5 Molecular Genetics of Inherited Red Cell Membrane Disorders

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Inherited red cell membrane disorders constitute a diverse group of disorders which are characterized by wide clinical and molecular heterogeneity. They are nonimmune hereditary hemolytic anemia, and patients present with variable degrees of pallor, episodic jaundice, splenomegaly, and elevated lactate dehydrogenase (LDH) levels. The underlying cause is the defects either in the organization of membrane structure or membrane transport function arising because of mutations in genes encoding erythrocyte membrane proteins essential for stable structure and function. The commonest disorder is hereditary spherocytosis (HS) followed by relatively uncommon conditions such as hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP). Disorders of alterations of hydration include hereditary stomatocytosis (HSt) where cation permeability in the red cell membrane is disturbed, leading to overhydrated HSt and hereditary xerocytosis/dehydrated HSt. Extensive biochemical, biophysical, and genetic studies of the red cell membrane in the decades have provided detailed molecular insights into the structural basis for normal red cell membrane function and for altered function in various inherited red cell membrane disorders [1–5].

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5.1 Laboratory Diagnosis of Red Cell Membrane Disorders

Laboratory diagnosis of a patient suspected to have red cell membrane disorder is done by hierarchal testing based on automated red cell indices, the morphology of RBCs, and reticulocytosis in the context of an appropriate clinical presentation. Different diagnostic tests are performed to find the cause of the underlying hemolysis in a systematic manner as shown in Fig. 5.1.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, thalassemia syndromes, and other hemoglobinopathies like sickle cell disorders and hemoglobin E syndromes, unstable hemoglobins, autoimmune hemolytic anemia (AIHA), pyruvate kinase (PK) deficiency, and sometimes congenital dyserythropoietic anemia (CDA) type II need to be appropriately excluded. Diagnosis for patients with classic HS is straightforward, based on a positive family history especially when the inheritance is autosomal dominant, the presence of spherocytes on peripheral blood film, increased reticulocyte count, increased incubated osmotic fragility test (iOFT), and decreased mean channel fluorescence for eosin 5′ maleimide (EMA) dye by flow cytometry. Similarly, other disorders like HE, HPP, or HSt could be diagnosed by the morphology of red

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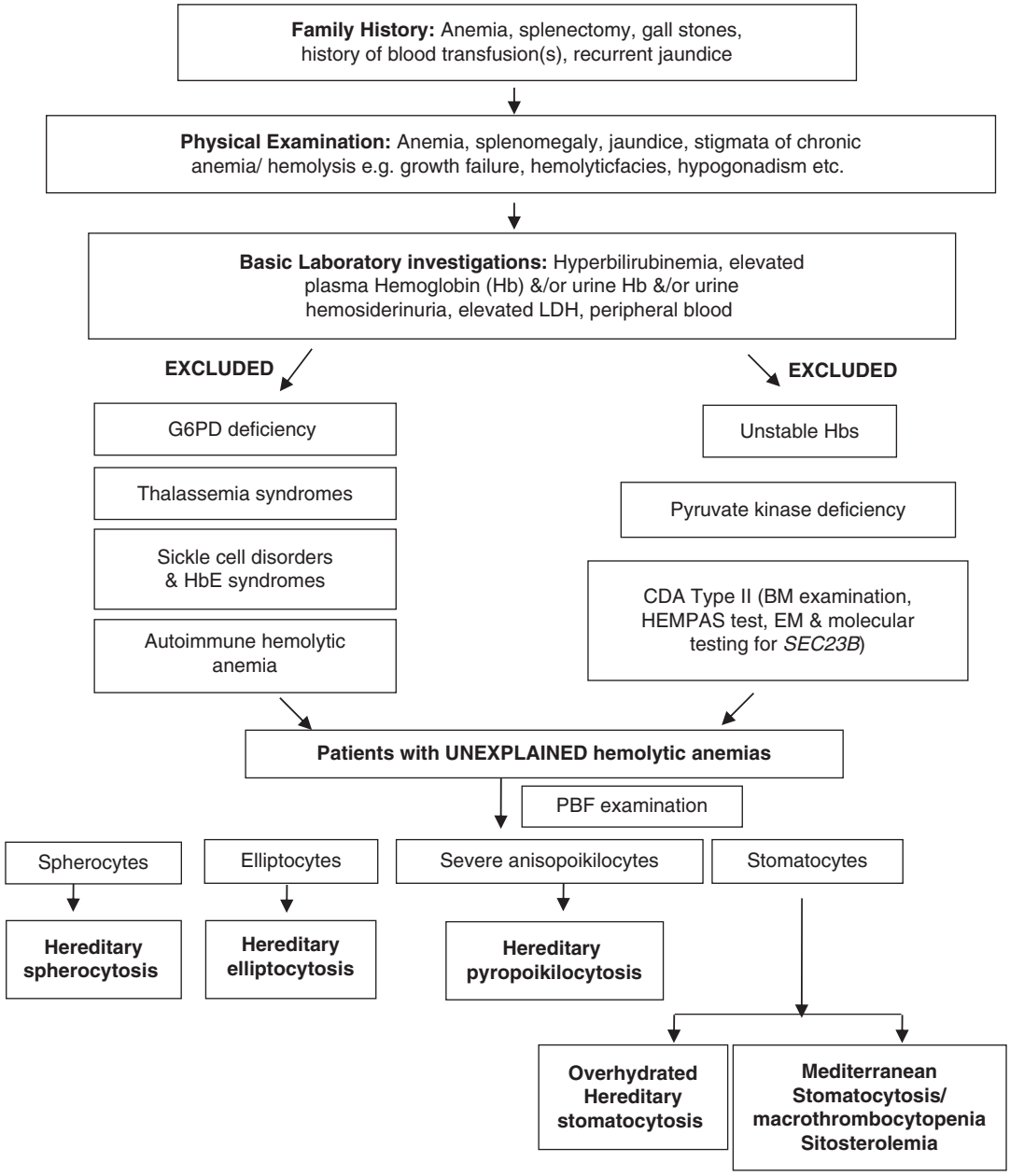

