Genotype/phenotype correlation in hereditary spherocytosis

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Red blood cells are biconcave under physiological conditions but their shape changes when navigating narrow blood vessels or confined spaces in tissue and organs (such as the spleen). The ability of a red cell to maintain its discoid shape, elasticity and deformability in the circulation, under constant mechanical shear and stress forces, is attributed to the components of the cell membrane.

This membrane consists of a lipid bilayer, a variety of proteins studded therein, and the glycans that protude outwards, being linked covalently to either proteins or lipids. In a wider sense, the red cell membrane includes an unusually thick, two-dimensional protein network that provides the red cell with its mechanical properties of both resistance and flexibility.[1,2] This protein network, named the red cell skeleton (or cytoskeleton), is mainly composed of spectrin α- and β-chains, proteins 4.1, or 4.1R, and actin. These are connected with one another at two sites. (i) Two or more spectrin αβ dimers articulate head-to-head (α-chain N-terminus *vs.* β-chain C-terminus) at the spectrin self-association site (Figure 1). Spectrin α and β chains contain 22 and 17 repeating segments of approximately 106 amino acids, respectively. The chains assemble side-to-side into a heterodimer beginning with

a nucleation site located near the N-terminus of β spectrin and the C-terminus of α spectrin. (ii) The ends of several tetramers converge toward a protein complex, the junctional complex. Upon stretching the skeletal network, electron microscopy showed that this cytoskeleton consists of inter-penetrating hexagons in which spectrin tetramers form the sides and the radii (Figure 1). The spectrin self-association site lies in the middle of each tetramer. The points of convergence represent the junctional complex (mainly consisting of actin, 4.1R, 4.9, p55 and tropomyosin).[3] The spectrin tetramers define triangles, within which there are a lot of band 3 molecules (approximately one million per cell) that act as anion transporters (chloride/bicarbonate exchange). The capability of these latter to form aggregates could determine the half-life of red cells, making the cells susceptible to attack from antibodies and removal by the spleen.[1,2] The vertical interaction involving cytoplasmic domains of band 3, RhAG, ankyrin, protein 4.2 and β-spectrin are particulary relevant for spherocyte formation (Figure 1).

Defects that interrupt the vertical interaction are the biochemical and molecular basis for hereditary spherocytosis (HS), whereas defects in horizontal interactions cause hereditary elliptocytosis.[1,3,4] HS is a group of inherited disorders characterized by the presence of spherical-shaped erythrocytes in a peripheral blood smear. HS is found worldwide but is the most common cause of non-immune hemolytic anemia among people of Northern European ancestry, with a prevalence of approximately 1 in 2000. However very mild forms of the disease may be much more common.[1]

The primary cellular defect is loss of membrane surface area relative to intracellular volume, leading to the spheroidal shape and decreased cell deformability of affected red blood cells. Membrane loss is due to defects in one of several membrane proteins that derive from mutations in one of the following genes: *ANK1, ELB42, SPTA1, SPTB* and *SLC4A1* encoding, respectively, for ankyrin, protein 4.2, α spectrin, β spectrin and band 3.[1,3,4] HS is clinically, biochemically, and genetically heterogeneous. In about 75% of the cases the inheritance follows an autosomal dominant pattern and one parent usually has the disease. However, the remaining 25%, of cases occur sporadically. About half of the sporadic cases are probably caused by a genuine recessive form of HS, as suggested by reports of families in which apparently normal parents have had more than one affected child. The remaining cases are probably due to spontaneous new mutations, particularly in the *ANK1* and *SPTB* genes.[1,5]

To understand the role of each gene or protein in the pathogenesis of the spherocyte it is essential to look at the biogenesis of the membrane cytoskeleton during erythopoiesis. The synthesis of erythroid plasma and skeletal membrane proteins is detectable during the early stages of erythroid development. However the synthesis is asynchronous: the synthesis of spectrins and ankyrin is initiated well before that of band 3. It is noteworthy that about 2-3 more α spectrin is synthesized than β-spectrin. This implies that we could hypothesize a mutational event in the β-spectrin (or band 3) gene in dominant forms and in the α-spectrin gene in recessive forms of HS.[6]

