Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 6, 2021, Pages. 20995 – 21006

Received 25 October 2021; Accepted 20 November 2021.

Recent Concepts in Lab Diagnosis of Inherited Red Blood Cell Membrane Disorders

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Abstract

Hereditary red cell membrane disorders occur due to mutation in genes responsible for coding the membrane proteins, cytoskeletal protein, and transmembrane transporter or channel resulting in decrease in the permeability and deformability and premature removal of RBC from the blood stream. These rare disorders show marked clinical and laboratory heterogeneity. Diagnosis is often challenging despite the availability of many laboratory tests. Hereditary red blood cell membrane disorders present as hemolytic anemias occurring due to defective structural proteins and anomalous cations permeability of RBC membrane. Majority of patients present with characteristic red cell morphology and clinical feature which along with the first line of diagnostic tests helps in making an appropriate diagnosis. Multiple laboratory indicators have evolved that will allow for accurate diagnosis of the disease. A combination of 2 screening tests can be used and molecular testing should be done when all screening tests are negative. Tests like Osmotic fragility, Flow cytometry, Ektacytometry, RBC membrane protein electrophoresis and genomic analysis are used in combination with family history, red cell morphological examination and red cell indices for the diagnosis of various red cell membrane disorders.

Key words:
Anemia; Red blood cell membrane; Hereditary spherocytosis

Introduction

RBC membrane disorders consists of list of chronic hereditary hemolytic anemias distributed worldwide [1] These are inherited conditions which occur due to mutation in genes responsible for coding the membrane proteins, cytoskeletal protein, and transmembrane transporter or channel resulting in decrease in the permeability and deformability and premature removal of RBC from the blood stream [2]

The normal red cell life span is 120 days and their survival in circulation depends on their major capacity of deformability which is particularly crucial in splenic cords [3] The deformability of erythrocytes is decided by factors like high surface to volume ratio, intrinsic viscoelasticity of erythrocytic membrane and the internal viscosity of erythrocyte. The hydration status of RBC determines the internal viscosity [4,5]. These rare disorders show marked clinical and laboratory heterogeneity. Splenectomy is curative in a few disorders whereas long-term risks and complications are a concern. Diagnosis is often challenging despite the availability of many

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laboratory tests. We present an overview of the inherited red cell membrane disorders, the available diagnostic modalities,and current concepts in the methods for laboratory diagnosis of inherited red blood cell membrane disorders.

The RBC membrane is made of two distinct layers with varying composition and physiological functions [6]. A two-dimensional elastic network of skeletal proteins is anchored to the plasma membrane [1,3]. RBC membrane is composed of a lipid bilayer which has embedded into it approximately 850 minor proteins and 20 major proteins [2]. Red cell membrane is capable of large scale rapid and reversible deformation in circulation [7]. The main skeletal protein which maintains a biconcave shape of RBC are spectrin alpha and beta chains, protein 4.1 or 4.1R and actin [8]. The transmembrane protein provides mainly the function of transport and a structural support to a lesser extent [2]. Inspite of all deformities, the red cell membrane maintains a constant surface area without undergoing cell fragmentation. Spectrin tetramer is the major structural component of the two-dimensional skeletal network. Maintenance of both mechanical stability of membrane and cohesion are critical for the red cell to maintain its redundant surface which is essential for external deformities. Mutations in membrane as well as skeletal protein results either in recurrent membrane cohesion or membrane mechanical stability leading to membrane surface area loss, decreased life of red cells and anemia which is severe in many red cell membrane disorders [9]

Currently, based on the genetic configuration, the Online Mendelian Inheritance in Man (OMIM) lists 15 different types of RBC membrane disorders as shown in table 1 [2]   These disorders show clinical and laboratory heterogeneity as well as genetic heterogeneity [10]

