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Hereditary red cell membrane disorders and laboratory diagnostic testing

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SUMMARY

This overview describes two groups of nonimmune hereditary hemolytic anemias caused by defects in membrane proteins located in distinct layers of the red cell membrane. Hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP) represent disorders of the red cell cytoskeleton. Hereditary stomatocytoses represents disorders of cation permeability in the red cell membrane. The current laboratory screening tests for HS are the osmotic fragility test, acid glycerol lysis time test (AGLT), cryohemolysis test, and eosin-5′-maleimide (EMA)-binding test. For atypical HS, SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins is carried out to confirm the diagnosis. The diagnosis of HE/HPP is based on abnormal red cell morphology and the detection of protein 4.1R deficiency or spectrin variants using gel electrophoresis. None of screening tests can detect all HS cases. Some testing centers (a survey of 25 laboratories) use a combination of tests (e.g., AGLT and EMA). No specific screening test for hereditary stomatocytoses is available. The preliminary diagnosis is based on presenting a compensated hemolytic anemia, macrocytosis, and a temperature or time dependent pseudohyperkalemia in some patients. Both the EMA-binding test and the osmotic fragility test may help in differential diagnosis of HS and hereditary stomatocytosis.

INTRODUCTION

The human erythrocyte membrane is a laminated structure consisting of an outer lipid bilayer and a two dimensional network of spectrin-based cytoskeleton (1–3). Connections of the two layers depend on different linker proteins with binding sites, respectively, for the cytoplasmic domains of the integral membrane proteins (band 3 and glycophorin C) embedded in the lipid bilayer and specific regions of spectrin proteins in the cytoskeleton (Figure 1a). Yet the process maintaining the biconcave shape of human red cell remains unclear (4, 5). Most often, an erythrocyte membrane defect is suspected when the blood smear of a patient with a nonimmune hemolytic anemia presents with red cells of different

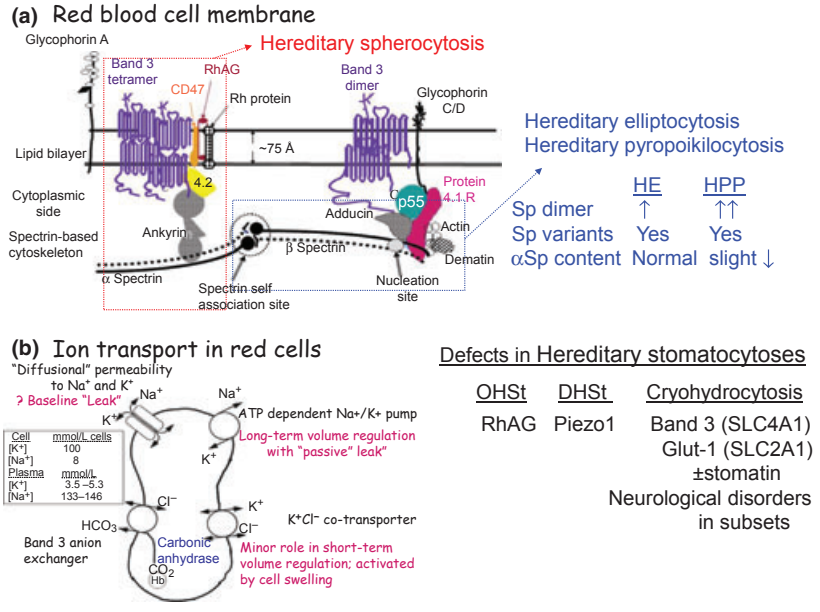

Figure 1. Erythrocyte membrane, ion transport, and associated membrane protein defects. Panel (a): The assembly of red cell cytoskeletal proteins showing the band 3 macro-complex comprising of band 3 (exisiting as dimer and tetramer), CD47, Rh protein, and Rh-associated glycoprotein (RhAG) in the lipid layer. Protein 4.2 and ankyrin (on the cytoplasmic side) form a bridge between the Band 3 macro-complex and the spectrin-based network. The GPC-P55-protein 4.1R-actin form a junctional complex. Panel (b): Selected ion pumps and passive diffusion of cations that maintain the red cell volume and intracellular Na^+/K^+ gradients. The concentrations of intracellular K^+ and Na^+ in red cells are reversed in plasma. DHSt, dehydrated hereditary stomatocytosis; Glut1, Glucose transporter 1; Hb, hemoglobin; OHSt, overhydrated hereditary stomatocytosis; RhAG, Rh-associated glycoprotein; Sp, spectrin.

