Arch Iran Med. March 2020;23(3):207-210

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Case Report

Variable Presentation of Hereditary Spherocytosis in an Iranian Family

Reza Vahidi, PhD[1,2,3]; Zahra Sheikhrezaei, MSc[4]; Zahra Ameri, PhD[5,3,4]; Morteza Khaleghi, MSc[6]; Alireza Farsinejad, PhD[3,4*]

[1]Student Research Committee, Kerman University of Medical Sciences, Kerman, Iran
[2]Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran
[3]Cell Therapy and Regenerative Medicine Comprehensive Center, Kerman University of Medical Sciences, Kerman, Iran
[4]Department of Hematology and Medical Laboratory Sciences, Faculty of Allied Medicine, Kerman University of Medical Sciences, Kerman, Iran
[5]Department of Tissue Engineering and Regenerative Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
[6]Pathology and Stem Cell Research Center, Kerman University of Medical Sciences, Kerman, Iran

Abstract

Hereditary spherocytosis (HS), a familial defect involving red blood cell (RBC) membrane proteins, is associated with reduced deformability, increased fragility, and progressive destruction of spherical cells. The present study focuses on three subjects of a family showing a history of repeated episodes of lethargy and pallor of unknown etiology. All patients displayed reticulocytosis and spherocytosis and one of them had anemia and splenomegaly. The patients underwent screening tests to rule in/out possible underlying disorders, and deficiency/dysfunction of RBC membrane proteins was suspected. Definitive diagnosis can be made on the basis of membrane protein analysis by quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Interestingly, all patients showed marked decrease in the protein 4.2 expression and therefore, HS was confirmed. This case report highlights the simultaneous occurrence of protein 4.2-dependent "typical" and "atypical" HS in a family and serves as a reminder to clinicians to consider RBC membrane disorders in patients presenting with suspicious and unexplained clinical signs.

Keywords: Anemia, Hereditary Spherocytosis, Membrane Proteins

Cite this article as: Vahidi R, Sheikhrezaei Z, Ameri Z, Khaleghi M, Farsinejad A. Variable presentation of hereditary spherocytosis in an Iranian family. Arch Iran Med. 2020;23(3):207–210.

Received: July 12, 2018, Accepted: February 16, 2019, ePublished: March 1, 2020

Introduction

Hereditary spherocytosis (HS) is one of the most important disruptive conditions of red blood cell (RBC) geometry that leads to the reduction of membrane surface area, abnormal red cell morphology (cell sphericity), compromised structural integrity, and greater tendency for premature removal from the circulation.[1,2] The principal defect is deficiency or dysfunction of membrane proteins including skeletal (spectrin), anchoring (ankyrin or protein 4.2), and linking (band 3) proteins.[3,4] Severe or partial deficiency of some proteins (spectrin, band 3, and ankyrin) is likely to cause a moderate to severe clinical phenotype when compared with alterations in other membrane proteins.[4] Hemoglobin concentration, reticulocytes percentage, and number of spherocytes in peripheral blood smear examination are cardinal factors used for clinical classification of this disorder.[5] Various studies have proven that the clinical expression (disease severity) of this defect is relatively uniform within a given family but varies considerably across families.[1,3] In the present paper, we present the heterogeneous clinical, hematological, and laboratory findings of one suspected family with protein 4.2 deficiency.