Disease Symbol	Phenotype	Protein name	Inheritance
HS1	Hereditary Spherocytosis 1	Ankyrin-1	AD
HS2	Hereditary Spherocytosis 2	Spectrin β chain, erythrocytic	AD
HS3	Hereditary Spherocytosis 3	Spectrin a chain, erythrocytic 1	AR
HS4	Hereditary Spherocytosis 4	Band 3 anion transport protein	AD
HS5	Hereditary Spherocytosis 5	Erythrocyte membrane protein band 4.2	AR
HE1	Hereditary Elliptocytosis1	Band 4.1	AD
HE2	Hereditary Elliptocytosis2	Spectrin α chain	AD
HE3	Hereditary Elliptocytosis3	Spectrin β chain	AD
HPP	Hereditary Pyropoikiolcytosis	Spectrin α chain	AR
SAO	Southeast Asian Ovalocytosis	Band 3 anion transport protein	AD
OHS	Hereditary stomatocytosis (Overhydrated)	Rh type A ammonium transporter	AD

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DHS1	Hereditary stomacytosis1(dehydrated)	Piezo-1	AD
DHS2	Hereditary stomatocytosis2 (dehydrated)	Calcium-activated potassium channel protein4	AD
FP	Familial pseudo-hyperkalemia	ATP-binding cassette sub-family B	AD
CHC	Cryohydrocytosis	Band 3 anion transport protein	AD

Table 1: Classification of erythrocyte membrane disorders by OMIM database [2]

Laboratory diagnosis of inherited red cell membrane disorders.

The approach to the diagnosis of inherited erythrocyte disorders includes first line investigations like hemoglobin (Hb), mean corpuscular volume (MCV),mean corpuscular hemoglobin(MCH), mean corpuscular hemoglobin concentration(MCHC), peripheral blood smear, family history and transmission pattern. Second line of investigations include osmotic fragility (OF), Acidified glycerol lysis time test (AGLT), EMA binding test and Ektacytometry. In a very small subset of cases, confirmation will need third line investigations like SDS-Page, Direct sequencing of causative genes and NGS custom panels [2]. The following tests are used in the screening and diagnosis of the RBC membrane disorders [24, 4].

1. Osmotic Fragility (OF)
    - Osmotic fragility (in hypotonic saline)
    - Incubated Osmotic fragility (iOF)
    - Flow Cytometric (Dye-binding test)/EMA test.
2. Glycerol lysis time tests (GLT)
    - Acidified Glycerol lysis-time test (AGLT)
3. Cryohaemolysis
4. Autohemolysis
5. Membrane protein analysis
6. Ion flux measurements/intracellular ionic content
7. Red cell deformability measurement on Ektacytometry and Laser-assisted optical rotational cell analysis (Lorrca)
8. Molecular genetic analysis

Osmotic fragility (OF)

This test gives an indication of the surface to volume ratio of erythrocytes. It is a very useful test for the diagnosis of HS. It is conducted at room temperature and can provide satisfactory results if done carefully. The method involves mixing of a small volume of blood in large volume of buffered saline solutions. The spherocytes rupture earlier than normal red cells as they take up less water in hypotonic solution before, they rupture. The fraction of red cells lysed at each saline concentration is detected using a colorimeter.

Increased osmotic fragility is seen in HS, HE and normal in non-hemolytic HE [24] Decreased osmotic fragility is seen in Hereditary xerocytosis. OF test is more of an indicator of normalcy of

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red cells because abnormal results invariably indicate abnormality whereas the results within normal range does not mean that the red cells are normal. It lacks specificity and sensitivity and may fail to detect atypical or mild HS [24]

OF test is a time-consuming test and needs technical skills. The test results are not reproducible and there is lack of uniformity in establishing cutoff for positive cases [25] Incubated osmotic fragility (iOF) test is conducted using defibrinated blood samples incubated for 24 hours and fragility is noted [24] Conventional incubated OF has low sensitivity, particularly in cases with milder phenotypes and smaller number of spherocytes [11] For HS, best combination of test with highest sensitivity and specificity is association of EMA binding test and an acidified glycerol lysis test [13]. Osmocells device is a semiautomatic system allowing the determination of RBC osmotic fragility using a dialysis method. This test allows rapid screening and offers a very fast and reliable assessment of osmotic fragility [26]