sizes and shapes. The results of patient studies and basic research in the period of 1980–1990s had given us a good understanding on the pathophysiology of hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP) (6).

In the last 15 years, the research on rare red cell disorders has made progress in two fronts. The identification of genes responsible for type I and type II of congenital dyserythropoietic anemia (CDA)(7) has allowed confirmation of their diagnoses in addition to electron microscopic examination of the erythroblasts in the bone marrow (8, 9). Research into hereditary stomatocytoses (Figure 1b) has revealed the involvement of very different transmembrane proteins localized to the lipid bilayer (10–12), and the molecular basis of monovalent cation transport in this group of rare red cell disorders (13, 14).

HEMOLYTIC ANEMIAS CAUSED BY DEFECTIVE RED CELL SKELETON

This category of red cell disorders arises from either a quantitative or a qualitative defect in the membrane protein(s) constituting the red cell cytoskeleton (Figure 1a). Hereditary spherocytosis occurs at a higher incidence among Northern Europeans (1 in 2000–5000 births) than in other ethnic groups. Seventy-five percent of the cases have a dominant mode of inheritance. Some HS patients can have asymptomatic parents. HS is heterogeneous in every aspect: clinically (asymptomatic, mild, moderate, to severe hemolysis requiring blood transfusion), biochemically, and genetically (15). Asymptomatic or mild HS condition can be exacerbated by an infection (e.g., Parvovirus B19, Herpes 6, CMV, or gastroenteritis) or pregnancy. On postpartum, the HS patient's condition will return to baseline level.

Despite accumulation of massive data from biochemical and molecular analyses on membrane protein defects, it remains difficult to delineate the basis of variable clinical expression occasionally observed in a family or kindred (16). Co-inheritance of a low expression spectrin polymorphism $Sp\alpha^{LEPRA}$ allele was found to have contributed to a more severe hemolysis in HS patients having a $HS^{\alpha Sp}$ allele. Membrane proteins involved with the vertical interaction of the red cell membrane are affected, namely spectrin (α and β chain), ankyrin, band 3 (anion exchanger), and protein 4.2 (Figure 1a).

Hereditary elliptocytosis is a dominantly inherited condition caused by either a protein 4.1R deficiency or spectrin abnormalities (17), affecting the horizontal interaction of the red cell cytoskeleton (Figure 1a). A milder hemolytic anemia is often found in patients with a partial protein 4.1R reduction than in those patients having spectrin variant(s) (18). A complete absence of protein 4.1R (null phenotype) is expected to result in a severe hemolytic anemia. A high proportion of HE patients with spectrin defects are of African origin. A majority of the spectrin mutations are localized to the spectrin self-association site, where spectrin heterodimers cross-link to form tetramers and higher oligomers.

Hereditary pyropoikilocytosis is a severe form of HE, and it is often diagnosed in early childhood as the patient requires regular transfusions soon after birth. However, transient poikilocytosis in the first year of life is a different condition. The affected infant becomes transfusion-independent a year after birth and will take on the clinical phenotype of the HE parent. The red blood cells of typical HPP have low MCV (50–60 fL), and the blood smear shows predominantly (micro) spherocytes and some elliptocytes. HPP is a biallelic disorder, in that the patient is either a homozygote for spectrin mutation or a compound heterozygote for a Sp^{HE} allele together with a low expression spectrin polymorphism known as $Sp\alpha^{LELY}$ allele. The poikilocytes as seen in HPP represent increased red cell fragmentation due to a greater amount of spectrin dimer content in these red cell membranes (Figure 1a).

