreased capacity of iron export by FPN, which was somehow similar to FD.[34,35] Notably, the mode of iron overload in FD is different from other hemochromatosis and T cells are not heavily dependent on iron as that of erythroid cells. Thus, there might be just a slightly increased intracellular iron level in T cells with this novel variant and a proliferation promotion effect could be expected. Nevertheless, the role of T cell-derived iron metabolism in human diseases is relatively under-investigated, and further studies are warranted to discover novel therapeutic strategies for iron metabolism associated diseases.

Based on these evidence, a novel pathological mechanism for this triad is proposed (Fig. 6), in which a decreased iron export capacity of FPN and iron accumulation in cells account for all the disease phenotypes.

Different from other hemochromatosis, phlebotomy is not suitable for the treatment of FD, especially in the context of associated PRCA. Iron chelating agents were indicated and a remarkable response was observed in this case. However, it is not known yet whether iron removal treatment could improve anemia and lymphocyte proliferation. If so, the focus of treatment for this triad would be iron removal. In addition, unchanged sensitivity to hepcidin of this novel variant indicates that a higher level of hepcidin in some cases, such as inflammations and infections, may aggravate the dysfunction of FPN. In view of these situations, we investigated an inhibitor of hepcidin, however, the inhibitory effect of GDP was not as obvious as one previous study.[36] The reason for it was not probed yet.

In conclusion, the functional data observed in this study together with the iron overload syndrome in the index patient suggested that this novel variant c.1256C > T (p.T419I) in the SLC40A1 gene resulted in a loss-of-function phenotype of FPN, and was causative of the classical form of FD with an incomplete penetrance. Considering the coexistence of LGLL, PRCA, and FD, our findings now offer the appealing possibility to reveal a novel pathological mechanism for this triad in which an increased intracellular iron level was likely to be a common pathogenic factor. Future studies are warranted to test this possibility in clinical settings.