Glycerol lysis time tests (GLT)

These are tests which measures the rate of erythrocytic lysis when they are suspended in buffered glycerol solutions. This group of tests includes the conventional glycerol lysis time test, acidified glycerol lysis time test (AGLT) and the pink test. The limitations of these tests are that they cannot pick the milder disease forms and do not differentiate hereditary spherocytosis from autoimmune hemolytic anemias. The AGLT can also give positive results in pregnant females, chronic renal failure patients and myelodysplastic syndromes. But association of AGLT with an EMA binding test helps in identification of almost all cases of hereditary spherocytosis including the milder forms [6, 13]

Cryohaemolysis test

Cryohemolysis test is another test and has been suggested as a specific test for HS but increased lysis is also observed in hereditary stomatocytosis [24]

Autohemlysis

This is a test of spontaneous hemolysis developing in blood incubated at 37^{o} C for 48 hours. It is useful as an initial screen in suspected cases of hemolytic anemia but this test lacks specificity [24]

Flow cytometric (Dye-Binding test)/EMA Binding test

The fluorescence intensity of intact red cells labelled with Eosin-5-maleimide (EMA) is measured by this test. The dye Eosin-5'-maleimide (EMA) binds covalently to band 3 protein. Decreased fluorescence is observed due to deficiency of band 3 protein. The observation can be seen in HS red cells, HPP, SAO, CDA type II and Cryohydrocytosis. Each laboratory should set the reference range and cut off value for its own instrument [24]

When adequate clinical and laboratory information of patient is available, the EMA can be used alone. Application of EMA binding test along with another screening test can possibly detect all patients with HS [6]. The EMA binding test results are almost normal in all forms of hereditary stomatocytosis [22]

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This test is used as an alternative for the diagnosis of HS [12]. The EMA test has a better reproducibility and sensitivity for the diagnosis of HS. It is less laborious and cost effective [25, 27]. EMA binding test in cases of HS can show a sensitivity of 92.7% and a specificity of 99% [25,13]. The disadvantage for this test includes lack of universal reference ranges for normal and HS cases, a higher cost, difficulty in preserving the reagent in aliquots at -20 deg C and light sensitive [11, 6]. SAO cases give a false positive result with EMA test because of failure of the truncated band 3 to bind to EMA [28]. HS due to defect in band 3, spectrin and 4.2 can be determined using EMA. Ankyrin defects in HS cannot be identified by EMA [25]

Flow Cytometric Osmotic Fragility (FCM OF)

The flow cytometric osmotic fragility test is a new method based on the assessment of the left-over red cells or residual RBCs after hemolysis is induced with a hypotonic solution.[29]. This test is more sensitive and cost effective. It can effectively screen HS cases and correlate better with clinical disease severity [11]. FC-OFT is a newer test and for each technical setting, different cut-off values have to be devised and software to be selected for analysis. Technical modifications can help in standardization and acquiring results comparable to other studies [11]. A red cell suspension is prepared and installed at the sample injection port after thorough mixing. The degree of hemolysis is expressed as a percentage of red cells. Increased OF is indicated by low percentage of residual cells [30]

Membrane Protein Analysis:

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-Page)

This is a standard technique to identify qualitative membrane structural protein defects and can also quantitate the relative membrane protein contents [6, 24]. It is available for accurately diagnosing the protein defects. But it is more expensive and cumbersome [11]. This technique cannot detect GLUT1, RhAg, and Piezo 1 for hereditary stomatocytosis. But it is a useful technique to assess the protein content in HS, HE and HE/HPP. SDS-Page can also reveal the abnormal proteins present in the red cells in conditions such as CDA II. For HE and HPP, one dimensional SDS-Page is very useful in detecting 4.1 R defects and spectrin variants [6]. SDS-Page has limitations in the diagnosis of HS. In case of ankyrin defects, it may be difficult to detect it due to the presence of large number of reticulocytes as reticulocytes contains excess amounts of ankyrin which masks the reduced erythrocytic ankyrin content. In 20% cases which are asymptomatic carriers or individuals with very mild HS, the molecular defects cannot be detected [12]