RARE RED CELL DISORDERS ASSOCIATED WITH CATION PERMEABILITY AND TRANSPORT

In human red blood cell, the monovalent cation gradients and the cellular shape are maintained and regulated by the actions of a passive leak process and the ATP-driven Na/K pump (Figure 1b). Earlier studies could not delineate an association of membrane protein defects in HS red cells with the altered cation permeability (19, 20). A subset of spherocytosis with low temperature leak (also having a band 3 deficiency) was found to have a series of mutations in the intramembrane domain of band 3 protein. The mutated band 3 protein had reduced anion transport together with an additional unregulated cation transport activity (21).

Unlike the low-temperature HS, sickle cell disease, thalassemia, and pyruvate kinase deficiency, hereditary stomatocytoses (HSt) represent a category of diverse red cell disorders with a common lesion, a significantly altered Na^+/K^+ fluxes or 'leaky' red cells. The incidence of HSt in a population is estimated about 40- to 50-fold lower than HS. Although isolated variants of hereditary stomatocytosis have been reported (14, 22), four main types are identified: overhydrated (OHSt), dehydrated (DHSt), cryohydrocytosis (CHC), and Familial Pseudohyperkalemia (FP). Only FP has almost normal routine hematology, but a blood specimen will give a raised plasma K^+ level after standing at room temperature for a few hours. The affected members in three generations of one FP kindred were initially thought to have HS because of a mild dominantly inherited hemolytic anemia (Hb between 119 g/L and 169 g/L). However, their red cells had abnormal intracellular $[Na^+]$ (42.6–77 mmol/L cells) and $[K^+]$ (28–58 mmol/L cells).

The first case report on OHSt presented many key features for this type of red cell disorder (23). The red cells were bowl shaped on fresh unstained blood smear, and they became stomatocytic (cells with slit-shaped central pallor) after staining. Atypical HS was the initial diagnosis because these macrocytic red cells gave grossly increased osmotic fragility. Only when both patients (mother and daughter) were nonresponsive to splenectomy was a new type of red cell disorder confirmed.

Dehydrated HSt is an autosomal dominant compensated hemolytic anemia, more commonly found than OHSt. Its clinical presentations represent a pleiotropic syndrome: DHSt only showing target cells and/or stomatocytes, DHSt with pseudohyperkalemia, or DHSt with pseudohyperkalemia and perinatal edema (14). The rate of intracellular $[K^+]$ efflux into plasma exceeds

that of Na^+ influx due to a temperature-dependent leak of K^+ from red cells after venesection (e.g., blood specimen being left at room temperature). The total intracellular Na^+ and K^+ content is lower than the normal individuals, leading to water loss and the production of dehydrated cells, which are osmotically resistant (i.e., reduced osmotic fragility). Splenectomy is not advised due to an increased risk of thromboembolic complications, including pulmonary hypertension and death (24). Mutations in the protein PIEZO1 (a mechanotransduction protein) were associated with DHSt (25).

Cryohydrocytosis (CHC) was the first noted red cell disorder that shows very marked swelling and autohemolysis when stored at refrigerator temperature (50–70% cell lysis after 24–48 h). Stomatin is present in CHC type 1 but absent in CHC type 2 (14). A reduction in band 3 was detected in some CHC patients.

LABORATORY DIAGNOSIS OF RED CELL MEMBRANE DEFECTS

When investigating a patient suspected to have a membrane-associated hemolytic anemia, a range of information is required: family and clinical history, red cell indices and peripheral blood smear examination, and results from relevant laboratory tests for indicating a hemolytic process and a membrane defect (26). Although the diagnosis of typical HS is straightforward, the current difficulty lies with making a firm diagnosis of HS for those patients presenting with an intermittent hemolysis and occasional spherocytosis.