Fig. 5.1 Algorithm used in the diagnosis of inherited red cell membrane disorders

blood cells on peripheral smear. However, the difficulty lies when there is overlapping clinical features, protean manifestations, post-blood transfusion samples, and co-inheritance of other disorders. Traditional diagnostic testing approaches fail to identify the cause in such cases, and therefore, genetic testing to identify the underlying defect could be implemented. Sanger sequencing for the region of interest is the first approach to proceed to find the defect. However, the number and complexity of the genes involved make it cumbersome and time-consuming. Next-generation sequencing (NGS)/targeted re-sequencing of the panel of implicated genes provides a rapid and cost-effective approach to the molecular diagnosis of hemolytic anemias [6].

5.2 Hereditary Spherocytosis (HS)

HS is a heterogeneous disease characterized by the presence of microspherocytes in the peripheral blood smear (PBS) with increased osmotic fragility which was first described in 1871. Spherocytes show reduction of normal surface area and increased rigidity. During circulation, the abnormal erythrocytes get trapped in the spleen where they encounter in a metabolically unfavorable environment in the splenic pulp and are phagocytosed. The hemolysis in the spleen results directly in varying degrees of anemia and hyperbilirubinemia, which in turn results in pallor, fatigue, and jaundice in the patient. The loss of surface area results from increased membrane fragility due to defects in the erythrocyte membrane proteins, like ankyrin, band 3, beta spectrin, alpha spectrin, and protein 4 [3, 7].

5.2.1 Geographical Distribution

HS occurs universally in all populations and happens to be the commonest cause of inherited chronic hemolytic anemias among people of North European descent, affecting approximately one case in 2000 people. It is also prevalent in Japan and less frequently in the Southeast Asian and African-American populations; however, comprehensive data is not available for them [7]. In India, HS has been reported chiefly from the northern states (Punjab, Delhi, Uttar Pradesh, and Maharashtra) as well as sporadically from eastern and southern states like Andhra Pradesh and West Bengal [8–10]. Although precise incidence data is unavailable, anecdotal evidence suggests that it is a relatively frequently encountered membrane disorder.

5.2.2 Genetics of HS

Majority of the cases (~70%) of HS is autosomal dominant (AD) in inheritance, and others show autosomal recessive (AR) inheritance or *de novo* mutations [11–13]. Homozygosity for dominantly inherited HS gene has not been known, suggestive of the incompatibility of the homozygous state with life. Dominant HS is predominantly associated with null phenotypes due to nonsense or frameshift mutations resulting from insertions or deletions, whereas recessive HS is caused by promoter or missense mutations, which are probably *de novo* mutations [14–17]. Mutations causing HS are in genes like ankyrin, β-spectrin, band 3, α-spectrin, and protein 4.2 that act as transmembrane proteins, membrane skeletal proteins, and mediating proteins helping in the attachment of the transmembrane proteins to the skeletal proteins of erythrocyte membrane respectively as shown in Table 5.1.

HS may be subclassified depending upon the genes implicated as given in Table 5.2.

Table 5.2 Classification of HS depending upon the implicated genes

Type	MIM ID #	Gene implicated
HS type 1 (SPH 1)	# 182900	*ANK1*
HS type 2 (SPH 2)	# 182870	*SPTB*
HS type 3 (SPH 3)	# 270970	*SPTA1*
HS type 4 (SPH 4)	# 612653	*EPB3/SLC4A1*
HS type 5 (SPH 5)	# 612690	*EPB42/ELB42*

Compiled from OMIM [19]

Table 5.1 Main features of the genes involved in hereditary spherocytosis

Gene symbol	Chromosome location	Gene size (kb)	No. of exons	AA
SPTA1/EL2	1q23.1	80	53	2429
SPTB/EL3	14q23.3	100	38	2137
ANK1	8p11.21	160	49	1880
EPB3/SLC4A1	17q21.31	18	21	911
EPB42/ELB42	15q15.2	20	16	691