Molecular Genetic Analysis

There is a great advancement in recent years in the field of erythrocyte membrane defects due to the next generation sequencing technologies. Genetic testing is a very valuable tool for the diagnosis of hereditary anemias with overlapping clinical features such as HS and CDA II [29]. Customized multigene panels (targeted NGS) and whole genome sequencing has been introduced as the latest techniques for genetic testing of RBC defects. Recent studies with Targeted-NGS and whole genome sequencing studies showed that nearly 3% of the inherited diseases are oligogenic which in turn has helped in the better understanding of the pathogenesis of various erythrocytic membrane disorders [29]. In severe transfusion-dependent cases where RBC phenotype assessment is difficult,

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targeted sequencing using a NGS platform is a very efficient tool to confirm the diagnosis of HE and HPP [31]. NGS is an advanced technique that allows one to overcome the limitations of previous techniques [2]

Ektacytometry

The ektacytometry is a laser diffraction viscometer that measure RBC deformability at constant sheer stress as a continuous function of suspending osmolarity [28]. A very small amount of blood is needed, and erythrocytes are suspended in a media with high viscosity and sheared into a couette system. The shape of RBCs attains elongation in the direction of flow. The extent of elongation depends upon the deformability of RBCs [32]. A laser beam produces an intensity pattern which is projected on a screen. Healthy red cells produce isointensity curves forming ellipses with an axial ratio equal to that of a normal healthy erythrocyte. Whereas poorly deformable cells result in circular isointensity curves [33]. Osmotic gradient ektacytometry, was originally designed in the seventies. This was considered as a reference method for red cell deformability measurements although it was not useful for clinical purposes due to its complex technicalities [1]. At present, the osmotic gradient ektacytometry is accepted as the reference technique for the diagnosis of most common erythrocytic membrane disorders [32]. Multiple changes in the cellular properties can be detected with great precision by ektacytometry [34]. Ektacytometry is only simple and reliable screening test for DHSt although sickle cell disease also gives similar profile. Difference between HS and DHSt is straightforward due to left shift of the DHSt curve. Challenge is to differentiate by ektacytometry between HS and CDA II as same profiles are obtained. The SDS-Page is useful in such cases [6]. The RBCs hydration status and heterogeneity of deformability can be assessed by osmotic gradient deformability index (DI) as depicted in figure 1. [4, 6]. The laboratory test findings in various red cell membrane disorders is presented in table 2.

The three principal features of the osmotic gradient ektacytometry profiles are:

1. The Omin point: This represents the surface to volume ratio. It points to the osmolarity in classical osmotic fragility test at which 50% of the red cells are lysed.

2. DImax: The maximum ability of cell to deform given by deformability index or elongation value.

3. O’ or Hyperpoint. This points to the osmolarity at DImax/2 and reflects the red cells hydration status [2, 4, 12]

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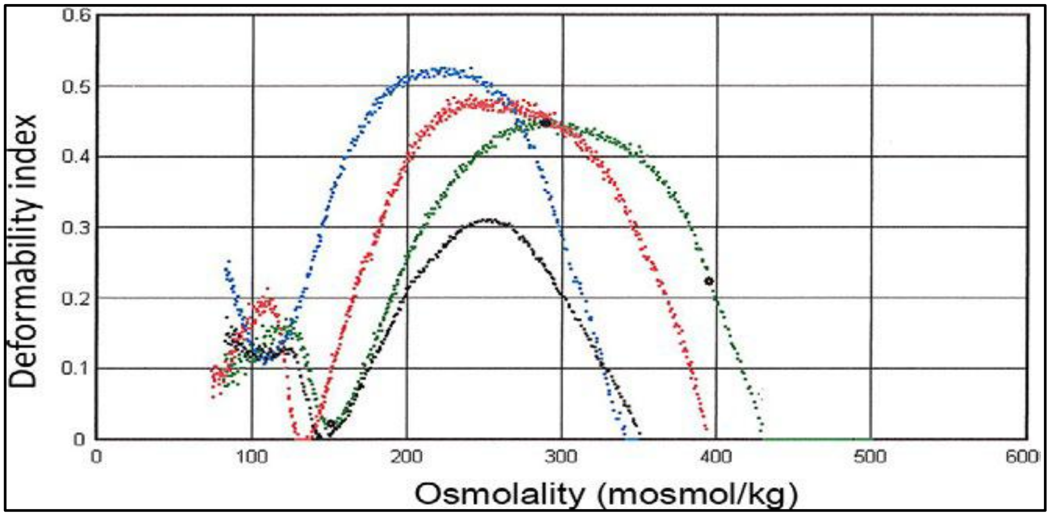

