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Hereditary spherocytosis: from clinical to molecular defects

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Abstract

Resistance and elastic deformability of red cells are due to a protein network (cytoskeleton) that laminates the lipid bilayer and to proteins that span the latter. All proteins are interconnected. Their structure as well as the structure of the corresponding genes are now well known. Hereditary spherocytosis (HS) is the most common hemolytic anemia due to a red cell membrane defect. It derives from alterations of the following genes: ANK1, EPB3, ELB42, SPTA1 and SPTB. This condition is clinically, biochemically and genetically heterogeneous. The osmotically fragile spherocytes are selectively trapped in the spleen and destroyed. Increased red blood cell destruction causes the three main clinical signs of HS: anemia, jaundice and splenomegaly. In this review we analyze the most recent advances concerning the molecular basis and the clinical course of HS. In particular, we examine the major individual proteins that constitute the skeleton, which are now known to play an essential role in the pathogenesis of HS. This paper also includes a review of the therapeutical approach to HS. Concerning the diagnosis we provide a flow chart from the clinical aspects to the molecular diagnosis.

Key words: hemolytic anemia, spherocytosis, membrane, band 3, cytoskeleton, ankyrin, spectrin

Historical approach

Vanlair and Masius presented the first report on hereditary spherocytosis (HS) in 1871.[1] The authors described the occurrence of microcythemia under the light microscope, recognized the hereditary nature of the disease, and ventured in a farsighted manner that the abnormal red cells were generated by the enlarged spleen. Later on, Wilson and Stanley, Hayem, and Minkowski confirmed and refined the original findings. It has been Chauffard's major merit early in this century was discovering the reduction of the osmotic resistance associated with the abnormal erythrocytes. This finding has become the cornerstone for the diagnosis of HS ever since. Micheli introduced in Italy the use of a splenectomy as an efficient treatment of HS. That complete the early history of HS, thereafter, entering the long stage of its Middle Ages.

The basic frame sketched out by the pioneers has remained largely unaltered over decades. Rather, it was filled in by a number of observations that generally broke limited new ground and were sometimes flawed with some degree of inaccuracy. Metabolic studies disclosed the proportional reduction of phospholipids and of cholesterol, that is the molecular expression of the loss of microvesicles. Jacob and Jandl,[2] and among others, described spherocytes as more *leaky* with respect to Na^+ and K^+ ions, a point likely to account for an increase in the Na^+, K^+-ATPase activity. The reduction of membrane protein phosphorylation was repeatedly reported,[3-6] yet its significance remains elusive. A specific phosphorylation-induced gelation of membrane skeletons was demonstrated[7] but, likewise, is still not understood.

HS has also been the subject of early genetic and epidemiological studies. Morton *et al*.[8] claimed that HS affects approximately one in 5,000 individuals, and is probably present in all ethnic groups. As a genetic disease, it sounds more appropriate to express the proportion of affected nuclear families, should some kindreds appear related upon subsequent examination; otherwise, the frequency is biased by the sizes of the families. It is now known that all ethnic groups do not harbor HS. This is the situation among Black people. As a matter of fact, the absence of genetic disease in determined ethnic groups is more puzzling than its prevalence, and the mechanisms that *counterselect* the illness are completely unknown. Morton *et al*.[8] also stated that HS is dominant in about 75% of overall cases. Variable clinical expression within a given kindred, e.g. for a given mutation, was only recently recognized.[9-11] Along a different line, it has been established over the past few years that apparently recessive HS could indeed be the outcome of *de novo* mutations, particularly as regarding the ANK1 gene and the SPTB gene.[12-16]

The modern times of HS begins with the development of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE).[17,18] As early as 1974, several Japanese authors tentatively

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described what was to become HS with protein 4.2 deficiency.[19,20] Yet, electrophoretic procedures were to attain their full capacity when coupled with hypersensitive optical scanning. In this respect, a spectacular breakthrough was achieved by Agre *et al.*,[21,22] who showed that a number of HS cases were associated with spectrin deficiency. It turned out, however, that spectrin deficiency could be the result of mutations affecting the SPTA1 gene and more often the SPTB gene (encoding the α- and β-chains of spectrin, respectively), and that the most common situation was created by mutations in the ANK1 gene and leading to a secondary reduction of both spectrin chains.

The heyday of HS probably came about with the isolation of the first cDNA clones encoding membrane proteins in the mid 80's. Hardly a few years later, the first mutations responsible for HS were elucidated. Chronologically, the changes initially found concerned the ELB42 gene,[23]), the EPB3 gene,[24] the SPTB gene,[25,26] the ANK1 gene,[13,27] and the SPTA1 gene.[28] Despite powerful screening methods, such as the denaturing gel gradient electrophoresis (DGGE) or the search for single strand conformational polymorphisms (SSCP), elucidating a mutation remains a time consuming and expensive task. They cannot be considered for routine diagnostic purposes, especially since most of the mutations are private, that is, are found once or at the best sporadically.

Today, the basic diagnosis of HS is best provided by ektacytometry performed in an osmolality gradient. This techniques[29] provides unmistakable curves, as has been recently surveyed in a very clear fashion.[30] It is noteworthy that this procedure still explores, among other parameters, the osmotic resistance of the spherocytes nearly a century after their discovery. The identification of the mutated protein is deduced from a number of well characterized electrophoretic patterns. In practice, these tests are in order enough to characterize the subset of HS one is dealing with.

HS has indeed split into a number of subsets depending primarily on the altered gene and, even more subtly, on the location of the mutations within these genes. The effects of HS mutations on red cells converge to a common, still largely hypothetical, mechanism best described at the cellular scale. A provisionally accepted view is that the protein defects, whichever they are and whichever mechanisms they set in motion, leave microscopic patches of the lipid bilayer inner surface bare of proteins. Protein-deprived areas are the starting point of microvesiculization. The blebs are *aspirated*, for so to say, by the macrophages, particularly in the spleen. Hence, the loss of surface area without any substantial reduction in the cell volume.

Clinical findings

HS is an inherited hemolytic anemia characterized by a broad spectrum of clinical severity, ranging from asymptomatic condition to life-threatening anemia with transfusion-dependence and rarely to hydrops fetalis and fetal death.[9] This variability is related to different molecular defects underlying HS and to bone marrow compensation. Clinical severity of the disease is quite homogeneous in a given family; but sometimes it is possible that in the same kindred there is an heterogeneity of the clinical phenotype.

During the neonatal period HS is commonly symptomatic. The Italian pediatric survey on HS, which enrolled 468 subjects, showed that the disease was clinically evident during the neonatal period in 65% of the cases.[31] Jaundice was present in almost all cases, requiring phototherapy to control hyperbilirubinemia and sometimes exchange-transfusion. Anemia was present in 44% of neonatal HS, two thirds of the patients requiring blood transfusion.

Presence of neonatal symptoms were not strictly predictive of the adulthood form. Nevertheless, it could be observed that in 100% of severe cases there were neonatal symptoms of the disease; whereas only 20% of mild adult form had neonatal symptoms.[32] Thus, the relative risk of a newborn with neonatal symptoms of HS to have a severe adult form is two times that of the general HS population (10% versus 5%).

Two of the most puzzling questions in patients with HS are what causes the neonatal symptoms and why the majority of HS cases have a neonatal onset. It appears that the biochemical defect is not related to neonatal onset. So, the problems must be in the association of the r.b.c. neonatal apparatus with the membrane defect. The worsening of 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 spectrin-protein 4.1 interactions, thereby further destabilizing the membrane skeleton.[33] The worsening of the jaundice is also due to the interaction between hemolysis of spherocytes and the reduced capacity of the neonatal liver to conjugate bilirubin; this association allows serum concentrations of unconjugated bilirubin to raise rapidly, with an increased risk of kernicterus. Furthermore a rapid increase of hyperbilirubinemia during the first (24 hours) of phototherapy administration has been described; this enhancement may be due to a light-induced oxidative effect or may reflect the natural course of hyperbilirubinemia in HS newborns.[34]

In the adulthood, the clinical features of HS, are anemia, jaundice and splenomegaly. Reticulocytosis, the presence of spherocytes in the peripheral blood smear and the increase in r.b.c. osmotic fragility are hallmarks of the disease. Peripheral blood smear observation may be useful in the suspicion of specific biochemical defects.[35] As a matter of the fact patients with β-spectrin abnormalities could show spiculated red cells (acanthocytes); whereas patients with band 3 deficiency show pincered red cells (mushroom shape).[36]

The severity of hemolysis and anemia differs markedly distinctly among HS patients, ranging from very mild (symptomless), compensated hemolysis to severe hemolytic anemia requiring frequent transfusions. There are three classification of HS in adulthood: mild, typical and severe, depending on the severity of the disease (classification based primarily on the Hb concentration, serum bilirubin and reticulocyte count).[32] Typical HS is characterized by incompletely compensated hemolysis with mild to moderate anemia, only sporadically requiring blood transfusions (50-60% of HS patients); the main clinical feature of severe HS is severe anemia which may be transfusion-dependent until splenectomy (5-7% of HS patients). On the contrary subjects with mild HS (20-30% of HS patients) are typically asymptomatic and show fully compensated hemolysis.