Case Presentation

A 49-year-old woman referred to the Internal Medicine Department with complaints of abdominal pain and repeated episodes of lethargy and pallor of unknown origin. She was only on iron supplementation and her physical as well as abdominal ultrasound examinations revealed moderate splenomegaly without hepatomegaly. Consequently, complete blood count (CBC) was performed and other hematological parameters were remarkable except for platelet and white blood cell counts (Table 1). Peripheral blood smear revealed anisocytosis, reticulocytosis, spherocytosis, and increased pincered cell count (Figures 1 and 2). The patient's uncle had splenomegaly; thus, the family history raised the intriguing assumption of an inherited disorder. Therefore, we conducted additional evaluations on this patient and her other family members to rule in/out this possibility. Among other family members, only one sibling (a 54-year-old male, patient 3 and a 47-year-old female, patient 2) exhibited repeated episodes of lethargy and pallor. Despite

***Corresponding Author:** Alireza Farsinejad, PhD; Department of Hematology and Medical Laboratory Sciences, Faculty of Allied Medicine, Kerman University of Medical Sciences, Kerman, Iran. Tel: +98-34-31325374; Fax: +98-34-31325375; Email: farsinezhad239@yahoo.com

Table 1. Blood Parameters Findings in Patients 1, 2 and 3

Parameters	Patient 1	Patient 2	Patient 3
Hct (%)	31.1	38.5	39.6
Hb (g/dL)	9.3	13.3	13.9
RBC (10^6/μL)	3.92	4.41	4.64
MCV (fL)	79.3	87.3	85.3
MCH (pg)	23.7	29.5	30
MCHC (%)	29.9	33.8	35.1
RDW (%)	17.7	17.7	14.3
Retic (%)	4.7	3.5	2.5
Spherocytes	1+	1+	1+
Ovalocytes	2+	1+	1+
Pincered cells	2%	<1%	<1%
WBC (10^3/μL)	4.4	7.63	5.6
PLT (10^3/μL)	176	229	189

Hct, hematocrit; Hb, hemoglobin concentration; RBC, red blood cell count; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; Retic, reticulocyte count; WBC, white blood cell count; PLT, platelet count.

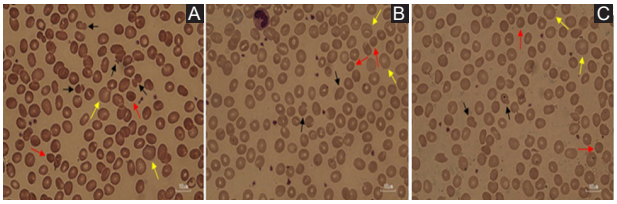

Figure 1. Morphological Findings in Peripheral Blood Smears (Wright-Giemsa, ×1000) in Patient 1 (A), Patient 2 (B) and Patient 3 (C). Pathomorphological changes include spherocytosis, anisocytosis, increased number of polychromatophilic cells, and pincered cells. Red arrow, Spherocytes; Yellow arrow, Polychromatophilic cells; Black arrow, Pincered cells.

mild spherocytosis, ovalocytosis, and increased reticulocyte count, other CBC and clinical examination profiles did not reveal any considerable finding in this sibling (Table 1). Family history and existence of these abnormalities (spherocytosis and reticulocytosis) prompted us to consider additional laboratory tests.

The diagnostic workup was followed by direct coombs (DAT) and osmotic fragility (OF) tests, with the former yielding a negative result (immune mediated hemolytic anemia was ruled out). The results of the latter are presented in Figure 3.

The patients' RBCs were subjected to Eosin-5-maleimide (EMA) analysis for better judgment. EMA binding assay is a new flow cytometric test which measures the fluorescence intensity of EMA-labeled RBCs and is used to diagnose some erythrocyte defects, especially HS.[6,7] The original method described by King et al was applied and EMA-incubated RBCs were analyzed using BD FACSCalibur™ flow Cytometer (Becton Dickinson).[6] Mean fluorescence intensity values (as mean channel fluorescence or MCF) for three independent measurements were plotted and significance values, in comparison with healthy control subjects, were given by one-way analysis of variance (ANOVA) (Figure 4).