Iolascon et al. [18]; Online Mendelian Inheritance in Man, OMIM [19]

Table 5.3 Clinical classification of HS

	Mild	Moderate	Moderately severe	Severe
Percent of cases	10–15	60–70	10–15	5–15
Hb; mean (range) in g/dL	11–15	8–12	6–8	<6
Reticulocyte count (%)	10–15	20–30	15–25	15–25
Bilirubin level (mg/dL)	1–2	2	2–3	3
Blood transfusions	No	0–3	1–4	3–8
Transmission pattern	AD or *De novo*	*De novo* and AD	*De novo* and AD	AR

5.2.3 Clinical Manifestations

Clinical manifestations of HS are quite variable among the patients and are marked by

1. Hemolysis with or without anemia
2. Elevated absolute reticulocyte count
3. Splenomegaly
4. Jaundice
5. Presence of gallstones
6. Increased mean corpuscular hemoglobin concentration (MCHC)
7. Presence of spherocytes on PBS
8. Increased osmotic fragility test
9. Family history of anemia, jaundice, and gallstones

The clinical presentation is extremely variable among the patients, and many patients may not be identified until later in life when an infection or other process aggravates hemolysis. The degree of hemolysis varies considerably from asymptomatic to severely transfusion-dependent patients. The anemia seen in HS is usually mild to moderate, but may be worsened with fatigue, cold exposure, pregnancy, deficiencies of iron, vitamin B12, folate in addition to co-inheritance of β-thalassemia, PK deficiency, G6PD deficiency or due to viral infection likely parvovirus B19. Jaundice in anemic patients is due to increased red cell destruction leading to hyperbilirubinemia. Some patients also develop pigment (calcium bilirubinate) gallstones due to chronic hemolysis. The disease worsens with the co-inheritance of Gilbert syndrome or G6PD deficiency especially in the neonatal period. HS is clinically subclassified based on the severity of disease as mild, moderate, moderately severe, and severe phenotypes which is shown in the Table 5.3 based on the classification given by Perrotta et al. (2008) [7]. The classification is modified according to Indian population (unpublished).

5.2.4 Laboratory Diagnosis of HS

The diagnosis of HS is based on clinical findings and various laboratory tests. Clinical features like variable degree of anemia, jaundice, and splenomegaly are suggestive in the patient. In laboratory diagnosis, the most important is PBS showing spherocytes, mushroom red cells, poikilocytosis, acanthocytes, or ovalostomatocytes with abnormal red blood cell indices (increased MCHC and increased RDW) and high reticulocyte count. Various laboratory tests including OFT (incubated and fresh), acidified glycerol lysis test (AGLT), and Pink test may be used as the first line of screening test for the diagnosis. These tests vary in their specificity and sensitivity in patients. Figure 5.2 shows the increased reticulocytes, presence of spherocytes and acanthocytes in PBS, and increased iOFT.

The sensitivity of these tests is low, and a rapid flow cytometric analysis by eosin-5-maleimide (EMA) binding dye for erythrocytes has been considered as a sensitive and specific screening test for the diagnosis of HS [20]. The maleimide moiety of EMA dye predominantly binds to band 3 protein at the Lys-430 (in the first extracellular loop). In addition, it also binds to sulfhydryl groups expressed by Rh, RhAg, and CD47. In HS, absent or decreased expression of