The parents of the recessive form of HS should be considered silent carrier state. They are not authentically normal subjects since a battery of tests (incubated osmotic fragility, reticulocyte count, haptoglobin level and autohemolysis tests) could detect this condition.[37]

Different cytoskeletal protein abnormalities could account for different clinical severity of HS. Some years ago Agree *et al.* showed that genuine recessive form of HS are clinically severe,[22,38] but this was not confirmed by us.[39]

While the clinical severity of HS is highly variable among different kindreds, it is relatively uniform within a given family in which HS is typically inherited as an autosomal dominant trait.[37] However, in several kindreds there is variability in clinical expression which could be due to variable penetrance or expression of unknown gene modifier. The latter usually do not appear if not associated with a spherocytogenic gene. Some of these are described in kindreds with band 3 deficiency[10] and with HS due to isolated spectrin deficiency.[28] The presence of another red blood cell defect, which could enhance or reduce the phenotypic aspect of spherocytosis,[40-42] could also explain interfamilial heterogeneity. A few years ago a similar kindred of Italian extraction was described.[40] Some members had HS with a typical clinical presentation of spherocytosis causing anemia, reticulocytosis and increased red cell osmotic fragility; others in the same family who had HS associated with β-thalassemia trait were not anemic and presented with mild well-compensated hemolysis. Detailed studies of membrane protein composition demonstrated the presence of a band 3 deficiency in all individuals with HS. In this family β-thalassemia trait evidently *silences* most of the clinical characteristics associated with HS due to band 3 deficiency. Hence, in some clinical cases coexistence of HS in association with another red blood cell defect could account for the phenotypic differences between different members of the same kindred.

Patients with HS, like patients with other hemolytic anemias, are subject to various problems: crises, gallbladder disease, hemochromatosis and rarely to indolent leg ulcers and extramedullary masses of hematopoietic tissue, which may be mistaken for mediastinal neoplasms. Some of these complications are due to the erythroid hyperplasia as consequence of chronic hemolysis. Interestingly, this aspect is also evident in the subjects with normal hemoglobin level. A decreased concentration of 2,3-DPG or lack of perfusion of the juxtaglomerular renal vessel could play a role in this finding. Very recently, Guarnone *et al.*[43] showed that the compensated hemolytic state was the result of up to 8 times normal erythropoietin (Epo) level and up to 3.9 times expansion of erythropoiesis. Therefore in HS patients compensation of the hemolytic state is produced by inappropriately high Epo level and the pattern of overproduction is a biological characteristic of the disease.[43]

Aplastic crisis was one of the more frequent complications of HS. They were reported in 17 of 400 subjects of the Italian registry. In 5 cases the aplastic crisis was the first sign of HS.[31] Human Parvovirus B19 was the causative agent of these transient intense erythroblastopenias. This virus by means of erythrocyte P antigen as receptor invades and destroys erythroblasts, causing aplastic crises.[44] Since the intensity of anemia required rbc transfusions, it was difficult to appreciate the spontaneous duration of aplastic crisis. It seemed short (7-10 days), at the end of which the reticulocyte count increased.[45] Viral syndromes are causes of hemolytic crisis with a mild transient increase in jaundice, splenomegaly, anemia and reticulocytosis. The third type of crises is the megaloblastic, which occurs when dietary intake of folic acid is inadequate for the increased needs of erythroid hyperplasia.

Gallstones are the most common complication of HS. In the Italian registry about 25-30% of the patients had gallstones, this percentage would be a minimal figure because not symptomatic adult HS patients could not receive periodic ultrasonographic control. Approximately 25% of our patients showed gallstones at the age of 6-7 years. It is not clear what the percentage of symptomatic gallbladder is; a first evaluation established that it was 7.5% of gallstones.[31]

Hemochromatosis has been reported in a number of unsplenectomized or late-splenectomized adults with HS in absence of blood transfusions or chronic iron administration. In these cases the heterozygous state for genetic hemochromatosis as been suggested.[46,47]

Structure and assembly of membrane

The lipid bilayer accounts for approximately 50% of the weight of the red cell membrane. In the bilayer lipids are asymmetrically distributed: choline phospholipids are concentrated in the outer half of the bilayer and amino phospholipids and phosphatidylinositols in the inner half. The hydrophobic lipid core is traversed side by side by the family of integral membrane proteins. This family include proteins such as glycophorins A, B, C and D, which carry membrane

receptor and antigens, and transport proteins such as protein 3, the anion channel for Cl-HCO_3^- transport.

Integral proteins usually have three specific domains: the extracellular, intramembraneous and cytoplasmic. The extracellualr is usually a receptor domain and is glycosylated. This part of the molecule contains the blood group antigens. The intramembraneous domain is very hydrophobic and is thought to extend the bilayer as single or multiple α-helices. There is some evidence that this part of the molecule binds phospholipids.[48] The third domain is the cytoplasmic domain by which the peripheral proteins are linked.

Band 3 or anion trasporter is the major integral protein and it accounts for 1.2 million copies per cell.[49] The extracellular part is composed of a single complex carbohydrate chain attached to Asn 642; this lactosaminoglycan chain is variable in length and contains Ii blood group antigens. The membrane domain is composed of 14 transmembrane helices, which form the transport channel. Functionally active molecules are formed by dimers and tetramers of band 3; in this way band 3 allows the efflux of bicarbonates from the cell in exchange for plasma chloride, so as to equilibrate bicarbonate between the red cell and plasma. Band 3 clustering is related to hemoglobin denaturation followed by hemochrome binding which promotes a red cell membrane oxidative damage and might be considered as the expression of erythrocyte senescence. On aged erythrocytes the autologous antibodies binding play a key role as markers for recognition and removal of aged red cells.[50]

The cytoplasmic domain is 404 amino-acids long, with a pliant *hinge* near the center. This acidic N-terminal sequence contains the binding sites for glycolytic enzymes, which are negatively regulated by this linkage. Furthermore three cytoskeletal protein are fixed to this domain: ankyrin, protein 4.1 and 4.2. Ankyrin binds to band 3 tetramers. The stoichiometric relation between band 3 and ankyrin is 6:1 respectively. So a vast majority of band 3 molecules are free to move in the exagons formed by spectrin tetramers.

The cytoskeleton is located on the inner surface of the erythrocyte plasma membrane and gives a red blood cell its unique properties of stability, durability and flexibility

Spectrin is the major protein component of the erythrocyte membrane skeleton. Native spectrin is composed of two large subunits, α-spectrin (MW 240,000) and β-spectrin (MW 220,000) (for review see ref. #51). Spectrin comprises about 20% of the mammalian erythrocyte membrane protein, with the α and β subunits present in equimolar amounts. α and β-subunits are intertwined with one another in an antiparallel manner. Heterodimers of α and β spectrin self-associate head-to-head to form heterotetramers, which at the tail bind to short actin filaments in the junctional complex (tropomyosin, 4.1, 4.9). In the membrane spectrin tetramers and medium sized oligomers are thought to be the prominent species. When the skeletal meshwork is spread onto a thin carbon film, clear images of a primarily hexagonal lattice of junctional F-actin complexes cross-linked by spectrin filaments are obtained.[52]

An abnormality in the dimers head-to-head interaction causes elliptocytosis (this variety shows increased dimers content), as well as the reduction of 4.1 protein (this variety of HS shows normal dimers content).

β-spectrin binds to ankyrin at a site 20 nm from the head-to-head interaction. Ankyrin binding requires almost the entire 15th repeat segment of β-spectrin and a small portion of the 16th.[53] Ankyrin in turn is bound with high affinity to the cytoplasmic domain of band 3. This vertical interaction is the principal anchor for the membrane skeleton. Disruption of this interaction causes spherocytosis, as a consequence result of the cytoskeletal and lipid bilayer destabilization and vesciculization. Pallidin or protein 4.2 binds to the cytoplasmic domain of band 3 and this interaction seems to play a role in the ankyrin-band 3 interaction. The tail of the tetramer at the level of β-spectrin binds actin, which in turn binds to protein 4.1 (Figure 1). So a second linkage of spectrin tetramers to the plasma membrane is mediated by the association of β-spectrin-actin-protein 4.1 that binds to the transmembrane protein glycophorin C. This vertical interaction does not appear involved in the pathogenesis of spherocytosis.