Figure 1: Osmoscan ektacytometric profiles showing normal red cell behavior(red), DHS (blue), OHS (green) and spherocytosis type profile(black) (Adapted from Caulier et al[4]).

New Generation Ektacytometer: LoRRca MaxSis Osmoscan

LoRRca MaxSis(mechatronics Instruments BR©,Zwaag, The Nethelands) is a new generation ektacytometer. It is a user-friendly equipment and allows improved diagnosis of red cell membrane disorders [1]. It combines red cell ektacytometry with an osmotic gradient and aggregometry and is basically designed to be used for hemo-rheological research and clinical application. The laser diode mounted on a bob is the light source and the reflected light is sensed by a photodiode. A video camera detects the laser beam diffraction pattern which is further analysed by a computer. Under the osmoscan application, three major RBC properties, RBC geometry, viscosity and deformability can be analysed. Previous versions of LoRRca did not have the ability to perform osmoscans [28]. Specialized laboratories use this as an intermediate step between the first screening and more specific diagnostic tests [34]. Small amount of blood is required (100microlitre) and can be done in neonates. Analysis has to be performed within 48 hours [12]. For producing continuous DI profiles, same day blood collection and testing are performed. Red cells stored over a 24 hour will give a profile of overhydration. If antibodies are bound to red cells, then each parameter can be affected. All blood sample should give a negative result in direct antiglobulin test before performing ektacytometry in order to avoid overlapping results [6]. LoRRca MaXsis osmoscan profiles clearly distinguishes among different RBC membrane defects [1].

The parameters defined in the LoRRca MaXsis and shown in figure 2 are [1]

I. Elmin: This is the minimal elongation index
II. Omin: Omin represents the osmolality at which minimum elongation index(Elmin) is attained and it correlates well with the osmolality of 50% of lysis point in OFT and represents the surface area/volume ratio
III. Elmax: Represents the maximal RBC deformability,
IV. Omax: Represents the osmolality at Elmax and further indicates the ion channel function
V. Ohyper: Indicates the Osmolality at 50% of Elmax in the hypertonic region and reflects the hydration state
VI. Elhyper: Elongation index at Ohyper

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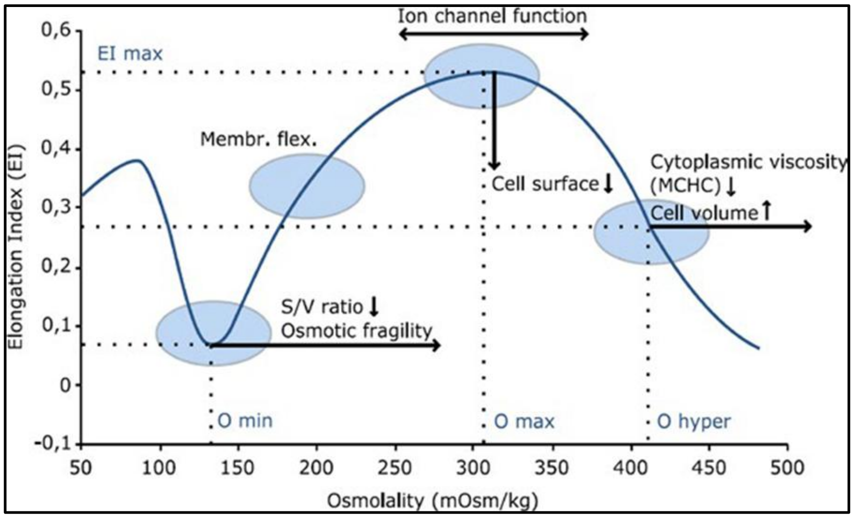