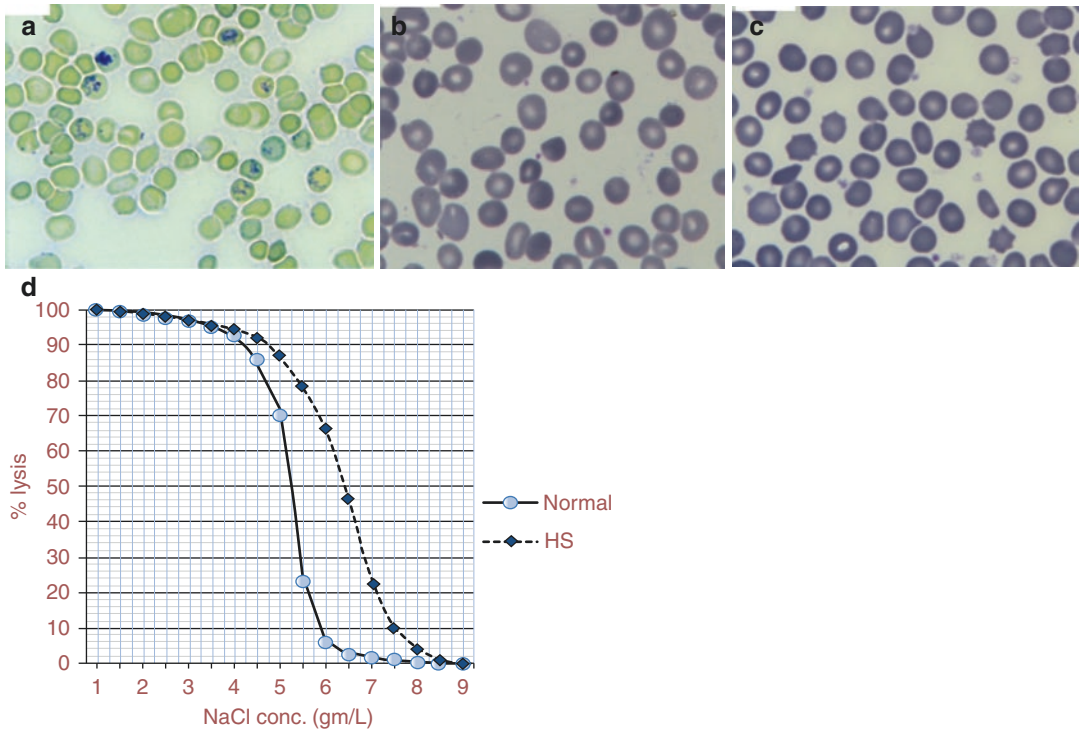

Fig. 5.2 (**a**) PBS showing reticulocytes (Azure B, 1000×); (**b**) PBS showing spherocytes and occasional stomatocytes (Leishman, 1000×); (**c**) PBS showing acanthocytes and spherocytes (Leishman, 1000×); (**d**) HS patient showing increased susceptibility to lysis using iOFT

red blood cell membrane proteins causes reduced binding of EMA to band 3 and thus shows decreased fluorescence emission. The sensitivity of the tests varies from 90 to 95%, whereas its specificity ranges from 95 to 99%.

5.2.5 Status of EMA Flow Cytometry Test in India

Studies are available from three different centers of India. In the study by Kedar et al., patients with HS and HE showed significant reduced MCF values ($P < 0.001$) than the control group and the other patient group of hemolytic anemia [21]. Another study by Kar et al. enrolled 114 subjects belonging to different categories of hemolytic anemias and showed the decreased MCF values of erythrocytes labeled with EMA dye in HS than other hemolytic and non-hemolytic anemias ($P < 0.01$). False-positive values were obtained in AIHA and CDA patients. Therefore, the sensitivity and specificity determined were 96.4% and 94.2%, respectively [22]. Joshi et al. established the cutoff value for MCF ratio of 0.79 and percent decrease of MCF as 17% in HS patients. Figure 5.3 describes the gating strategy and histograms depicting control and HS case. In their study, they proved the efficiency of EMA dye fluorescence test to capably diagnose the splenectomized cases of HS when hematological parameters improved considerably [23].

$$\text{MCF ratio} = \frac{\text{MCF of patient}}{\text{Mean MCF of normal controls}}$$

= <0.8 is consistent with the diagnosis of HS.

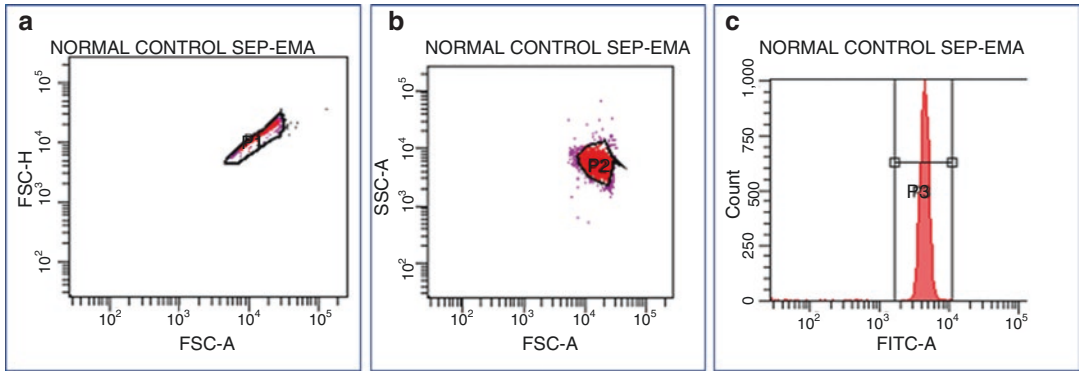

Fig. 5.3 (**a**) Gating strategy for RBCs; (**b**) Normal control subject showing population of RBC, stained with EMA; (**c**) Confirmed case of HS with reduced MCF and decreased CV

In few atypical cases where diagnosis cannot be made or in transfused patients, the underlying molecular diagnosis of HS may be detected using freshly prepared red cell ghosts on 4–12% gradient polyacrylamide gels. It can act as the third line of investigation in many subjects for analysis of erythrocyte membrane proteins but still in many cases it may not be helpful [3]. SDS-PAGE is useful to distinguish CDA type II from HS as gel electrophoresis of erythrocyte membrane proteins demonstrate the characteristic compact band 3 in CDA type II patients in comparison with HS patients [24].