The principal vertical interaction is composed of four proteins band 3, ankyrin, 4.2 and β-spectrin. This implies that a disruption of band 3 ankyrin interaction could cause a deficiency of ankyrin, 4.2 and spectrin. A problem in the β-spectrin could cause an isolated spectrin deficiency (Figure 1).

Cytoskeletal and transmembrane protein synthesis is a process developing very early in erythroid maturation. Cell culture studies established that this production is asynchronous (spectrin production begins before the synthesis of the other cytoskeletal components) and is quantitatively exuberant (production of α-spectrin exceeds four times that of β-spectrin). This pattern of production seems to play an important role in the genetics of HS. As a matter of fact only homozygous defects of α-spectrin could cause HS; whereas, due to its limiting amount (with respect to α-spectrin), deficiency of β-spectrin causes spherocytosis in the heterozygous state too.

Band 3 and ankyrin synthesis are the latest to appear and they seem to play a critical role in the assembly. Asynchronous expression of the major peripheral proteins and one of their major transmembrane receptors has been conserved in the erythroid differentiation programs from birds to mammals. Such a pattern of expression precludes band 3 serving as a nucleating site to initiate the assembly of the peripheral network. This hypothesis was recently refuted by the demonstration of the presence of a

cytoskeletal network in the red blood cells of knockout animals.[54]

Protein 4.1 and ankyrin are the last cytoskeletal protein components to continue to be synthesized and assembled. This is at least partly due to the fact that ankyrin and protein 4.1 mRNAs persist late in erythropoiesis when levels of the majority of cytoplasmic RNAs, including those for band 3 and spectrins, have declined precipitously.

The genes involved in hereditary spherocytosis

HS is caused by mutations in at least five genes: 1) the SPTA1 gene (encoding spectrin α-chain); 2) the SPTB gene (spectrin β-chain); 3) the ANK1 gene (ankyrin 1); 4) the EPB3 gene (protein AE1, or band 3), and 5) the ELB42 gene (protein 4.2) (for review, see ref. #55, and references therein). By decreasing order of frequency, the mutated genes are the ANK1, EPB3, SPTB, ELB42 and the SPTA1 genes. The main features of these genes are provided in Table 1. In the current state of knowledge, all genes belong to families,except for the ELB42 gene. Genes mapping to 9q34.1 (α-chain)[56] and 2p21 (β-chain),[57] respectively encode the chains of nonerythroid spectrin, or fodrin. The spectrin gene family is itself part of a superfamily, including the genes encoding α-actinin and dystrophin. The ANK2 and the ANK3 genes, which map to 4q25-q27[58] and 10q21,[59] respectively, encode nonerythroid ankyrins, ankyrins 2 and 3. Anion exchange (AE) proteins, AE2 and AE3, are encoded by genes mapping to 7q35-q36[60] and 2q36,[61] respectively. Additional genes are likely to extend the list in the future.

Polymorphisms

Before we go over the main features of the above genes, we will say a few words about relevant polymorphisms. The SPTA1, SPTB, ANK1 and the EPB3 genes contain or are flanked by known polymorphisms that are helpful for linkage studies. They consist of point mutations, variable number of tandem repeats (VNTR) or dinucleotide repeats (VNDR). The most common polymorphisms are shown in Table 2[62-65] (See also ref. #55, and references therein).

The SPTA1 and the SPTB genes

The SPTA1 gene[66,67] encodes a single isoform of spectrin α-chain in the red cells. RT-PCR may happen to pick up ultraminor spliceoforms. They have no biological relevance in the erythrocytes, but may prevail in other cell types. This remark apply to all genes and shall not be repeated. The SPTB gene[68,69] also leads to a single major isoform of spectrin α-chain in the red cells.

A few words ought to be said about the SPTA1 and SPTB gene products. The α- and β-chains of spectrin consist of the recurrence of 22 and 17 linear repeats of about 106 amino acids each. Except for the N-terminal part of spectrin α-chain, the other distal regions have a nonrepeat structure. In time, free α-and β-chains start binding to one another at two complementary nucleation sites (C-terminal and N-terminal portions, respectively). They then zip together to form an αβ dimer, completing the so-called side-to-side interaction. Both chains are antiparallel within a dimer. Subsequently, two dimers self-associate through complementary sites (N-terminal and C-terminal portions of the α- and β-chains, respectively) into a $\alpha_2\beta_2$ tetramer. So is achieved the head-to-head interaction.

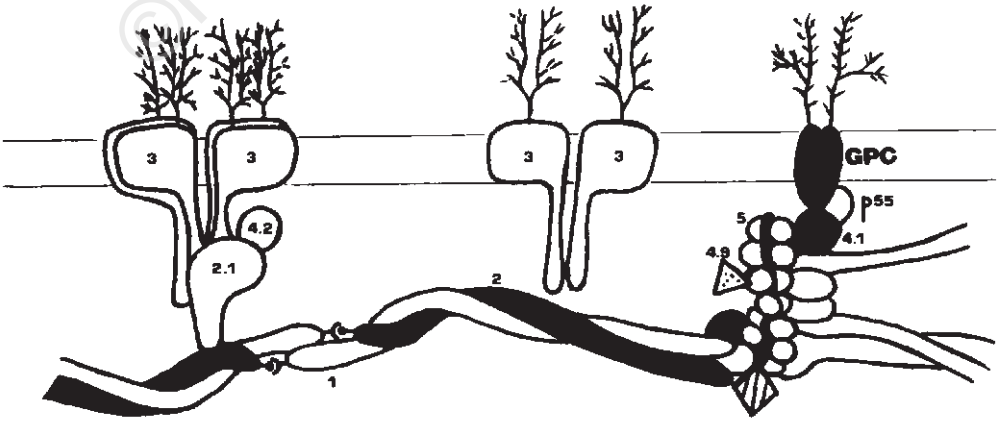

Figure 1. Schematic representation of the erythrocyte membrane architecture. The integral proteins are: band 3 and glycophorin C (GPC). The cytoskeleton consists mainly of: spectrin ($\alpha_2\beta_2$) tetramers, actin (5), protein 4.1, 4.9, 4.2 and ankyrin (2.1).

Table 1. Main features of the genes and the corresponding mRNAs involved in HS. Most of the relevant references are contained in a recent review article (Delaunay, 1995). Recent data on the ANK1 gene have been provided by Gallagher *et al.* (1997).

	GeneBank accession	*Gene symbol*	*Chromosome location*	*Gene size (kb)*	*Exon number*	*mRNA size (kb)*	*Coding sequences (amino acid number)*
Spectrin α-chain	M61887	SPTA1	1q22-q23	80	52	8.0	2429
Spectrin β-chain	J05500	SPTB	14q23-q24.2	>100	32*	7.5	2137
Ankyrin 1	X16609	ANK1	8p11.2	~160	42	9.0-7.2	1880
Protein AE1	M27819	EPB3	17q12-q21	17	20	4.7	911
Protein 4.2	M30646	ELB42	15q15-q21	20	13	2.4	691

**additional exons are spliced in within skeletal muscle (in the 3'-side of the coding sequence).*

Spectrin tetramers account for the operational form of spectrin *in situ*. Apart from the nucleation and self-association sites, numerous binding sites and specific domains mark out both dimeric halves of a tetramer. Starting from the middle (head-to-head interaction) and moving either way outbound, one finds the binding site for ankyrin, an *src* homology (SH3) domain, and, intermingled, the binding site for the actin-protein 4.1 complex, the occurrence of two EF-hands and of a plekstrin homology domain.

The SPTB gene is expressed in the skeletal muscle as well in the red cell. However, the protein product, though, is longer (+21.4kDa). This results from the use of four additional exons, numbered 33 to 36, in the 3' region of the gene.[70] Exons 33 to 36 share homologies with exons 29 to 32 (encoding the C-terminal part of spectrin β-chain in the red cell). Exon 33 stands 17 kb 3' of exon 32. In skeletal muscle, the use of these four additional exons stem from an alternative splicing in exon 32, that is supposedly triggered by muscle-specific trans-factors, and allows it to bypass the erythroid-specific stop codon. A subset of HS is due to premature stop codons which prevents spectrin β-chain to exist in the red cells.[71] One would expect the same fate for SPTB gene-encoded β-spectrin chain in muscle, and reasonably anticipate associated muscle disorders. Indeed, the mutations are clinically expressed in the heterozygous state in the red cell (HS), but not in the muscle. How the muscle gets around with only one haploid set of its SPTB gene-encoded spliceoform remains to be accounted for. Comparable and misunderstood paradoxes will be found in regard to other genes, but shall not be commented upon again.