As shown in Figure 4, EMA-labeled RBCs from patients showed reduced MCF levels compared with the healthy controls. As a confusing result, this reduction was only significant in patient 1 ($P<0.05$). In order to clarify ambiguities, membrane protein analysis was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, RBC ghosts were prepared and each membrane protein fraction was separated by electrophoresis (3% to 12% exponential gradient gel).[6] Thereafter, Coomassie brilliant blue R-250 was used for staining the gel and the density of each band was measured and analyzed using Image Lab.3.0 software (Figure 5). Based on the guideline presented by Suemori,[7] the density of each band/combined density of the total bands (bands 1-7) ratio was applied for calculation of each membrane protein fraction, and protein deficiency was determined when patient had ≥ 10 ± 2% difference relative to the mean density for each fraction among healthy subjects.

Discussion

The RBC cytoskeleton is a protein network comprised of major (α- and β-spectrin, actin, protein 4.1, ankyrin) and minor components (protein 4.2, dematin, tropomyosin, tropomodulin etc), which are in contact with each other and with other proteins and lipids of the membrane.[8]

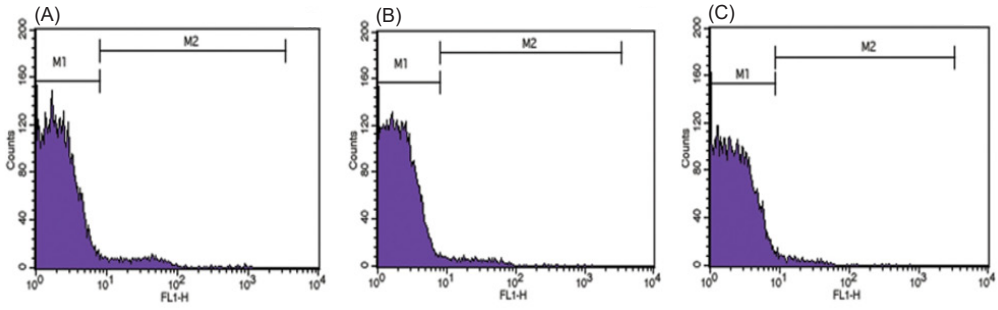

Figure 2. Flow Cytometric Enumeration of Reticulocytes Using Thiazole Orange Staining of Whole Blood in Patient 1 (A), Patient 2 (B) and Patient 3 (C). Representative histograms exhibit the reticulocytes analyzed by RNA content with those to the far right having the most RNA. It should be noted that the flow cytometric results were confirmed using New Methylene Blue staining of fresh samples (data not shown). The percentages of reticulocytes among the total RBCs were 4.7, 3.5, and 2.5% for a, b, and c, respectively. M1, Mature RBCs (%); M2, Reticulocytes (%).

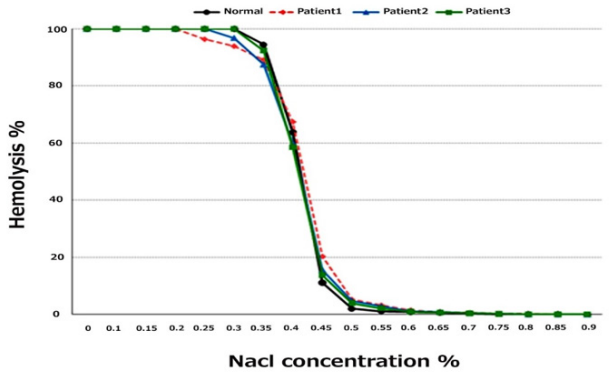

Figure 3. Osmotic Fragility Test in the Suspected Subjects Compared to a Normal Control. As shown, we did not find any significant differences between the fragiligrams, and the NaCl concentrations at which 5%, 50%, and 90% of the erythrocytes were hemolyzed (OF5, OF50, and OF90) between normal and patient subjects. A very mild and insignificant shift of fragiligram of patient 1 can be attributed to the minor subpopulation of erythrocytes with decreased osmotic fragility.