5.2.6 Molecular Spectrum of HS

Various types of mutations (point mutations, splice site mutations, small deletions, duplications, and insertions) occur throughout the lengths of *ANK1*, *SPTB*, *SPTA1*, *EBP3/SLC4A1*, and *EPB42/ELB42* genes. Protein-truncating mutations (frameshift and nonsense) are more frequently observed as compared to missense mutations, in these genes. Mutations in the ankyrin 1 (50%), β-spectrin (20%), and band 3 (15–20%) are most frequently found in autosomal dominant pattern of inheritance. In few cases, ankyrin defects can also cause autosomal recessive inheritance of HS. The production of α-spectrin is three- to fourfold greater than that of β-spectrin in normal erythroid cells; therefore, only homozygous/compound heterozygous defects of α-spectrin can cause HS. The mutations in protein 4.2 are rare and are mostly present in the Japanese patients in autosomal recessive pattern.

5.2.7 Co-inheritance of G6PD Deficiency, Gilbert Syndrome, or Thalassemia with HS

There is heterogeneity in the phenotypes of HS, and even within the same family, the clinical characteristics are varied due to other genetic conditions that can modify disease phenotype in hemolytic anemias. Concomitant genetic modifiers such as red cell enzymopathies, thalassemia syndrome, and Gilbert syndrome can attribute to this intra-familial heterogeneity. Several reports state that the presence of other simultaneous disorders may worsen [25, 26] or ameliorate [27] HS phenotype.

G6PD deficiency is the most common enzyme deficiency in erythrocytes present in India and marks the initial point of molecular testing [28]. There are reports showing HS occurring concomitantly with G6PD deficiency [25]. G6PD deficiency needs to be ruled out as this could lead to inappropriate therapeutic interventions.

HS with Gilbert syndrome [26, 29, 30] has also been described previously. In Gilbert syndrome, hyperbilirubinemia is the prime clinical feature which is associated with decreased activity of the UGT1A1 enzyme. Co-inheritance of Gilbert syndrome with HS can aggravate the symptoms in patients and is reported to have a greater tendency to form gallstones in these patients [26, 31]. Gilbert syndrome is caused by the insertion of [TA] repeat in the promoter region of *UGT1A1* gene (Fig. 5.4) where the reference sequence consists of six TA repeats [A(TA)6TAA].

Co-inheritance of Gilbert syndrome and G6PD deficiency with HS was also seen in the relatively severe phenotypes. Thalassemia is also seen in concurrence with HS [27, 32, 33]. One of the patients with HS carried the single gene alpha 4.2 deletion with G6PD deficiency and Gilbert syndrome and was less symptomatic [32]. Clinical observations and correct analysis of laboratory tests are required to diagnose such complex conditions.

5.3 Hereditary Elliptocytosis (HE)

HE is characterized by the presence of elliptocytes on the blood film with a variable degree of anemia, ranging from asymptomatic to severe. They may have reticulocytosis depending on the degree of hemolysis, but mostly HE patients are asymptomatic who rarely require therapy and are diagnosed incidentally. HE is caused by abnormalities in the membrane skeleton proteins like α-spectrin, β-spectrin, protein 4.1, and glycophorin C. Figure 5.5 shows a case of hereditary elliptocytosis and reticulocytosis.

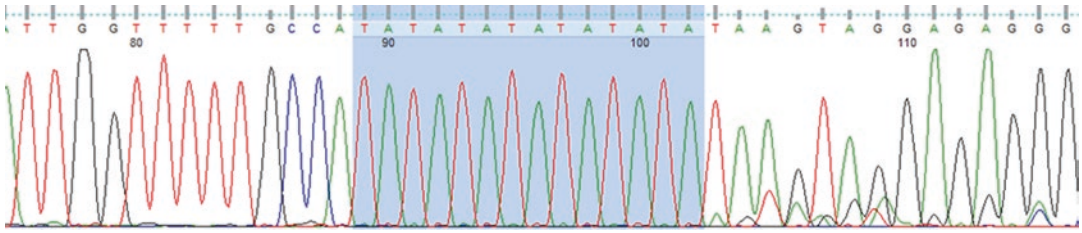

Fig. 5.4 Chromatogram showing homozygosity for TA (7/7) repeats in promoter of *UGT1A1* gene