Clinical manifestations

The osmotically fragile spherocytes are selectively trapped in the spleen and destroyed. Increased red blood cell destruction causes the main clinical features of typical HS: evidence of hemolysis with anemia, reticulocytosis, splenomegaly, jaundice, gallstone formation, spherocytes in a peripheral blood smear and increased osmotic fragility of erythrocytes. The degree of hemolysis varies widely, such that there are asymptomatic patients, who are incidentally diagnosed, through to severe, transfusion-dependent patients.[1,3,7,8] In a study published in this issue of the journal, Mariani *et al*. examined 300 consecutive HS patients in order to determine whethere there is a relationship between biochemical phenotype and clinical findings. Unfortunately, no information on genes involved or on the inheritance patterns is present. However, this paper could be useful to shed some light

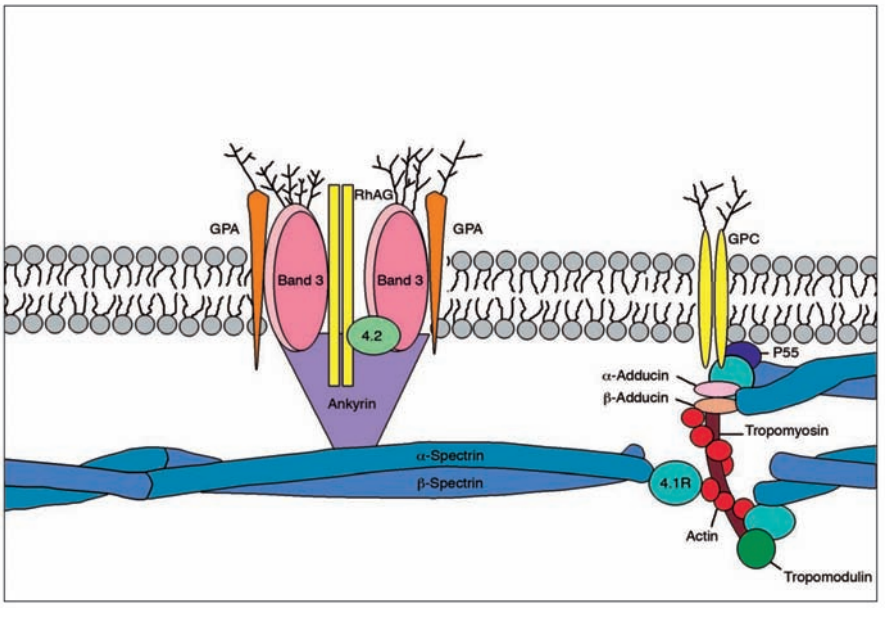

Figure 1. A simplified cross-section of the red cell membrane. The membrane is a composite structure in which a plasma membrane envelope composed of amphiphilic lipid molecules is anchored to a two-dimensional elastic network of skeletal proteins through tethering sites (transmembrane proteins) embedded in the lipid bilayer. The vertical interaction involves the cytoplasmatic domain of band 3 and RhAG, ankyrin, protein 4.2 and spectrin, while the horizontal interaction includes spectrin, which interacts with protein 4.1R, actin, tropomodulin, tropomyosin and adducin. Protein 4.1R also interacts with trasmembrane glycophorin C (GPC) and p55 in a triangular fashion.

on the relationship between biochemical and clinical patterns.[9]

During the neonatal period HS is commonly symptomatic. The Italian pediatric survey on HS showed that the disease was clinically evident during the neonatal period in 65% of cases.[17] Jaundice was present in almost all cases, requiring phototherapy to control hyperbilirubinemia and sometimes exchange transfusion. Anemia was present in 44% of neonates with HS and two-thirds of patients required blood transfusions.[10]