Using the red cell indices as a 'diagnostic tool' can be confounding when faced with a patient presenting an atypical HS condition. Traditionally, intermittent jaundice, occasional spherocytes on blood smear or an increased percentage of hyperdense red blood cells together with raised mean cell hemoglobin concentration (MCHC) are signs for suspected HS. However, the MCV and MCHC values are equally informative for diagnosing hereditary stomatocytoses. A decreased MCHC with a markedly increase in MCV ex vivo is indicative of OHSt (e.g., a fresh blood sample with MCV of 95-98 fL can increase to 110-120 fL after overnight storage at ambient temperature or at 4 °C). By contrast, a raised MCHC with MCV ($\geq$100 fL) indicates DHSt. A reduced fluorescence in the EMA-binding test(27) with a significant increase in red cell lysis after storage overnight at 4 °C is suggestive of cryohydrocytosis. The supportive evidence for this diagnosis may be elevated plasma [K^+] and a mild reduction in erythrocyte band 3 content, which could not explain the more severe hemolytic condition in the patient.

SDS-polyacrylamide gel electrophoresis (PAGE) of membrane proteins can detect isolated or combined protein deficiencies in HS patients (Figure 1a). However, SDS-PAGE has its limitations. About 10% of HS cases may have no detectable membrane protein deficiency. Reticulocytosis can mask an ankyrin deficiency in a patient because young red cells tend to have a higher ankyrin content than aged red cells. Confirmation of HE/HPP definitely requires SDS-PAGE for detection of a protein 4.1R reduction as well as in spectrin analysis, which includes quantitation of spectrin dimer content and identification of spectrin variant after limited trypsin digestion of spectrin extracted from the red cell membranes (28).

Ektacytometry is the technique that can identify all cases of HSt due to the production of distinct deformability profiles (14). In the absence of a simple screening test for hereditary stomatocytoses, two methods may offer some differential information as to whether the patient has a hemolytic anemia associated with HS or anomalous cation transport. Firstly, making use of the different results obtained from the osmotic fragility test and the EMA-binding test (Table 1) will differentiate HS from DHSt and OHSt (26). Secondly, determination of [K^+] in the plasmas from the heparinized whole-blood samples from the patient and a normal control, both having been kept on ice for overnight storage, can identify red cells with a [K^+] leak. A markedly raised plasma [K^+] is a proof of leaky red cells (as found in cryohydrocytosis). Confirmation of hereditary stomatocytoses usually involves measurements of intracellular [Na^+] and [K^+] using flame photometry and the determination of isotopic flux rates (29).

DIAGNOSTIC PERFORMANCE OF SCREENING TESTS FOR HEREDITARY SPHEROCYTOSIS

The principle for the traditional laboratory diagnostic tests of hereditary red cell membrane defects exploits the reduced surface area-to-volume ratio, as found in spherocytes. These tests measure the rate of red cell lysis in different incubation media. The red cell

Table 1. Application of screening tests in the differential diagnosis of hereditary spherocytosis and other membrane-associated red cell disorders

Diagnosis	Osmotic fragility test	Acid glycerol lysis time test	EMA-binding test
Hereditary spherocytosis	↑ fragility	Shortened lysis time	↓ fluorescence
Auto-immune hemolytic anemia	↑ fragility	Shortened lysis time	Normal or ↑ with some cases
Hereditary pyropoikilocytosis	?	?	↓↓ fluorescence
Overhydrated hereditary stomatocytosis	↑ fragility	?	↑ fluorescence
Dehydrated hereditary stomatocytosis	↓ fragility	Normal lysis time	Normal or ↑ fluorescence
Cryohydrocytosis	?	?	↓ fluorescence
Congenital dyserythropoietic anemia type II	↑ fragility	Shortened lysis time with some cases	Normal or ↓ Fluorescence with some cases
Southeast Asian ovalocytosis	?	?	↓ fluorescence

?: No published data found.