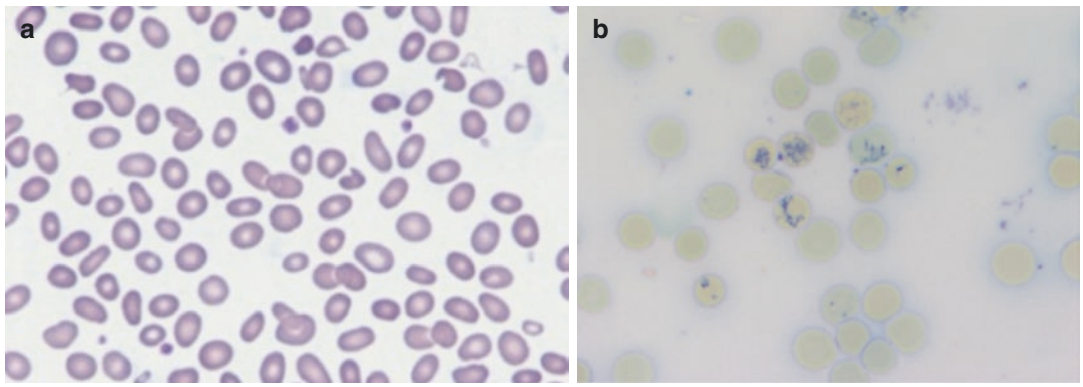

Fig. 5.5 (**a**) PBS showing elliptocytes; (**b**) Increased reticulocytes

5.3.1 Geographical Distribution

The incidence of HE is estimated as 1:2000–4000 globally, but is observed upto 1:100 in parts of Africa. As most of the patients are asymptomatic, the actual incidence of the disease remains unknown. It has been postulated that elliptocytes confer resistance to malaria.

5.3.2 Molecular Spectrum of HE

HE is usually an autosomal dominant disorder, and the leading causes are the mutations in genes coding for membrane skeleton, namely *EPB41*, *SPTA1*, and *SPTB* which disrupts self-association regions of spectrin dimers/tetramers (Table 5.4) [34, 35].

5.3.2.1 Hereditary Pyropoikilocytosis (HPP)

HPP is classified as a subtype of HE and its related disorders which characteristically shows poikilocytosis, fragmented erythrocytes, and microspherocytes. There is severe anemia with reticulocytosis and other hemolytic features. HPP is an autosomal recessive disease caused by compound heterozygous or homozygous mutations in *SPTA1* gene often inherited from asymptomatic HE parents. Severe poikilocytosis results in low MCV (50–60 fL).

5.3.2.2 Southeast Asian Ovalocytosis (SAO)

SAO is similar to but distinct from hereditary elliptocytosis in which membrane permeability of erythrocytes is affected [36]. It is commonly found in Thailand, Malaysia, Philippines, Brunei, Cambodia, Indonesia, Papua, and New Guinea. Cases are diagnosed incidentally as majority remain asymptomatic. Rarely patients are reported to have mild hemolysis and jaundice [37]. It is an autosomal dominant condition and is caused by heterozygous deletion of 27 nucleotides in *SLC4A1* gene. SAO is known to provide resistance against malaria [38]. Only one case of homozygosity for the 27 bp is known, and the phenotype is severe with the requirement of intrauterine transfusions [39].

5.4 Hemolytic Anemias Caused by Defects in Red Cell Cation Permeability and Transport

Red cell membrane disorders arising because of the defects in genes encoding red cell membrane channels or transport proteins primarily have defective cation permeability or transport mechanism across the membrane. The distinct feature is the presence of stomatocytes in the peripheral blood film. Stomatocytosis could be the manifestation of underlying genetic defect or can occur in association with several acquired conditions. Stomatocytes can be artifacts, so multiple evaluations are required. There is wide heterogeneity in the clinical and laboratory manifestations in the stomatocytic syndromes ranging from compensated hemolysis to severe anemia. There is a varied degree of clinical, laboratory, and genetic heterogeneity in hereditary stomatocytosis which are summarized in Table 5.5. Significant overlap is seen in clinical phenotypes.

Table 5.4 Classification of HE depending upon the gene implicated

Type	Symbol	MIM ID #	Gene implicated
Elliptocytosis-1	EL1	# 611804	*EPB41*
Elliptocytosis-2	EL2	# 130600	*SPTA1*
Elliptocytosis-3	EL3	–	*SPTB*
Elliptocytosis-4	EL4	# 166900	*SLC4A1*

Compiled from OMIM [19]

Table 5.5 Classification of defects in red cell cation permeability and transport

Type	MIM ID #	Inheritance	Gene
Overhydrated hereditary stomatocytosis(OHS)	# 185000	AD	*RHAG*
Cryohydrocytosis/stomatocytosis, cold-sensitive (CHC)	# 185020	AD	*SLC4A1*
Stomatin-deficient cryohydrocytosis with neurologic defects	# 608885	AD	*SLC2A1*
Pseudohyperkalemia, familial, 2, due to red cell leak PSHK2	# 609153	AD	*ABCB6*
Dehydrated hereditary stomatocytosis with or without pseudohyperkalemia and/or perinatal edema (DHS1)	# 194380	AD	*PIEZO1*
Dehydrated hereditary stomatocytosis 2 (DHS2)	# 616689	AD	*KCNN4*
Sitosterolemia Mediterranean stomatocytosis/macrothrombocytopenia	# 210250	AR	*ABCG5/ABCG8*

Compiled from OMIM [19]