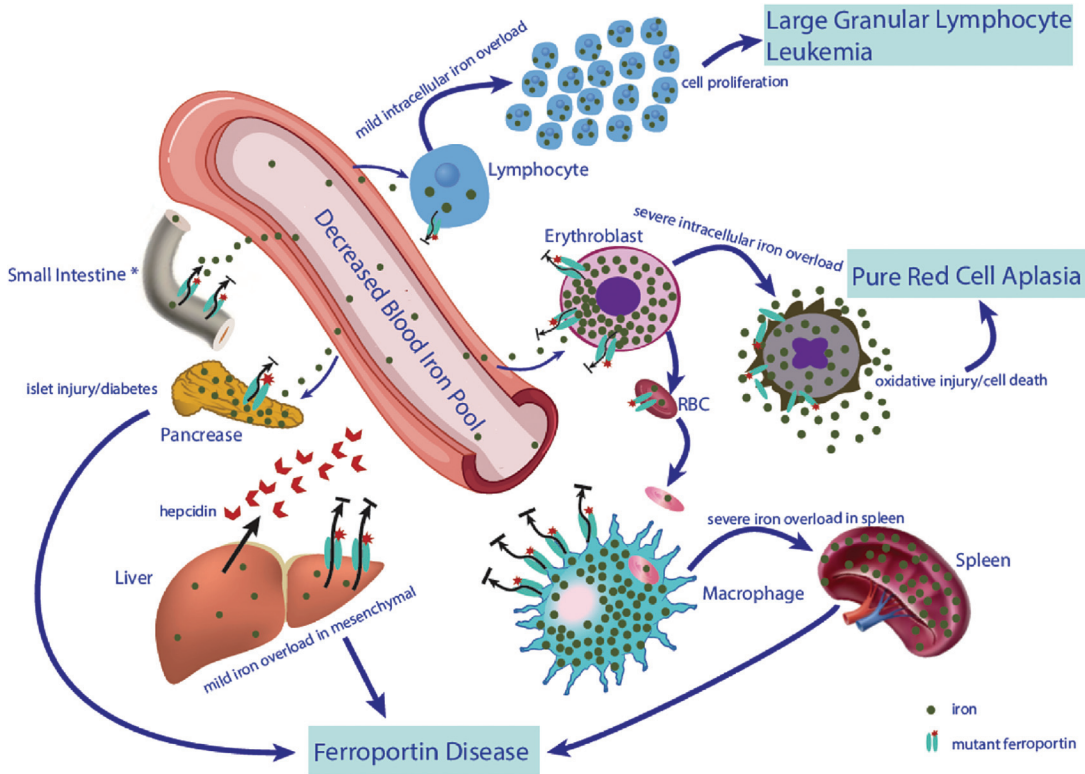

Figure 6. One possible novel pathogenic mechanism of a triad of ferroportin disease, pure red cell aplasia, and large granular lymphocytic leukemia, proposed on the basis of functional impairment of FPN, leading to an excessive increase in intracellular iron. FPN=ferroportin, RBC=red blood cell.

ACKNOWLEDGMENTS

The authors would like to thank all the doctors and nurses in the Diagnostic and Therapeutic Center for Anemic Diseases and the researcher team of the Clinical Laboratory/Imaging Center for their professional assistance.

REFERENCES

[1] Pietrangelo A. Ferroportin disease: pathogenesis, diagnosis and treatment. *Haematologica* 2017;102:1972–1984.
[2] Cianetti L, Segnalini P, Calzolari A, et al. Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation. *Haematologica* 2005;90:1595–1606.
[3] Pinto JP, Dias V, Zoller H, et al. Hepcidin messenger RNA expression in human lymphocytes. *Immunology* 2010;130:217–230.
[4] Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin: a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 2001;276:7806–7810.
[5] Vecchi C, Montosi G, Zhang K, et al. ER stress controls iron metabolism through induction of hepcidin. *Science* 2009;325:877–880.
[6] Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004;306:2090–2093.
[7] Ross SL, Tran L, Winters A, et al. Molecular mechanism of hepcidin-mediated ferroportin internalization requires ferroportin lysines, not tyrosines or JAK-STAT. *Cell Metab* 2012;15:905–917.
[8] De Domenico I, Ward DM, Langelier C, et al. The molecular mechanism of hepcidin-mediated ferroportin down-regulation. *Mol Biol Cell* 2007;18:2569–2578.
[9] Wang CY, Babitt JL. Liver iron sensing and body iron homeostasis. *Blood* 2019;133:18–29.
[10] Gordeuk VR, Caleffi A, Corradini E, et al. Iron overload in Africans and African-Americans and a common mutation in the SCL40A1 (ferroportin 1) gene. *Blood Cells Mol Dis* 2003;31:299–304.
[11] Wallace DF, Clark RM, Harley HA, Subramaniam VN. Autosomal dominant iron overload due to a novel mutation of ferroportin1 associated with parenchymal iron loading and cirrhosis. *J Hepatol* 2004;40:710–713.
[12] Sham RL, Phatak PD, West C, Lee P, Andrews C, Beutler E. Autosomal dominant hereditary hemochromatosis associated with a novel ferroportin mutation and unique clinical features. *Blood Cells Mol Dis* 2005;34:157–161.
[13] Wallace DF, Harris JM, Subramaniam VN. Functional analysis and theoretical modeling of ferroportin reveals clustering of mutations according to phenotype. *Am J Physiol Cell Physiol* 2010;298:C75–C84.
[14] Schimanski LM, Drakesmith H, Merryweather-Clarke AT, et al. In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations. *Blood* 2005;105:4096–4102.
[15] Moreno-Carralero MI, Muñoz-Muñoz JA, Cuadrado-Grande N, et al. A novel mutation in the SLC40A1 gene associated with reduced iron export in vitro. *Am J Hematol* 2014;89:689–694.
[16] Pietrangelo A, Montosi G, Totaro A, et al. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. *N Engl J Med* 1999;341:725–732.
[17] Montosi G, Donovan A, Totaro A, et al. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. *J Clin Invest* 2001;108:619–623.
[18] Chen SR, Yang LQ, Chong YT, et al. Novel gain of function mutation in the SLC40A1 gene associated with hereditary haemochromatosis type 4. *Intern Med J* 2015;45:672–676.
[19] An P, Jiang L, Guan Y, et al. Identification of hereditary hemochromatosis pedigrees and a novel SLC40A1 mutation in Chinese population. *Blood Cells Mol Dis* 2017;63:34–36.
[20] Le Lan C, Mosser A, Ropert M, et al. Sex and acquired cofactors determine phenotypes of ferroportin disease. *Gastroenterology* 2011;140:1199–1207.
[21] Ueda N, Takasawa K. Impact of inflammation on ferritin, hepcidin and the management of iron deficiency anemia in chronic kidney disease. *Nutrients* 2018;10 (9):1173.
[22] Ohtake T, Saito H, Hosoki Y, et al. Hepcidin is down-regulated in alcohol loading. *Alcohol Clin Exp Res* 2007;31:S2–S8.
[23] Means RJ. Pure red cell aplasia. *Blood* 2016;128:2504–2509.