osmotic fragility (OF) test determines the concentration of sodium chloride at which the fresh and incubated red cells give 50% cell lysis. The Glycerol Lysis (GLT), Acidified Glycerol Lysis Time (AGLT) test (30), and the Pink test determine the extent or the rate of lysis of red cells suspended in buffered glycerol solutions. However, these tests do not differentiate HS from secondary spherocytosis associated with other conditions, mainly autoimmune hemolytic anemias. The cryohemolysis test(31) utilizes an increased susceptibility of HS red cells to rapid cooling from 37 to 0 °C in hypertonic conditions. The relatively high sensitivity of the eosin-5′-maleimide (EMA)-binding test(27) for detecting abnormal red blood cells of varying sizes can be attributed to the use of flow cytometry as a detection system, which analyzes single red cells in a sample (28). By contrast, all the other screening tests measure hemoglobin released from red cells using spectrophotometry. The relatively high specificity of EMA-binding test for HS is due to the specific membrane molecules to which this fluorescent dye binds. The EMA-binding proteins (Band 3, CD47, Rh protein, and Rh-associated glycoprotein) happen to form a part of the band 3 macro complex in the red cell membrane (Figure 1a).

In atypical HS cases and other membrane defects, the diagnostic workout may be more complex requiring specific diagnostic tools such as SDS-PAGE of red cell membrane proteins, ektacytometry or molecular investigations (32). SDS-PAGE analysis is also required when a congenital dyserythropoietic anemia type II is suspected (28).

A recent survey by the European Network for Rare Red Cell Anemias (ENERCA, www.enerca.org) aimed at understanding which laboratory tests were used for the diagnosis of RBCs membrane defects in 25 European reference centers showed that the most frequently adopted tests were EMA binding (60% of centers) followed by 50% of the centers using NaCl curve on fresh blood (i.e., the osmotic fragility test) and AGLT. Less than 20% of the centers used the cryohemolysis test. The ektacytometry, SDS-PAGE and molecular analysis were performed only in selected atypical cases. The survey outcome has revealed that there was no consensus on the screening test(s) having the best specificity and sensitivity for detecting hereditary spherocytosis and membrane-associated red cell disorders. The majority of centers indicated the use of a combination of tests, greatly variable from center to center, rather than relying on a single method (33).

In an independent study of 150 patients with known clinical phenotypes and membrane protein defects for comparing the diagnostic performances of current screening tests for HS (34), the findings showed that none of the current laboratory tests could detect all of the HS cases. Although the EMA-binding test was found to have a 93% sensitivity and 98%

specificity, the AGLT (95%) and Pink test (91%) also gave a similar sensitivity for HS. The sensitivity of NaCl osmotic fragility tests, traditionally considered as the gold standard for the laboratory diagnosis of HS, was 68% on fresh and 81% on incubated blood. Its sensitivity was lower with compensated HS cases (53% and 64%, respectively, for fresh and incubated blood). When used together, the EMA-binding test and the AGLT could detect all of the HS patients used in this study. Thus, this approach may represent at present an effective diagnostic tool for HS, especially those with mild/compensated HS.

CONCLUSION

The effects of defective structural proteins and anomalous cation permeability on red blood cells are manifested as hereditary hemolytic anemias, which are not single-gene diseases. The association of band 3 in HS and hereditary stomatocytoses has confirmed its multifunctional role in the red cells: as a structural protein as well as an anion (or unregulated cation) transport (35). Although a majority of patients present with adequate characteristic red cell morphology and clinical features for making an appropriate diagnosis without resorting to additional laboratory testing, occasional overlapping clinical features of HS and hereditary stomatocytoses can occur. As we have sufficient experience in applying the range of laboratory tests for the diagnosis of HS, HE, and HPP, a diagnosis of hereditary stomatocytosis should be considered when a patient has a negative direct antiglobulin test, a compensated hemolytic anemia, high MCV, and possibly elevated plasma [K^+] (26, 28, 33). The proviso is that HS, enzymopathy, and macrocytic anemia are already excluded.

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CONFLICT OF INTEREST

M-JK and A.Z. have declared no conflict of interest.

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