Figure 2: Osmoscan curve relevant parameters: Omin, Elmin, Omax, Elmax, Ohyper and Elhyper (Adapted from Llaudet-Planas E et al[1].).

Laser-Free optical marker for red blood cell phenotypes of inherited anemias

This is a new technique, and it is used to detect new red blood cell (RBC) phenotype. This technique demonstrates that RBC can be modeled as a biological lens and all optical parameters can clearly identify a signature that is directly related to RBC disease. This appears to be a promising method for diagnosis and sorting out of several inherited anemias including HS and CDA [29, 35]

Imaging flow cytometry (IFC)

In this technique, microscopy and image flow cytometry are combined and fluorescent images of large number of cells are captured and quantified for morphometric and fluorescent characteristics. Recently, this approach has been used to study RBC morphology and detection and differentiation of red cell membrane disorders based on morphological characterization of RBCs. This technique provides high content image analysis and measures parameters such as circularity of shape ratio, allows differentiation of red cell membrane disorders and has advantage over standard flow cytometry [36] Most cases of red cell hydration disorders can be quickly and accurately diagnosed by osmotic scan ektacytometry. Deformability profiles can distinguish erythrocyte hydration disorder from other cytoskeletal abnormalities. In the primary red cell hydration disorders, intracellular ionic content can be measured by various techniques, like ion-sensitive fluorescent indicators and fluorescence microscopes, electrophysiological methods using ion electrodes or mini electrodes and most recent proposal like the HPLC to measure the Na+/k+ in RBC. Most of the tests have high complexity due to time consuming sample processing and hence are used only in research area [4]

	OF Test	AGLT	EMA binding test	Ektacytometry

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Received 25 October 2021; Accepted 20 November 2021.

HS	Increased Fragility	Shortened lysis time	Decreased fluorescence	Decreased DImax Increased Omin Decreased Ohyper
AIHA	Increased Fragility	Shortened lysis time	Normal or increase with some cases	normal to HS like DI
HE (nonhemolytic)	Normal	Normal	Normal or borderline Mean channel fluorescence	Decreased DImax (trapezoidal profile)
HPP	Increased	Decreased lysis time	Decreased fluorescence	Decreased DImax, trapezoidal profile
SAO	Reduced fragility	Data not available	Decreased fluorescence (similar to that of HS)	No deformability
OHSt	Fragility increased (HS like)	No data	Normal or Increased fluorescence	Normal deformability DI profile right of normal deformability (over-hydration)
DHSt	Decreased fragility	Normal lysis time	Normal or increased fluorescence	Normal DImax, decreased Ohyper, DI profile left of normal (dehydration)
Cryohydrocytosis	No data	No data	Decreased fluorescence	No data
CDA II	Increased fragility	Shortened lysis time with some cases	Normal or decreased fluorescence	Isolated decreased deformability to HS-like DI profile

Table 2. Laboratory test findings for various red cell membrane disorders

Conclusion:

Hereditary red blood cell membrane disorders present as hemolytic anemias occurring due to defective structural proteins and anomalous cations permeability of RBC membrane. Majority of patients present with characteristic red cell morphology. Clinical feature which along with the first line of diagnostic tests helps in making an appropriate diagnosis in most cases Tests like OF, Flow cytometry, Ektacytometry, RBC membrane protein electrophoresis and genomic analysis are used in combination with family history, red cell morphological examination and red cell indices for the diagnosis of various red cell membrane disorders.

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