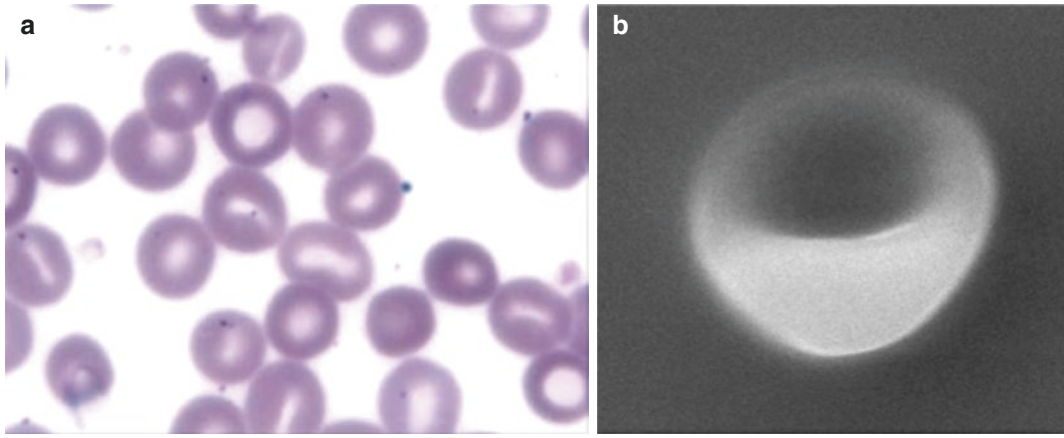

Fig. 5.6 (**a**) Blood film showing stomatocytes (Leishman stain, 1000×); (**b**) Scanning electron microscopy showing stomatocyte

5.5 Overhydrated Hereditary Stomatocytosis (OHS)

OHS is characterized by the presence of stomatocytes (Fig. 5.6), and clinically most patients present with compensated hemolysis to mild macrocytic anemia, osmotic fragile red cells, splenomegaly, and unconjugated hyperbilirubinemia.

OHS is caused by heterozygous mutations in the *RHAG* (Rh-associated glycoprotein) gene. It is also caused by heterozygous mutations in the membrane channel protein-coding genes *SLC4A1*and *SLC2A1* [40]. It is relatively rare and mostly underdiagnosed condition with approximately 20 cases reported worldwide so far [3]. De novo mutations are very frequent in this disorder. Only two studies from India describe the phenotype of hereditary stomatocytosis [41, 42]. The SDS-PAGE analysis shows OHS to be stomatin (EPB72) deficient; however, no mutation in the *EPB72* gene is reported so far.

Correct diagnosis of OHS is however critical since postsplenectomy thrombotic complications have been documented in affected individuals and have been fatal in a few. Splenectomy has been shown to have limited therapeutic benefit in hereditary stomatocytosis and should be performed after careful consideration [43]. Iron overload is usually seen in patients of OHS despite being transfusion-independent.

5.6 Cryohydrocytosis/ Stomatocytosis, Cold-Sensitive (CHC)

CHC is an extremely rare form of stomatocytosis and is characterized by the presence of stomatocytes (overhydrated erythrocytes) that have defective activity at lower temperatures [44]. Major laboratory finding is mild anemia and pseudohyperkalemia. This is an autosomal dominant disorder and occurs due to heterozygous mutations in the *SLC4A1* gene. The same gene is known to cause HS type 4 and SAO. Splenomegaly can be present, but splenectomy is known to have no therapeutic benefits to the patients.

5.7 Stomatin-Deficient Cryohydrocytosis with Neurologic Defects (SDCHCN)

SDCHCN is also known as GLUT1 deficiency syndrome with pseudohyperkalemia and hemolysis and is an asyndromic form of stomatocytosis. Clinical presentation includes mental retardation, movement disorders, seizures, cataracts, and massive hepatosplenomegaly. Hemolytic anemia is characterized by the presence of stomatocytes on blood film and pseudohyperkalemia resulting from defects in the red blood cell membrane. The cause is found to be heterozygous mutations in the *SLC2A1* gene. The disorder combines the neurologic features of GLUT1 deficiency syndrome-1 resulting from impaired glucose transport at the blood–brain barrier.

5.8 Dehydrated Hereditary Stomatocytosis (DHS)/ Hereditary Xerocytosis

5.8.1 DHS With or Without Pseudohyperkalemia and/or Perinatal Edema (DHS 1)

DHS1 also known as hereditary xerocytosis or hereditary desiccytosis is caused by a heterozygous mutation in the *PIEZO1* gene with an autosomal dominant inheritance. Clinical features include jaundice and hepatosplenomegaly. It is characterized by primary erythrocyte dehydration (dessicytes cells with their hemoglobin puddled at the periphery). The red cell indices show a very high MCHC and decreased red cell osmotic fragility. Stomatocytes are rarely seen [45]. Macrocytosis is observed with mild to moderate compensated hemolytic anemia. Some patients may also have perinatal edema or pseudohyperkalemia. Like OHS, iron overload may be present without the history of blood transfusion and may require chelation [40, 46].