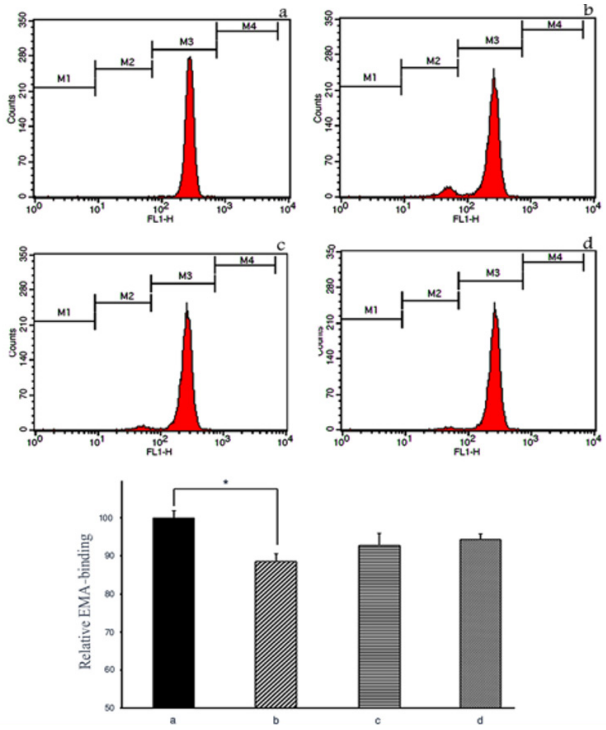

Figure 4. Flow Cytometric Analysis of Eosin-5-Maleimide Binding in Control (a), Patient 1 (b), Patient 2 (c) and Patient 3 (d). Packed RBCs were stained by EMA and assessed using flow cytometer. The results are depicted as histograms of cell number on the ordinate vs. fluorescence intensity on the abscissa. Relative EMA-binding histograms at the end of Figure 4, represent one of three independent measurements (*$P<0.05$).

These linkages (vertical and horizontal) enable the RBC to undergo multiple reversible deformations while maintaining its mechanical stability. Any defects of membrane proteins give rise to morphological changes and hemolytic anemias of varying severity.[1,3] HS is defined as an inherited disorder caused by RBC membrane protein (especially spectrin, ankyrin, protein 4.2, and band 3) defects. Mild to very severe hemolytic anemia, jaundice, splenomegaly, reticulocytosis, and the presence

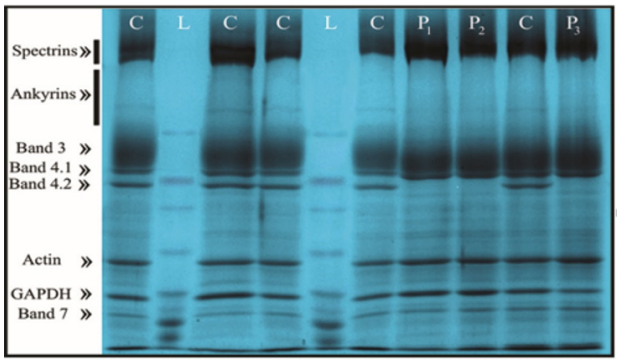

Figure 5. SDS-PAGE of RBC Membrane Proteins. The electrophoretic results of patients and controls are presented. As the results show, protein 4.2 expression is almost completely absent in RBCs of our patients. C, Controls; P1, Patient 1; P2, Patient 2; P3, Patient 3.

of spherocytes on peripheral blood smears are the most dramatic manifestations of "typical" HS.[1] This form needs to be distinguished from other hemolytic anemias manifesting with spherocytes, such as immune-mediated hemolytic anemia. However, one of the principal clinical challenges faced at the time of HS diagnosis is recognition of "asymptomatic" patients. These cases have neither typical laboratory findings nor pathognomonic clinical signs, and additional laboratory tests should be used for definitive diagnosis. Clinical expression was unremarkable among the studied patients (especially patients 2 and 3), and the presence of spherocytes and reticulocytosis suggested an underlying disorder in RBCs. Novel to our cases, Mushroom-shaped cells (pincered cells) could be seen on blood film examination (Figure 1). This finding conflicts with previous studies, in which these cells are claimed to occur only in band-3-deficient patients.[1,9]