The presence of neonatal symptoms was not strictly predictive of the adult form. Worse hemolysis in the neonatal period has been attributed to the presence of fetal hemoglobin, which binds 2,3-diphosphoglycerate (2,3-DPG) poorly. The ensuing elevation of free 2,3-DPG levels has a marked destabilizing effect on the spectrin-protein 4.1 interaction, thereby further destabilizing the membrane skeleton.[10] Anemia of HS patients during the neonatal period or soon after is due to *sluggish* erythropoiesis during the first months of life. The lack of an appropriate erythropoietic response to compensate for increased red cell destruction necessitates blood transfusions in a large quantity of HS-affected infants during their first year of life. After this period less than 20% of affected subjects require regular transfusion support. This transient requirement for transfusions led to the question of whether anemic infants with HS, like premature anemic infants, could benefit from recombinant erythropoietin therapy, which appears to be effective in the management of anemia and could serve as a valuable alternative to transfusions of packed red blood cells.[11] Mariani *et al*. found that exchange transfusion was necessary in 14/82 cases (17%) and noted a relationship between anemia, neonatal jaundice and transfusion requirement with spectrin/ankyrin deficiency but not with exchange transfusion.[9]

Although HS is often diagnosed in childhood and young adulthood, it may be diagnosed at any time, even in the seventh to ninth decades of life.[1] HS in adulthood can be divided according to the severity of the disease, into three clinical forms: mild, moderate/severe and severe.[7,8] Patients with mild HS have compensated hemolysis without anemia, whereas those with moderate/severe HS have incompletely compensated hemolysis and anemia, with hemoglobin levels ranging from 7 to 9 g/dL, reticulocytes 10% or higher and mild to moderate splenomegaly. Mariani *et al*. found that among splenectomized patients, those with spectrin deficiency had significantly higher reticulocyte counts compared to those with band 3 deficiency.[9] The main clinical features of severe HS are anemia, which may require transfusions, severe splenomegaly, which occasionally must be treated with splenectomy, and jaundice, which causes a high incidence of gallstones.

One of the main problems of HS is its clinical heterogeneity, which can be expressed at different levels: (i) the clinical appearance can be different during the neonatal period with respect to during adulthood; (ii) when analyzing family trees, it is possible to observe that two offsprings or a parent and a child can have very different clinical features. The biochemical and genetic heterogeneity of this condition could represent the basis for its clinical heterogeneity. However, the inheritance of several modifier genes could also account for intrafamilial heterogeneity. One such modifier gene is *UGT1A1*. This gene is involved in bilirubin metabolism and its reduced or abnormal expression could cause Gilbert's syndrome, which affects approximately 12-18% of the population.[12] The *UGT1A1* gene promoter contains a TATAA variant element that is the binding site for transcription factor IID. The wide TATAA element has the A(TA)6TAA sequence while the Gilbert-associated promoter has the A(TA)7TAA sequence. It has been demonstrated by luciferase assays that the presence of this polymorphic sequence in the upstream promoter region reduces hepatic UGT1A1 activity to about 30% of normal.[12] The co-inheritance of this type of polymorphism could account for increased HS findings during the neonatal period, elevated bilirubin levels in HS patients during adulthood and the early clinical appearance of gallstones as well as an increased incidence of gallstones in these patients.[13] There is public evidence that *HFE* mutations could also enhance iron overload in this condition.[14] Furthermore, particularly in the Mediterranean area, it is possible that coinheritance of another red blood cell defect, such as beta-thalassemia or G6PDH deficiency, could account for different clinical manifestations of HS.[1,15,16]

Diagnosis

Laboratory findings include those common to all hemolytic processes, such as an increased number of reticulocytes, a slight to moderate rise in indirect bilirubin concentration, an elevated fecal excretion of urobilinogens, and hyperplasia of erythroid precursors in the bone marrow. Red cell indices show characteristic changes.[1] The mean corpuscular hemoglobin concentration (MCHC) is increased, because of mild cellular dehydration and exceeds the upper limit of normal (35-38%) due to relative cellular dehydration in approximately 50% of patients, although all HS patient have some dehydrated cells. Mean corpuscular volume (MCV) and mean hemoglobin concentration (MCH) can be in the normal range, but there is usually an increased red cell distribution width (RDW).[1]