[24] Cornec D, Devauchelle-Pensec V, Jousse-Joulin S, et al. Long-term remission of T-cell large granular lymphocyte leukemia associated with rheumatoid arthritis after rituximab therapy. *Blood* 2013;122:1583–1586.
[25] Hara T, Mizuno Y, Nagata M, et al. Human gamma delta T-cell receptor-positive cell-mediated inhibition of erythropoiesis in vitro in a patient with type I autoimmune polyglandular syndrome and pure red blood cell aplasia. *Blood* 1990;75:941–950.
[26] Ergas D, Tsimanis A, Shtalrid M, Duskin C, Berrebi A. T-gamma large granular lymphocyte leukemia associated with amegakaryocytic thrombocytopenic purpura, Sjögren's syndrome, and polyglandular autoimmune syndrome type II, with subsequent development of pure red cell aplasia. *Am J Hematol* 2002;69:132–134.
[27] Gaur S, Mansoor S, Aish L. T-cell large granular lymphocytic leukemia and hereditary hemochromatosis: a fortuitous association? *Am J Hematol* 2005;78:299–301.
[28] Zhang DL, Ghosh MC, Ollivierre H, Li Y, Rouault TA. Ferroportin deficiency in erythroid cells causes serum iron deficiency and promotes hemolysis due to oxidative stress. *Blood* 2018;132:2078–2087.
[29] Zhang DL, Wu J, Shah BN, et al. Erythrocytic ferroportin reduces intracellular iron accumulation, hemolysis, and malaria risk. *Science* 2018;359:1520–1523.
[30] Oppenheimer SJ. Iron and its relation to immunity and infectious disease. *J Nutr* 2001;131:616S–633S. 633S-635S.
[31] Motamedi M, Xu L, Elahi S. Correlation of transferrin receptor (CD71) with Ki67 expression on stimulated human and mouse T cells: The kinetics of expression of T cell activation markers. *J Immunol Methods* 2016;437:43–52.
[32] Cardier JE, Romano E, Soyano A. T lymphocytes subsets in experimental iron overload. *Immunopharmacol Immunotoxicol* 1997;19:75–87.
[33] Melo RA, Garcia AB, Viana SR, Falcão RP. Lymphocyte subsets in experimental hemochromatosis. *Acta Haematol* 1997;98:72–75.
[34] Ishida F, Matsuda K, Sekiguchi N, et al. STAT3 gene mutations and their association with pure red cell aplasia in large granular lymphocyte leukemia. *Cancer Sci* 2014;105:342–346.
[35] Hose D, Beck S, Salwender H, et al. Prospective target assessment and multimodal prediction of survival for personalized and risk-adapted treatment strategies in multiple myeloma in the GMMG-MM5 multicenter trial. *J Hematol Oncol* 2019;12:65.
[36] Angmo S, Tripathi N, Abbat S, et al. Identification of guanosine 5'-diphosphate as potential iron mobilizer: preventing the hepcidin-ferroportin interaction and modulating the interleukin-6/Stat-3 pathway. *Sci Rep* 2017;7:40097.