As presented in Figure 3, we did not find any significant differences between the fragiligrams of normal and patient subjects. Certainly, spherocytic cells hemolyzed at higher NaCl concentrations than normal RBCs, but it should be considered that unremarkable OF does not exclude HS and may occur in 10%-20% of patients.[5]

Another measured parameter was EMA-binding. EMA is a fluorochrome that primarily binds to RBC membrane proteins (band 3, CD47, and Rh protein); thus, any defects in these proteins (especially band 3) cause reduction of MCF level. In other words, EMA-flow cytometry has been considered as the effective screening test for RBC membranopathies.[7] Despite the near-normal OF result, measurement of MCF level revealed a significant ($P<0.05$) decrease of EMA labeling in patient 1. To our surprise, MCF levels were not significantly ($P>0.05$) different between control and the patients (Figure 4).

A definitive diagnosis requires confirmation and specification of the underlying membranous perturbation. In this regard, SDS-PAGE is considered to be a useful and practical approach. Our electrophoresis results revealed that protein 4.2 expression was significantly decreased in

patients' RBCs compared to healthy control samples and thereby, protein 4.2 deficiency was confirmed (Figure 5). This protein is a 72-kDa component of the RBC membrane skeleton that has reciprocal interaction with band 3-ankyrin-spectrin linkage. Protein 4.2 binds to both band 3 and ankyrin and can regulate the avidity of the band 3-ankyrin interaction.[8] On the other hand, band 3 is critical for stable incorporation of protein 4.2 into the skeleton. As far as the authors of the present study are aware, protein 4.2 defects are less common than other RBC membrane protein abnormalities and usually occur simultaneously with decrement of band-3-content.[3] However, our findings are in contrast to this claim and there was no significant difference between band 3 expression in healthy and patient subjects (Figure 5). Previous studies revealed another critical interaction for protein 4.2.[1,8] This protein binds with an integrin-associated protein (CD47), which actively inhibits engulfment of RBCs by macrophages (the "do not eat me" signal). Interestingly, RBCs lacking protein 4.2 nearly lack CD47 and are prone to premature clearance from the blood stream.[8] Regarding the protein 4.2 expression in RBCs of our patients, impairment of the cytoskeletal stability and lack of CD47 are proposed mechanisms causing an accelerated removal of RBCs from the blood circulation.

As a confounding finding, our patients with the same and severe single protein deficiency exhibited non-uniform and specific clinical presentation with mild hemolytic anemia (patients 2 and 3 had compensated hemolysis). Therefore, it is still unclear what defines heterogeneity in disease severity among patients with the same disturbance. Interestingly, in agreement with the previous study, the hemoglobin/MCHC ratio decreased with increasing clinical severity of the disease in our patients.[10]

In conclusion, our data indicate the simultaneous occurrence of protein 4.2-dependent "symptomatic" and "asymptomatic" types of HS in a suspected family. Moreover, this case report serves as a reminder for clinicians to consider the wide heterogeneity of HS clinical presentation, and highlights the need for confirming a suspected RBC membrane disorder using supplementary laboratory workup.

The treatment options for our HS patients included supportive care and pharmacological treatment (folic acid, 1 mg/d). This combination can ameliorate anemia and save the patients from surgical intervention. All patients are currently monitored by the Hematology and Internal Medicine Departments.

Authors' Contribution

AF and RV proposed the original concept and designed the experiment and supervised all aspects of the work. RV, ZS, ZA, and MK equally participated in the data acquisition and analysis. All authors contributed to writing the manuscript. AF and RV provided critical reviews in order to promote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interest.

Ethical Statement

Informed consent was obtained from all the participants prior to enrolment. The study was approved by Ethical Committee of Kerman University of Medical Sciences.

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