Other causes of hemolytic anemia should be excluded, particularly autoimmune hemolytic anemia. This latter can usually be excluded by a negative direct antibody test (DAT); furthermore, it is uncommon in children and would not show a strong family history, particularly in adults. In neonates hemolysis caused by irregular maternal antibodies must be excluded, at least by a negative DAT. The diagnosis of HS may be difficult in the neonatal period. If the baby is well, testing can be postponed until the child is at least 6 months of age or older when the morphology may be less confusing.[1] A combination of a high MCHC, an increased RDW and a shift in distribution curves is often enough to suggest the diagnosis of HS.[2]

Careful examination of peripheral blood smears can give some clue to the correct diagnosis by demonstrating spherocytes: dense, round and hyperchromic red cells, lacking central pallor. In the severe form of HS there is a large quantity of spherocytes, but in the mild form these usual-

ly account for less than 10-20% of red blood cells. Molecular studies have shown that specific morphological findings are associated with certain membrane protein defects such as pincered erythrocytes (band 3), spherocyte acanthocytes (β spectrin), or spherostomatocytes.[17]

It is important to differentiate HS from hereditary stomatocytosis and related disorders with abnormal permeability to sodium and potassium ions.[18] These disorders are rare: morphology may not be typical, particularly if blood films are not freshly made.[1]

Evaluation of osmotic fragility is considered the most useful test for diagnosing HS in a patient with Coombs'-negative spherocytic hemolytic anemia with a positive family history of undiagnosed anemia. In about 25% of case of HS the osmotic fragility lies within the normal range and in most of these cases the disease is revealed after incubation of the sample at 37°C for 24 hours.[19]

If the diagnostic criteria are not met, for example if there are a few spherocytes on the film but no other laboratory, clinical or family evidence of HS, a screening test with a high predictive value for HS is helpful. The acid glycerol lysis test has a higher detection rate in asymptomatic relatives of known affected individuals than has the osmotic fragility test.[20] Osmotic gradient ektacytometry[21] and eosin-5-maleimide (EMA) binding tests[22] have a higher predictive value in diagnosing HS because there are no reports of positive results in immune or non-membrane-associated disorders. Mariani *et al*. performed different tests to evaluate osmotic fragility and established that the association of the acid glycerol lysis test and the NaCl test on incubated blood reached a sensitivity of 99%, even considering patients with atypical disease. This sensitivity is higher than that observed for cryohemolysis and the EMA binding test.[9]

The demonstration of the causative membrane protein defect would confirm the diagnosis of HS. Analysis of erythrocyte membrane proteins by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE) remains an unmatched element of orientation toward the primarily mutated protein. However, in the vast majority of cases, the diagnosis of HS can be reached by the first step described. Indeed, in dominant forms there is no need to combine the biochemical and molecular approaches. This type of analysis demonstrated that band 3, spectrin and ankyrin deficiencies are present in the vast majority of cases (Table 1). Very few studies have used the biochemical approach to HS in large series of patients.[23-27] We have summarized the more relevant in Table 1. It is very difficult to comment on an analysis of literature data. There is very marked heterogeneity in the percentages of the different biochemical defects underlying HS. There are several reasons for this. The first is the large difference in the numbers of samples. A second reason is the difference in the methods for SDS-PAGE preparation and evaluation. Thirdly, some authors did not consider reticulocytosis in the analysis. It is well known that the amount of ankyrin-1 must be considered in relation to the reticulocyte count because it is more represented in young cells. A high reticulocyte count may mask a reduction of ankyrin-1. This bias could explain why in some series ankyrin (and secondary spectrin) deficiency is understimated. Finally, it is also possible that ethnic origin of the patients could influence the percentage of biochemical defects, as demonstrated by the large percentage of 4.2 deficiencies in oriental populations.

Considering the percentage of the proteins produced in the erythroblasts it can be hypothesized that α-spectrin mutations would have a relevant role only in recessive HS, whereas band 3, ankyrin and β-spectrin mutations are the molecular basis for dominant HS. Ankyrin is produced in large quantities in reticulocytes and this could lead to an underestimate of ankyrin deficiency in the pathogenesis of HS.

The majority of mutations leading to HS are found in ankyrin, band 3, protein 4.2 and spectrin genes: mutations of α spectrin have been associated with recessive spherocytosis while mutations of β spectrin have been described in dominant forms. This is most likely due to the overproduction of α spectrin in erythroid progenitors.