The ANK1 gene

In contrast to the SPTA1 and SPTB genes, the ANK1 gene[72,73] gives rise to a profuse bundle of spliceoforms in the red cells, some of which encode detectable products and are biologically significant. The major protein species accounts for band 2.1. It is comprised of three distinct domains (N→C termini): the 89, 63 and 55 kDa domains. The 89 kDa domain harbors 22 repeats of about 33 amino acid each, and contains the binding site for the cytoplasmic domain of the protein AE1. The above repeats, termed repeats of the ankyrin type, are found in a variety of proteins whose function has little to do with ankyrin 1's (for review, see ref. #74). The 63 kDa domain carries the binding site for spectrin β-chain. The 55 kDa domain is said to be *regulatory*: it modulates the strength of the binding of ankyrin 1 to its partner proteins, yet in an ill-defined fashion.

The part of the transcript that encodes the 55 kDa domain is the target of intensive alternative splicing.[73] One relatively important spliceoform loses nearly the entirety of exon 38, of which only a tiny segment of the 3' region remains. The corresponding protein product is well seen on gels as band 2.2 (195 kDa). At least, eight spliceoforms of exons 40 to 42 exist in trace amounts. Minor spliceoforms lacking exons 38-39, 36 to 39 and 36 to 41 have been recorded. The physiological meaning of so many spliceoforms is unknown and their protein products are not visible on gels for most of them.

The nb/nb mouse appears with severe HS and, in a differed manner, with a cerebellum syndrome underlain by the degeneration of Purkinje cells. The causative mutation involves the ANK1 gene and yields a truncated form of ankyrin 1.[75] This is a spectacular example of how the alteration of a gene, initially known as to be expressed in the red cell only, also generates disorders in nonhematological cells.

The EPB3 gene

The EPB3 gene[76-79] is expressed as a single polypeptide isoform in the red cells, polypeptide AE1. It has three domains (N→C-termini): 1) the cytoplasmic domain (residues 1 to 403), mostly involved in the binding of hemoglobin and some cytosolic enzymes, and, as far as we are concerned, of ankyrin 1, protein 4.2 and possibly protein 4.1; 2) the membrane

Table 2. Some useful polymorphisms. Most of the relevant references are contained in a recent review article (Delaunay, 1995). Polymorphisms published or completed since then are indicated by letters in superscripts. The three mutations marked by an asterisk are in constant linkage disequilibrium and constitute the common allele α^{LELY} that is not relevant to SPTA1-related HS, in contrast with SPTA1-related hereditary elliptocytosis.

Genes	*Restriction sites, if any*	*Characteristics, when known*
***SPTA1* gene**		
αI domain	*Xba*I (0.36/0.33+0.03)	Intron 2; tc<u>t</u>aga/tc<u>c</u>aga[a]
	*Pvu*II (1.4+1.1/2.5)	Intron 2; c<u>a</u>gctg/c<u>g</u>gctg[a]
	*Msp*I (2.5/1.4+2.1)	Intron 2; cc<u>a</u>g/c<u>g</u>gg[a]
αII domain		CGC/CAC; R701H
		ATC/GTC; I809V
		ACA/AGA; T853R
		GAT/GCT; D970A[b]
αIII domain		AGA/ATA; R1333I[c]
αV domain		CTA/GTA; L1857V*
	*Ava*I (8.40/5.3+3.2)	Intron 45; 6714(-12c→t)*
		Intron 46; 6732(-12t→a)*
3'UT		GT repeats and VNTR
***SPTB* gene**	*Hind*III (17/14)	
	*Stu*I (8/4.8+3.2)	
	*Taq*I (3.5/2.7+0.8)[d]	
***ANK1* gene**	NcoI (3.8/3.6)	
3'UT		VNDR

***EPB3* gene:** included in Table 3.

[a]*Gallagher et al., in press;* [b]*Tse et al., 1997;* [c]*Glele-Kakai et al., 1996;* [d]*Beeton et al., 1995.*

domain (residues 404 to 882), consisting of 14 segments spanning the lipid bilayer and primarily involved in the Cl^-/HCO_3^- exchange as part of the respiratory process; 3) another cytoplasmic domain, yet very short (residues 883 to 911) and whose function is unknown.

In spite of the fact that polypeptide AE1 is unique, protein AE1 is heterogeneous due to a major glycan that is N-linked to residue asparaginyl 642. This glycan displays a great variety of composition from one AE1 molecule to the other. However, such a variability is under strict genetic control, based on the glycosyltransferase and glycosidase panel that is necessary to its synthesis. As a consequence, the distribution of ilycans is invariable (except in the perinatal period when a switch in the glycan structure takes place).

The EPB3 gene is expressed in the α-intercalar cells of the kidney distal tubule as well in the red cell. It contributes to the acid-base equilibrium of the blood. The kidney AE1 protein lacks the red cell's 66 first amino acids. This makes sense insofar as the kidney isoform has presumably less binding *duties* than its erythrocyte counterpart. The truncation of the AE1 protein stems from an alternative initiation of transcription, for there is a kidney-specific promoter in intron 3. The initiator methionine (number 66 in erythroid protein AE1) is located in exon 4.

The ELB42 gene

The ELB42 gene[80,81] gives rise to two one major and two minor spliceoforms in the red cell. The major spliceoform, yielding a protein 4.2 isoform of 72kDa, results from the loss of 90 out of exon 1 96 bases. Initiator methionine being itself removed cotranslationally, leaves only glycine and glutamine remaining from exon 1-encoded amino acids. The 72 kDa isoform of protein 4.2 is cotranslationally N-myristoylated[82] and post-translationally palmitoylated.[83] One minor spliceoform (74 kDa) retains exon 1. Another minor spliceoform (68 kDa), much less represented, loses exon 3 in addition to the largest part of exon 1, as above-decribed. No homologue for the ELB42 gene has been definitely found in the genome, however the odds for such a homologue remain open. The ELB42 gene is expressed in a variety of cell types.

Protein defects

Mutations responsible for hereditary spherocytosis (HS) lie in a variety of genes encoding transmembrane proteins (i.e., band 3), membrane skeletal proteins (i.e. β- and α-spectrin) and proteins mediating the attachment of the latter to the former (i.e., protein 4.2 and ankyrin). Analysis of erythrocyte membrane proteins by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) remains an unmatched element of orientation toward the primarily mutated protein (and gene).[35] In order to analyze both high molecular weight proteins (i.e.; spectrin and ankyrin) and lower molecular weight proteins (i.e. band 3 and protein 4.2), two different electrophoretic systems have to be performed. They are the continuous buffer system of Fairbanks[18] with exponential gradient of acrylamide from 3.5% to 17% and the discontinuous buffer system of Laemmli[17] with acrylamide linear gradient from 5% to 15%. Although direct quantitation of red cell membrane proteins by radioimmunoassay has been recently proposed as a more accurate technique than SDS-PAGE,[84] considering his its particular complexity, it should be performed in selected cases only. Moreover, polyacrylamide gel electrophoresis also allows the identification of abnormally migrating protein (truncated or elongated peptides) which may be further identified by Western blotting.

By studying SDS-PAGE membrane proteins in HS patients, American and European groups detected an abnormality in more than 80% of cases.[39, 85-87] Four different protein deficiencies were described. They were combined spectrin and ankyrin deficiency, isolated spectrin reduction, band 3 reduction and

defects of protein 4.2. Rate of hemolysis has been related to the spectrin content of erythrocytes.[38] This variety of the biochemical phenotypes reflects the wide heterogeneity of the underlying molecular alterations. When a combined spectrin and ankyrin deficiency is detected, ANK1 gene should be investigated, whereas α- or β-spectrin genes are generally implicated in HS with isolated spectrin deficiency. AE1 gene is usually screened in the patients showing band 3 reduction.