5.8.2 Dehydrated Hereditary Stomatocytosis 2 (DHS2)

In DHS2 clinical and laboratory features are similar to *PIEZO1*-associated DHS. However, no mutation was detected in the *PIEZO1* gene. Recently with the advent of whole exome sequencing, a novel gene, *KCNN4*, was identified as causative and a second form of DHS was found [47–49]. Rapetti-Mauss et al. (2015) studied French and Polish families with same heterozygous mutation R352H and noted the varying degree of anemia between the two families. Variation in the clinical phenotypes carrying the same mutation could be explained by the differences in *PIEZO1* polymorphisms carried by those individuals. Splenectomy offered no therapeutic benefits as it did not improve the symptoms; however, no thrombotic complications were observed [47].

5.8.3 Pseudohyperkalemia, Familial, 2, Due to Red Cell Leak (PSHK2)

This disorder is clinically benign, a non-hemolytic variant of DHS1. Red blood cells show a "passive leak" of K+ cations into the plasma upon storage at room temperature (or below). It is an asymptomatic autosomal dominant disorder caused by heterozy-

gous mutations in *ABCB6* gene [50]. Peripheral blood smear usually does not show features of hemolytic anemia including stomatocytes.

5.8.4 Sitosterolemia/ Phytosterolemia

Sitosterolemia is an autosomal recessive metabolic condition caused by homozygous or compound heterozygous mutation in *ABCG8* or *ABCG5* gene. It results from an excess of plasma phytosterols arising as a consequence of unrestricted intestinal sterol absorption. Plant sterols in the patient plasma are markedly elevated, and clinically tendon and tuberous xanthomas with accelerated atherosclerosis and premature coronary artery disease are noted. Mediterranean stomatocytosis/macrothrombocytopenia was recognized as the hematologic presentation of sitosterolemia by Rees et al. in 2005 [51]. It is characterized by chronic hemolysis, stomatocytic red cells, macrothrombocytopenia, and varied systemic manifestations (Fig. 5.7). Recently, three siblings affected in the same family with *ABCG5* [c.727C>T (p.Arg243Ter), rs119479066] mutation with autosomal recessive inheritance were reported for the first time from India [52].

5.9 Recent Advances in Diagnosis of Red Cell Membrane Disorders

Diagnosis of red cell membrane disorders is usually based on laboratory testing followed by morphological screening as described in Fig. 5.1. Genetic diagnosis becomes important where

- Traditional testing has failed.
- Family history is lacking.
- Clinical phenotype is unexplained.
- Patient has required multiple transfusions, confounding the results of laboratory tests.

Establishing a molecular diagnosis offers insight into the pathophysiology and etiology of the disease and is crucial for patient management. Sanger sequencing is a method of choice when finding the molecular defect. Since the number of causative conditions are numerous and includes many and large genes, gene-by-gene approach is not helpful. Recently, targeted resequencing is becoming a popular approach for providing rapid and accurate diagnosis, as the limited panel of genes facilitates the data interpretation [6, 53–55].

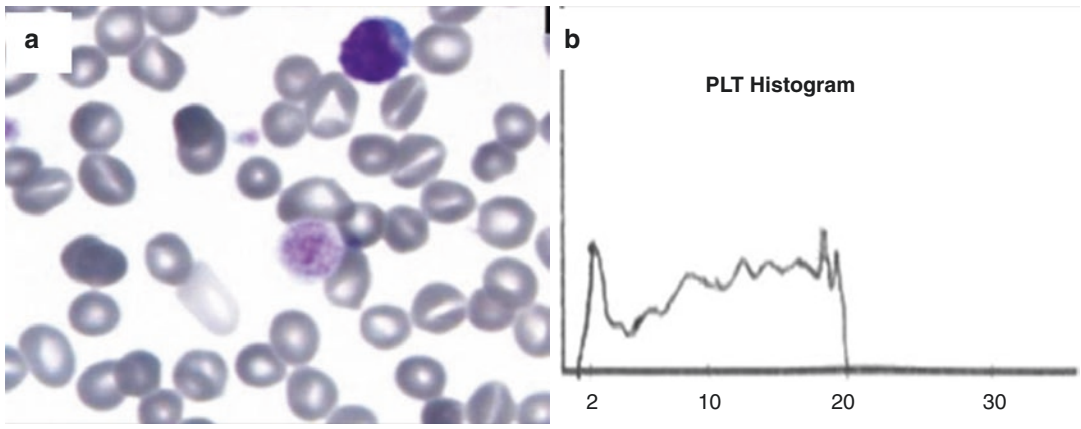

Fig. 5.7 (**a**) PBF showing stomatocytes, spherocytes, and giant platelet (May Grunwald-Giemsa, 1000×); (**b**) Platelet histogram showing the presence of giant platelets

Different laboratories have utilized different customized hemolytic anemia panels of next-generation sequencing to provide a rapid and sensitive assay [6, 54]. Hence, due to the genetic heterogeneity and complexity of the genes involved, red cell membrane disorders are perfect candidates for next-generation sequencing/targeted resequencing. Targeted resequencing is efficient to detect mutations causing hemolytic anemia, including novel variants and is ready for application in clinical laboratories.

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