Molecular studies are recommended in HS patients with normal parents. Such patients could have a genuinely recessive pattern of inheritance (homozygosity or double heterozygosity for an α-spectrin mutation) or an apparently recessive one with their disease actually due to *de novo* mutational events affecting ankyrin or β-spectin genes. To discriminate between these possibilities SDS-PAGE can be performed followed by an evaluation of ankyrin or β spectrin gene expression. Analysis of reverse-transcribed amplified cDNA from the region of several polymorphisms contained in ankyrin and beta spectrin genes suggests that in HS patients with an isolated reduction of ankyrin or β spectrin and normal parents, the apparently recessive inheritance pattern is, in fact,

Table 1. Biochemical defects (%) in HS: analysis of literature data.

Biochemical alteration	*Narla*[4]	*Families Iolascon**	*Patients Iolascon**	*Mariani*[9]	*Rocha*[23]	*Sanchez-Lopez*[24]	*Saad*[25]	*Yawata*[26]	*Lee*[27]
Spectrin-ankyrin	50-60	60	56	3	23	6	18	7	29.6
Band 3	15-20	17	19	54	66	10	13	20	11.1
Spectrin	20	15	16	31	8	16	39	0	7.4
Protein 4.2	<5	0.4	0.2	1	1	6	0	45	15
Not detectable	10	8	9	11	0	10	30	28	33
Number of patients	n.a.	220	580	139	81	31	23	60	27

**Unpublished data from Iolascon A and Perrotta S.*

frequently associated with *de novo* monoallelic expression of these genes.[1,7]

Finally, α spectrin alleles of patients with isolated spectrin deficiency and bi-allelic expression of β spectrin gene have to be investigated in order to determine the mutation (e.g., spectrin αLepra) responsible for the recessive pattern of inheritance.[3]

Conclusions

The clinical phenotype of HS is very heterogenous. It is possible to correlate the anemia with the disruption of the cytoskeleton of red blood cells, there being a good correlation between anemia and deficiency of one of the cytoskeletal components (spectrins, ankyrin, band 3, etc.).

The biochemical phenotype is not strictly linked to the molecular defects: for example mutations of spectrin or ankyrin genes can lead to spectrin deficiency. Furthermore co-inheritance of different red cell abnormalities (such as thalassemia or enzyme deficiency) as well as the inheritance of modifier genes could cause phenotypic variability.[12-16]

In general, recessive forms appear to cause more symptoms than dominant forms. HS mutations in the *SPTA1* gene show a recessive pattern of inheritance. The incidence of the αLepra allele among Caucasians is about 5%.[3] On the basis of its relative frequency, it is no wonder that it is involved in the appearance of severe forms of HS interacting with null mutation *in-trans*. HS associated with mutations in the *EPB4.2* gene shows a recessive mode of inheritance with a usually moderate clinical picture.

HS mutations in the *SPTB* gene show a dominant pattern of inheritance, in spite of the fact that β-spectrin is also manufactured in excess, although not as conspicuously as α-chains. *ANK1* gene mutations are also dominant and both these gene are frequently mutated *de novo*. The clinical picture, on average, is rather pronounced, although not requiring transfusions, at least after infancy.[1,5,7,8]

Mutations in *SLC4A1* (band 3) are the most frequent genetic alteration (along with those of ankyrin 1) and generally cause a clinical picture of moderate severity. Upon SDS-PAGE the amount of band 3 is decreased by 20-30%. There may be variations of expression from one member to another within a given family when two mild to moderate alleles criss-cross.[28] So far, three cases of homozygosity for band 3 mutations have been observed in humans within consanguineous kindreds.[29,30,3] It is noteworthy that these individuals have severe anemia, while the lack of either spectrin chains or ankyrin 1 seems to be incompatible with life in humans.

Unfortunately, the biochemical defeet escapes definition in 7-10% of cases of HS although approaches such as two-dimensional-PAGE and new proteomic techniques could resolve this situation since other candidate genes are waiting to be shown to cause HS (e.g. β-adducin).

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