Most HS patients have combined ankyrin and spectrin deficiency.[85] Because spectrin deficiency is usually secondary to a primary reduction of ankyrin these patients, differing from subjects with isolated spectrin deficiency, very likely have mutations of ANK1 gene. For this reason a major tool is to discriminate between these two biochemical alterations. Human erythrocyte ankyrin is a mixture of different size proteins. The major form (protein 2.1) is a 206-kd protein. Other faster migrating ankyrin species, designated proteins 2.2 (195-kd), 2.3 (170-kd) and 2.6 (145-kd) appear as faint bands on stained gels. Band 2.3 and 2.6 are thought to stem from band 2.1 through proteolysis. The conversion is incomplete in reticulocytes.[88] Thus protein 2.1 concentration in the membrane is partially affected by the number of circulating reticulocytes. Because protein 2.1 is the only ankyrin isoform commonly evaluated with SDS-PAGE, many unsplenectomized HS patients with ankyrin mutations could misleadingly show normal ankyrin levels. To avoid subsequent mistakes approaching the mutated gene we calculated the extent of the distortion of band 2.1 and its derivatives due to high reticulocyte count. The higher the reticulocyte count, the more the ankyrin level appeared to be increased. A simple and practical equation was elaborated to obtain the real ankyrin level even in HS patients with a high number of reticulocytes.[89]

Molecular defects

Defects of the ANK 1 gene

Ankyrin is an 1880-amino acid and globular protein, divided in three functional domains in the N→C terminal direction.[72,90] The 89 kD domain contains 24 homologous repeats of about 33 amino acids including the binding subsites for the cytoplasmic domain of band 3.[88] The central 62 kD domain contains the binding domain for spectrin β-chain. Finally, the 55 kD domain can modulate the affinity for band 3, depending on the amino acids sequences retained or eliminated following complex alternative splicing of the ANK1 trancript 3'-region.[91,92]

More than two-thirds of HS patients have a combined spectrin and ankyrin deficiency.[85,93] As established by studies of spectrin and ankyrin synthesis in erythroblasts, when ankyrin is deficient spectrin in excess, unable to assemble in the membrane due to the loss of ankyrin attachment sites, is degraded.[94] For this reason frequently in patients showing reduction of both proteins spectrin deficiency is secondary to a primary reduction of ankyrin.

Using variations in the number of AC dinucleotides in the 3' untranslated region of ankyrin mRNA as a marker, Jarolim *et al.*[12] showed that about 20% of patients with dominant HS had no detectable mRNA from one ankyrin allele, even though both alleles were present in genomic DNA. The likely explanation was the synthesis of an instable mRNA, which often occurs in frameshift or nonsense mutations, or a defect in the ankyrin promoter. More recently, Eber, by screening by single strand conformational polymorphism (SSCP) all 42 coding exons of ankyrin gene plus the 5' untranslated/promoter region in 46 HS families demonstrated that ankyrin mutations are a major cause of dominant and recessive HS (from 35% to 65%, taking into account that the SSCP method recognizes between 40% and 75% of single base changes in DNA).[27] Basically, dominant inherited cases were associated with frameshift or nonsense mutations. Recessive inherited cases were associated with changes including single amino acid substitutions or a single base substitution in the promoter region. However, it must to be understood that a great proportion of the missense mutations as well as those located in the promoter region have to be cautiously considered since some of them may just be rare polymorphisms. In fact, structural and functional consequences of most amino acid substitutions as well as the rate of transcription of the ankyrin alleles with the promoter mutations were not tested.[27]

Recently, 5 unrelated HS children with combined ankyrin and spectrin deficiency and an apparently recessive inheritance pattern on a clinical and hematological basis have been reported.[13,16,95] These patients showed different deletional frameshift mutations affecting mRNA stability. None of the parents carried these out-of-frame deletions strongly suggesting the presence of *de novo* mutations.

Around 25% of HS patients' have both parents are clinically and hematologically normal. They probably could have an autosomal recessive form of the disease, even if it is not possible to exclude a dominant HS resulting from *de novo* mutation. For this reason they constitute an important and unsolved problem during genetic counseling. In order to evaluate the frequency of *de novo* ankyrin mutations we studied 33 children with HS due to spectrin and ankyrin deficiency and with normal parents.[14] The distribution of the dinucleotide repeat (AC)n polymorphisms was evaluated by PCR-amplification of the ANK1 gene appropriate segment. Four different sizes of the PCR products were observed, corresponding to the A1, A2, A3 and A4 alleles, characterized by 14, 13, 12 and 11 AC repeats, respectively. Nineteen children were heterozygous for the AC repeat lengths and therefore we were able to observe two distinct ankyrin alleles. Remarkably, however, in 12 of them, we found a PCR product detect-

able for only one ankyrin allele in the cDNA. This discrepancy strongly suggested the absence of one of the two ankyrin mRNAs. We inferred the occurrence of *de novo* mutations inactivating the ANK1 gene in about 60% of HS patients with combined spectrin and ankyrin reduction. It is not well understood why some genes, such as ANKI gene are apparently so prone to *de novo* mutations. Why *de novo* changes are usually small deletions is also puzzling. However most of these mutations occur in exons with a high G+C content and may result from slipped mispairing during DNA replication.[95]

Surprisingly, although many patients with combined ankyrin and spectrin deficiency had the same molecular defect, i.e. the inactivation of one ankyrin allele, they unexpectedly showed heterogeneous clinical features as well as different ankyrin levels. This phenomenon could be explained by a variable compensation of the normal ANK1 allele occurring *in trans*.[27]

Defects of the EPB3 gene

Now, we will consider the defects of the EPB 3 gene, encoding protein AE1. We have mentioned that the EPB 3 gene is expressed in both in the red cells and in intercalar α-cells of the renal distal tubule. Its mutations yield hematological manifestations, that is, HS, and nonhematological manifestations, namely a variety of distal renal tubule acidosis (DRTA).

EPB3 gene mutations expressed in the red cell

Two subsets of HS are associated with EPB3 gene mutations: 1) the AE1(–) HS, in which one EPB3 allele yields no protein AE1; and 2) the 4.2(–) HS. Since the lack of protein 4.2 may also result from mutations in the ELB42 gene, we must be more explicit here, and using the terminology of *4.2(–) HS due to a EPB3 gene mutation*, in contrast to *4.2(–) HS due to a ELB42 gene mutation* which will be treated elsewhere in this review. Both kinds of mutations are provided in Table 3;[11, 96-103](for review, see ref. #104 and references therein).

AE1(–) HS

This is the second most common subset of HS (far behind the HS subset associated with ANK1 gene mutations and close to that related to SPTB gene mutations). It accounts for approximately 20% of all HS cases. One haploid stock of protein AE1 is not synthesized or, if synthesized, cannot be inserted into the lipid bilayer. The clinical picture is usually that of a mild, well compensated hyperhemolysis. Splenectomy, when performed, comes about rather late, possibly in the same time as cholecystectomy. Blood smears exhibit spherocytes and, specifically yet rarely (one in 50-100 red cells), mushroom-shaped cells, also named pincered red cells.[36] SDS-PAGE shows that 20 to 30% of protein AE1 is missing in the membrane (the wild type EPB3 allele *in trans* provides only partial compensation). This deficiency is accompanied by a roughly proportional deficiency in protein 4.2, in accord with the fact that protein AE1 represents the major anchoring points to protein 4.2. The mutations are expressed in the heterozygous state, and the inheritance pattern is dominant.

The genetic changes causing AE1(–) HS may be of two major kinds (Table 3):

1) the first are nonsense mutations, laying all over the coding sequence of the protein AE1 mRNA. They prematurely interrupt the synthesis of protein AE1. Protein AE1 Prague was the first reported example.[105] Truncated molecules are degraded. Nonsense mutations next to the physiological stop codon could, at least theoretically, give rise to partially functional molecules, but no variant of that sort has ever been reported. Quite often, the presence of a premature nonsense mutation is accompanied by a reduction of the corresponding mRNA;

2) the second mutations are point mutations, or deletions or insertions of variable sizes, which do not disrupt the reading frame whatsoever. They involve positions conserved not only through the species but also among the other AE proteins (AE2 and AE3). These positions are critical for the incorporation of protein AE1 into the lipid bilayer. Of note, the change of several arginyl residues is critical in that respect.[106] Other types of mutations can be encountered. Most EPB3 alleles responsible for HS fail to yield DRTA in the heterozygous state, except for a few cases: protein AE1 Campinas,[99] and protein AE1 Pribram[97] (Table 3).

Within a given family, i.e. for a given mutation, the clinical presentation is fairly constant. This statement must pondered, however, by the fact that some members may be sicker than the other affected members on the average. The present issue has been addressed by Alloisio *et al.*[10,11] and Kanzaki *et al.*[103] Aggravation results from the compound heterozygosity for a common AE(–) EPB3 allele and a weak allele that is not clinically expressed in the simple heterozygous state. The corresponding mutations have been found so far as missense mutations in the cytoplasmic domain of the AE1 protein. Once, the change was a single base substitution in the 5' untranslated region of mRNA.[10]

In stark contrast with a common belief, homozygosity for an EPB3 alleles causing HS is not necessarily lethal. A natural strain of cattle showed the ability to survive to a nonsense mutation in the bovine gene encoding protein AE1 in the homozygous state.[107] Two strains of mouse with an artificially inactivated gene encoding protein AE1 could also survive, and a few of them reached adulthood.[54,108] In all cases a severe HS was present. The total absence of protein AE1 causes a fullfledged DRTA. In humans, homozygosity for allele EPB3 Coimbra (Table 3) led to fatal hydrops fetalis. Nonetheless, appropriate obstetrical measures and a heavy transfusional regimen could compensate for severe HS, but did not prevent the development of DRTA (Tamagnini *et al.*, unpublished data).

Table 3. Main mutations associated with hereditary spherocytosis, and polymorphisms of the *EPB3* gene (August 31, 1997).

Names	*Change*	*Exon*	*Intron*	*Consequences*	*AE(–)HS: + 4.2(–)HS: •*	*Domains*
Genas	89G→A	1		Decreased synthesis		3' UT
*Pst*I			3			Cytoplasmic
'DA38A'[a]	227A→C	4		GAC→GCC; D38A		
Montefiore	232G→A	4		GAG→AAG; E40K	•	
Foggia[a]	275delC	4		Frameshift	+	
Hodonin (Prague IV)[a]	357G→A	5		TGG→TGA; W81X	+	
Memphis I	*280A→G*			*AAG→GAG; K56E*		
Bohain[b]	355delT	5		Frameshift	+	
Napoli I[a]	411insT	5		Frameshift		
Nachod (Hradec Kralove II)[c]	464(-3c→a)	5		Inframe deletion		
Fukuoka[d,e]	502G→A	6		GGA→AGA; G150R	•	
Mondego[f]	553C→T	6		CCT→TCT; P147S		
Lyon-Osnabrück	562C→T	6		CGA→TGA; R150X	+	
Worcester[c]	624insG	7		Frameshift	+	
Campinas[g]	809(-1g→t)	8		Altered splicing	+	
Princeton[c]	932insC	9		Frameshift	+	
Boston[c]	938(C→A)	9		GCT→GAT : A285T	+	
Noirterre[h]	1102(C→T)	10		CAG→TAG; Q330X	+	
Tuscaloosa	1094C→G	10		CCC→CGC; P327R	•	
Brüggen	1369delC	11		Frameshift	+	
						Membrane
Bicêtre II[b]	1475delG	12		Frameshift	+	
Benesof (Prague V)[c]	1478G→A	12		GGG→GAG; G455E	+	TM2
Pribram (Prague VI)[c]	1546(-1g→a)		12	Altered splicing	+	
Coimbra[f]	1576G→A	13		GTG→ATG; V488M	+	OL2
Biçêtre I[b]	1582C→T	13		CGC→TGC; R490C	+	OL2
Evry[b]	1600delT	13		Frameshift	+	
Milano[i]	1608ins69	13		Inframe insertion	+	TM4
Dresden	1666C→T	13		CGC→TGC; R518C	+	TM4
Smichof (Prague VII)[c]	1962delC	15		Frameshift	+	
Trutnov[c]	1998C→A	15		TAC→TAA; Y628X	+	OL4
Hobbart[c]	2051delG	16		Frameshift	+	
Osnabrück II	2101del3	16		DM663	+	TM8
Prague VIII[c]	2234T→C	17		CTG→CCG; L707P	+	TM9
Okinawa[e]	2254G→A	17		GGG→AGG; G714R	+	TM9
Hradec	2392C→T	17		CGG→TGG; R760W	+	IL5
Prague II	2393G→A	17		CGG→CAG; R760Q	+	IL5
Chur	2426G→A	18		GGC→GAC; G771D	+	TM11
Napoli II[a]	2462T→A	18		ATC→AAC; I783N	+	OL6
Jablonec	2536C→T	18		CGC→TGC; R808C	+	IL6
Prague I	2578ins10	18		Frameshift	+	
Birmingham[c]	2615A→C	19		CAC→CCC; H834P	+	IL6
Philadelphia[b,c]	2624C→T	19		ACG→ATG; T837M	+	IL6
Memphis II	*2675C→T*	*19*		*CCG→CTG; P854L*		*TM13*
Prague III	2722C→T	19		CGG→TGG; R870W	+	TM1

[a]Miraglia del Giudice et al., 1997; [b]Dhermy et al., 1997; [c]Jarolim et al., 1996; [d]Inoue et al., 1996; [e]Kanzaki et al., in press; [f]Alloisio et al., 1997; [g]Lima et al 1996; [h]Jenkins et al., 1996; [i]Bianchi et al., 1997.

Note. *The nucleotide numbering by Lux et al., 1989 is used. The nucleotide numbering by Tanner et al., 1988 is used for protein AE1 Genas. The references of many mutations are contained in a recent review (Delaunay et al., 1996). Mutations described or renamed since then are indicated by letters in superscripts after the names (or the second names) of the mutationts and are explicited at the bottom of the table. Changes in Italic characters correspond to more or less frequent polymorphisms. Of note, the Memphis II polymorphism is always associated in* cis *with the Memphis I polymorphism. Protein AE1 Mondego carries mutation Montefiore (E40K). AE1 (–) HS(+) is nearly always found in the heterozygous state. 4.2 (HS) (•) is, tentatively, always found in the homozygous state. TM: transmembrane segment.*
OL: outer loop; IL: inner loop.

4.2(–) HS due to mutations of the EPB3 gene

4.2(–) HS due to mutations of the EPB3 gene is ill-documend at the clinical and molecular levels, contrasting with 4.2(–) HS due to mutations of the ELB42 gene. We would only tentatively state that the inheritance pattern is strictly recessive. Very sensitive measurements evidence a minimal decrease of the AE1 protein (≤ 10%) The mutations are located in this part of the EPB3 gene that encodes the cytoplasmic domain of protein AE1: proteins AE1 Tuscaloosa,[24] Montefiore[109] and Fukuoka[96] (Table 3). 4.2(–) HS due to mutatons of the EPB3 gene were, assumedly, encountered in the homozygous state, but there is some doubt about protein AE1 Tuscaloosa. These mutations would disrupt the binding site of protein AE1 for protein 4.2. It is not sure whether the binding capacity is always entirely or sometimes partially (proteins AE1 Fukuoka) abolished. The scattering of the mutations over the cytoplasmic domain suggests that the binding site of protein AE1 for protein 4.2 is split into several subsites. To our knowledge, this issue has not been addressed in a definitive manner at this time.

EPB3 gene mutations expressed in the α-intercalary cells of the renal distal tubule

Some EPB3 gene mutations expressed in the α-intercalary cells of the renal distal tubule cause a rare genetic disorder of distal renal tubule acidosis (DRTA). The inheritance pattern of this subset of DRTA is dominant and so far has only been observed in the heterozygous state. The condition results in the inability of the kidney to acidify urine. Blood acidosis develops. Nephrocalcinosis and renal failure may ensue. Nevertheless, a lifelong daily supplement of sodium bicarbonate proves to be an efficient treatment. Tanner *et al.* have reported several mutations responsible for DRTA, which are not yet published. One of them occupies position 589 (R589H),[110] in the third cytoplasmic loop, next to K590 that is critical for the transport of anions.

One will naturally wonder what the expression of the above mutation (and comparable ones) in the red cell may be. Surprisingly enough, there are no clinical signs. It looks as if the domains of protein AE1 were functionally compartmentalized. The cytoplasmic domain fulfills mechanical tasks. The membrane domain ensures proper incorporation into the bilayer, of course, and achieves transport functions. It is significant, though, that the anion transport across the red cell membrane appears to be diminished by about 20% based on *in vitro* tests.

Backing up to AE1(–) HS, it is intriguing that those cases in which one haploid set of protein AE1 is missing do not engender DRTA as a rule in the heterozygous state. Taking the reasoning that a mutated haploid set can hardly be worse than the mere absence of the same set, it is not understood how α-intercalary cells get around this situation. Incidentally, the Southeast Asian ovalocytosis (SAO) provides an even more striking paradox. The 27 nucleotide deletion, which characterizes gene EPB3 SAO gene, removes amino acids 400 to 408 at the very junction of the cytoplasmic and the membrane domains. SAO is dominantly transmitted and has been observed in the heterozygous state only. This genetic change is functionally dual since it yields dramatic mechanical effects (at least *in vitro*) along with the complete inability to mediate anion transport. For so much, it yields no plasma acidosis. At first glance, the EPB3 mutations that yield hereditary anemia usually fail to engender DRTA, and *vice versa*. More detailed investigation will show to which degree this sort of mutual exclusion stands.

Defects of the ELB42 gene

Band 4.2 is a major component of the erythrocyte membrane and constitutes approximately 5% of total membrane proteins. This protein should be synthesized in the erythroblasts and then incorporated into the membranes by binding to the cytoplasmic domain of band 3.[111] Therefore, in determining the pathogenesis of band 4.2 deficiencies should be considered either an abnormality in band 4.2 itself or an abnormal band 3 for binding with band 4.2. Partial deficiency of band 4.2 with band 3 cytoplasmic domain abnormalities have been reported (B3 Montefiore, Tuscaloosa, Fukuoka). Few mutations of ELB42 gene have also been described and the protein appears absent by SDS-PAGE analysis. All reported cases of ELB42 gene mutations are homozygotes with clinically silent parents, suggesting autosomal recessive inheritance, and with few exceptions are typically of Japanese ascent. Various descriptions have been used to characterize the phenotypes of these reports, including hereditary spherocytosis, elliptocytosis and ovalostomatocytosis and some reports only refer to hemolytic anemia with no further characterization. At variance with a classical HS the absence of protein 4.2 generates rare typical microspherocytes. Mild elliptocytosis and ovalostomatocytosis are often present and the mean corpuscular hemoglobin concentration (MCHC) appears normal or only minimally elevated.[112]

A band 4.2 mutation (protein 4.2^{NIPPON}: GCT→ACT: Ala→Thr at codon 142) appears to be common in the Japanese population with a frequency around three percent. Recently, we found this mutation in an Italian subject, too (unpublished data). This allele is associated in the homozygous state to atypical HS with ovalostomatocytosis and uncompensated hemolysis which respond to splenectomy.[23] A similar hematological situation has been found in other protein 4.2 mutant (protein 4.2^{KOMATSU}, GAT→TAT: Asp→Tyr at codon 175).[113] Otherwise, in other reports, patients who are compound heterozygous for protein 4.2^{NIPPON} and another mutation (protein 4.2^{NOTAME}, G→A substitution at position +1 of intron 6, resulting in skipping of exon 6 and pre-

mature termination;[114] protein 4.2^{FUKUOKA}, Trp→Stop at codon 119;[115] protein 4.2^{SHIGA}, CGC→TGC: Arg→Cys at codon 317)[116] display a typical HS with spherocytes and increased osmotic fragility.

A distinct band 4.2 deficiency in Tunisian siblings has also been reported (protein 4.2^{TOZEUR}, CGA →CAA: Arg→Gln at codon 310). The proband suffered from hemolytic anemia which disappeared after splenectomy. The red cell morphology, ektacytometric analysis and pink test were normal.[117]

Another mutant protein 4.2 (protein 4.2^{LISBOA}, deletion of nucleotide 264 or 265 resulting in frameshift and premature termination), leading to a complete absence of protein 4.2, also appears to be associated with a typical HS.[118]

Defects of the SPTB and SPTA1 genes

Spectrin is the principal structural component of the erythrocyte membrane skeleton and its deficiency is the first biochemical defect recognized in patients with HS.[38] Furthermore, the degree of deficiency is reported to correlate with the severity of the hemolysis.[22] Mutations of a spectrin have been associated with recessive forms of HS while mutations of β spectrin have been described in families with autosomal dominant forms of the disease. This is most likely due to the fact that α spectrin is synthesized in more than twice the amount of β spectrin during maturation of mammalian erythrocytes with the unassembled α chains undergoing degradation[119] (see above).

The first reported α spectrin mutation is a nucleotide base substitution that causes the aminoacid replacement responsible for the variant α-spectrin chain, designated αIIa in preliminary reports (970 GCT→GAC: Ala→Asp).[120,121] Recently, this variant that is frequently coinherited with severe non-dominant HS and isolated spectrin deficiency, is renamed α spectrin Bughill or α^{BH}.[65] It now appears that this α^{BH} is probably not itself the cause of non-dominant HS, but represents a linked polymorphism. In fact, Witchterle *et al.* have recently described a low expression α spectrin variant designated α spectrin^{LEPRA} (Low Expression Prague) in a patient with a severe non dominant HS.[28] This α spectrin variant is associated with abnormal alternative splicing of α spectrin mRNA and decreased synthesis. The α^{BH} polymorphism is linked to the ^{LEPRA} mutation in a number of cases. Given the reported frequency of this polymorphism, one has a clue about the incidence of α spectrin deficient recessive HS.[65] This HS phenotype should be associated with either α spectrin^{LEPRA} homozygotes or compound heterozygotes with another α spectrin mutation *in trans*, such as α spectrin ^{PRAGUE}.[28] α spectrin^{LEPRA} homozygous HS subjects are clinically less severe than heterozygotes with another unknown α spectrin mutation. The hypothesis is that α spectrin^{LEPRA} represents only a partial inactivation of the mutant allele while the unknown mutations most likely result in nearly complete allelic inactivation leading to more pronounced spectrin deficiency and, consequently, to a more severe clinical phenotype.[122]

Several mutations involving the β spectrin gene have been described. They are responsible for a phenotype of mild to moderate autosomal dominant form of HS associated with a conspicuous spherocytosis with frequent spiculated cells (8% to 15% acanthocytes).[36] β spectrin Kissimmee represents the first β spectrin point mutation demonstrated in HS.[25] This *de novo* mutation results in the replacement of tryptophan by arginine in the N-terminal region of β spectrin leading to a defective binding of spectrin to protein 4.1. Based on bacterial expression of β spectrin peptides, it was presumed that the spectrin deficiency was the consequence of an increased protein degradation *in vivo*, as mutant peptides were unstable and highly susceptible to oxidative injury and proteolytic degradation.

A β spectrin variant, i.e. spectrin Durham, characterized by a truncated β chain and associated with HS, has also been reported.[26] It is due to an inframe genomic deletion of exons 22 and 23 that represents, in the protein, most of repeated segment 12 and part of repeated segment 13. It was shown that, despite its normal synthesis and stability, the abnormal β spectrin is inadequately incorporated into the membrane of erythroblasts. This inefficient incorporation results from conformational changes of the β spectrin subunit affecting the binding of the abnormal heterodimer to ankyrin, located in proximity of the deletion.

Another mutant β spectrin, i.e. spectrin Wiston-Salem, characterized by a truncated β chain and associated with HS and overall spectrin deficiency, has been extensively studied.[123] The truncation results from a single point mutation at position +1 (G→A) of the donor consensus splice site of intron 17, leading to an aberrant β spectrin transcriptional message which lacks exons 16 and 17. It was also shown that the mutant protein is synthesized in decreased amounts in the cytoplasm of erythroid progenitor cells and appears to be susceptible to proteolytic degradation.

Several other frameshift, nonsense and missense mutations of the β spectrin gene have been disclosed in patients with dominant HS associated with spectrin deficiency, using SSCP analysis of genomic DNA.[124]

In conclusion, HS patients with isolated spectrin reduction and normal parents have two different patterns of inheritance, i.e., genuinely recessive (homozygosity or double heterozygosity for a synthetically deficient α-spectrin allele) and apparently recessive (*de novo* monoallelic expression of β spectrin gene).

A proposal for a diagnostic flow chart

A remarkable effort has been produced in the last decade to clarify molecular basis of HS. As reported above, widely distributed mutations effecting the genes of spectrin, ankyrin, band 3 and protein 4.2 have been in turn uncovered. These data has been useful to better understand red cell membrane skeleton organization and consequently pathophysiology of HS. From a clinical perspective, however, only in

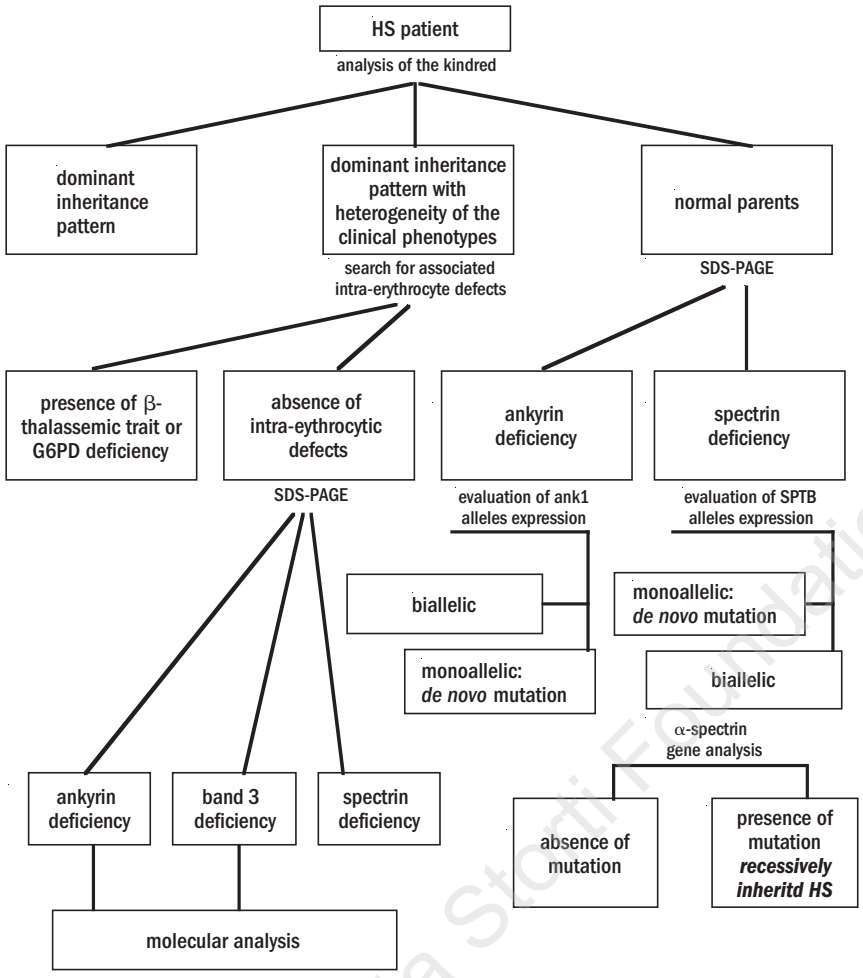

Figure 2. Diagnostic flow chart of HS. This is a proposal of diagnostic walk from the clinical data to the identification of molecular defects.

selected cases could biochemical and molecular analysis have a considerable impact.

HS patients with an appropriate clinical history in the kindred usually do not request further investigations. The identification of the erythrocyte membrane biochemical alterations and eventually of the underlying unlikely mutations may improve clinical management of these patients. On the contrary, dominantly inherited HS patients showing a wide heterogeneity of the clinical phenotypes among the affected members of the same kindred and HS subjects with clinically and hematologically normal parents represent two subsets of patients which molecular studies may benefit.

Clinical differences in the same HS kindred may be explained by the coinheritance in some members of another erythroid defect (e. g., β-thalassemic trait)[125,126] able to affect HS phenotype. When the presence of a superimposed erythrocytic defect is not found, biochemical analysis of membrane protein should be performed. If different degrees of protein deficiency are shown, molecular analysis has to be done to identify high or low expression alleles eventually occurring *in trans* to the HS allele (see above)(Figure 2). Molecular study is also recommended in HS patients with normal parents. They could have a genuinely recessive pattern of inheritance (i. e., homozygosity or double heterozygosity for an α-spectrin mutation) or an apparently recessive one due to *de novo* mutational events affecting ankyrin or β-spectrin genes (see above). To discriminate between these possibilities SDS-PAGE followed by the evaluation of ankyrin or β-spectrin gene expression, as reported in different paragraphs of this review, can be performed. 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 find mutations (i. e., spectrin α^{LEPRA}) responsible for a recessive pattern of inheritance (Figure 2).

Therapeutical approach

Spherocytes as a consequence of the surface area loss are very poor deformable cells. This means that they are selectively trapped in the spleen and destroyed. Splenectomy has been shown to abrogate or markedly decrease hemolysis.

The role of the spleen in the body's defence against infection is complex. One of the major is mechanical filtration; the spleen also serves as a major site of antigen presentation, lymphocyte activation, and antibody production. Finally, opsonins are produced in the spleen. Since the onset of this century it was shown that splenectomy increases the risk of infections and post-splenectomy sepsis was several fold described in children splenectomized for hereditary spherocytosis. For these reasons it has been proposed to post-poned splenectomy until the age of 5 to 9 years.[127]

One of the first question is why to splenectomize. Becker and Lux recommend splenectomy for all HS patients with either anemia or significant hemolysis (reticulocyte count repeatedly greater than 5%) or a strong family history of gallbladder disease. In patients with mild compensated hemolysis the presence of gallstones requiring cholecystectomy suggests splenectomy to minimize the recurrence of common duct stones.[84] Recently a combined laparoscopic cholecystectomy and splenectomy has been proposed.[128] Tchernia *et al.* proposed a partial splenectomy to decrease the hemolytic rate maintaining the phagocytic function.[129]

Estimates of the incidence of post-splenectomy sepsis vary.[130,131] Multistudy reviews involving large numbers of patients have found overall incidence rates for sepsis among all splenectomized patients ranging from 3.8 to 4.3% with overall mortality from sepsis ranging from 1.7 to 2.4%. In the Italian HS registry splenectomy was performed in 180 out of 460 patients; of these splenectomized patients only one developed a severe sepsis but no death occurred.[31]

OPSI was once thought to be a complication in patients with underlying hematologic disease; however, cases of OPSI have also been reported in previously healthy patients who had undergone splenectomy for trauma. It follows a characteristic course of fulminant infection preceded by a prodrome of non specific symptoms.[132] The mortality ranges from 50% to 70%. The causative agent more frequent is *S. pneumoniae*, present in about 50% of cases; other organisms include the encapsulated organisms *H. influentiae* and *Neisseria meningitidis*. However, group B Streptococcus, Staphylococcus, Salmonella, *E. coli*, *P. aeruginosa* and *Listeria monocytogenes* have also been mentioned in selected reports.[133]

Although the risk for the splenectomized patient of contracting an overwhelming infection is low, it is elevated in relation to the population at large. Thus, pneumococcal vaccine should be administered to all patients undergoing elective splenectomy, and preferably this should be done 2 weeks before the splenectomy. The current 23-valent vaccine covers 80% of the pneumococcal serotypes most commonly responsible for postsplenectomy infection.[134]

The need for booster vaccination is unclear, and suggested intervals for revaccination range from 3 to 10 years.[135] *The Centers for Disease Control and Prevention Advisory Committee on Immunization Practices* (ACIP) recommends revaccination after 3 to 5 years in asplenic children who would be less than 10 years old at the time of revaccination. For asplenic adults, the ACIP recommends revaccination approximately 6 years after the first dose. The antibody response to pneumococcal vaccine in young children is poor, and use of this vaccine before the age of 2 is in question.

Vaccination against *H. influenzae* type b and *N. meningitis* should also be offered to all asplenic patients.

Antibiotic prophylaxis

Given the high morbidity and mortality associated with such infection, antibiotics should be considered the first choice drug. However, use of antibiotics for continuous long-term prophylaxis is controversial. Reservations include questions about patient compliance, issues related to penicillin-resistant pneumococci, and the need for chemoprophylaxis in patients receiving pneumococcal vaccine. Some investigators recommend to the use of antimicrobial prophylaxis in the early post-splenectomy period and in children under 5 years of age, given the increased incidence and mortality of post-splenectomy sepsis in these circumstances. Lux and Palek advocate prophylactic antibiotics for at least for the first 5 years after surgery and preferably for life. Penicillin V 250 mg taken orally twice daily or amoxicillin 250 mg taken orally once daily[135] can be used for prophylaxis, with the choice based on individual patient characteristics. Erythromycin is recommended for penicillin-allergic patients. Ceftriaxone, which is active against penicillin-resistant pneumococci, can be added as part of the initial empiric regimen to cover the meningeal complications of pneumococcal sepsis, given the lesser concentrations of penicillin in the cerebrospinal fluid.

Several authors have suggested that self-administration of oral penicillin be considered in any splenectomized individual at the first sign of febrile illness.[136] Studies supporting a preemptive course of oral penicillin have not been performed. However, it may be a rational consideration for selected patients with splenectomy, since hypotension and disseminated intravascular coagulation with OPSI have occurred by the time that many patients seek medical attention. Regardless, splenectomized patients should be educated about their increased risk for infection and encouraged to seek prompt medical attention at the onset of any febrile illness.

Anemia appears to be a major problem during infancy; in our survey in fact, of 247 transfused subjects 154 received blood during the first year of life and 48 of these during the second year.[31] Some infants become progressively more anemic during the first months of life and require transfusion. This usually occurs because the marrow response to anemia is more sluggish than normal. Fortunately, the problem is transient in the majority of patients and usually remits after one or two transfusions. Subsequently,

the course of the disease depends on the equilibrium established between the rates of red-cell production and destruction.

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