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MOLECULAR HETEROGENEITY OF HEREDITARY ELLIPTOCYTOSIS IN ITALY

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ABSTRACT

Background. **Common HE is the most prevalent clinical form of hereditary elliptocytosis; its clinical findings vary considerably, ranging from an asymptomatic carrier state to a severe, even life-threatening hemolytic disorder. Structural modification and reduction of 4.1 protein, or abnormalities at the spectrin self-association site could lead to elliptocytes.**

Methods. **Sixty-one Italian HE patients belonging to 28 families were studied. Analysis of red blood cell cytoskeleton was performed by means of SDS-PAGE, and spectrin dimer percentage was assessed by non denaturing polyacrylamide gel electrophoresis. Limited tryptic digestion of spectrin was employed in patients showing an abnormal dimer increase, and the amount of abnormal α I peptide was estimated. Molecular defects were detected by means of PCR of α and β spectrin genes and direct sequencing of genomic DNA.**

Results. **We found a very heterogeneous spectrum of cytoskeletal alterations: 18 (29%) subjects showed partial protein 4.1 deficiency, whereas 31 (51%) displayed an increased amount of spectrin dimers; we were not able to detect any alteration in 12 (20%) HE patients. Patients enrolled in this study were widely distributed throughout Italy.**

Conclusions. **The subgroup of HE patients related to 4.1 deficiency is homogeneously asymptomatic, whereas forms due to disruption of the spectrin tetramerization site are very heterogeneous, and clinical severity appears to be related to spectrin dimers and especially to spectrin content. These two parameters in turn are related to the presence of a low expression α allele in trans and to the degree of disruption of head-to-head contact between α and β chains.**

Key words: anemia, elliptocytosis, spectrin, cytoskeleton, membrane

Hereditary elliptocytosis (HE) is a heterogeneous disorder characterized by the presence of a large number of elliptically-shaped red cells.[1] This condition has been observed in all racial groups, with a prevalence ranging from 1 to 0.25 per thousand with the exception of some Central West Africa regions where a much higher prevalence was shown.[2] Common HE is the most prevalent clinical form: its clinical findings vary considerably, ranging from an asymptomatic carrier state to a severe, even life-threatening hemolytic disorder.[3]

Red cell shape is under the control of a network of proteins on the inner surface of the red cell membrane. The major components of this complex cytoskeleton include α and β spectrin, ankyrin, protein 4.1 and actin, which can be separated by SDS-PAGE and revealed by Coomassie blue stain.[4] Spectrin chains (α and β) intertwine in an antiparallel manner to form heterodimers, which in turn self-associate in a head-to-head manner to form the tetramers that are the backbone of the erythrocyte membrane skeleton. The tails of the tetramers interact with the proteins of the junctional complex (actin, tropomyosin, protein 4.9 and protein 4.1); protein 4.1 in turn interacts with the integral protein glycophorin C, giving stability to the junctional complex and linking it to the

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erythrocyte membrane.[4,5] Structural modification and reduction of 4.1 protein, or abnormalities at the spectrin self-association site could lead to abnormally shaped erythrocytes (elliptocytes and/or poikilocytes).[5,6]

These two different molecular defects of HE are easily discriminated on the basis of spectrin dimer percentage evaluation. In fact, HE due to 4.1 decrease is associated with a normal percentage of spectrin dimers; whereas an abnormality of the tetramerization site is associated with a variable increase in spectrin dimers in 4°C crude spectrin extract. The latter can also be measured through the apparent association constant (Ka) of the tetramers.[6] Recent advances in the field of major cytoskeleton protein (spectrin and protein 4.1) cDNAs and /or gene structures allowed us to detect the primary molecular lesions in many cases.[7,8]

The aim of this work was to identify the pattern of red cell membrane protein alterations in 61 Italian HE patients and then to correlate the molecular defects found with clinical phenotypes of the disease. Finally, particular attention was given to identifying the molecular determinants of the severity of hemolysis in HE.

Materials and Methods

Clinical and hematological data

From January 1, 1991 to December 31, 1993 we studied 61 Italian HE patients belonging to 28 families: 26 children (mean of age 6 yrs; range 1-9 yrs) and 35 adults (mean age 41 yrs, range 18-63 yrs). Clinical pictures, biochemical and molecular data of some of these patients have been previously reported.[9-13]

Standard hematological procedures were used. Red cells were examined by light phase contrast microscopy after fixation of 2% glutaraldeyde in 5 mM/L phosphate buffer, 150 mM NaCl pH 7.4. Diagnosis of HE was made on the basis of detection of more than 10% elliptocytes in the blood smear.

We examined the clinical and hematological data regarding our patients and then classified each HE subject according to the particular clinical form of the disorder (asymptomatic HE, HE with moderate chronic hemolysis, and severe hemolytic HE).[5] In particular, severe HE was characterized by symptomatic hemolysis and pronounced anemia often requiring blood transfusions; subjects showing HE with moderate chronic hemolysis had a high reticulocyte count, but in the majority of cases hemolysis was compensated and anemia was absent. Lastly, standard hematological data (hemoglobin, reticulocyte count) for the subjects displaying asymptomatic HE were normal.

Membrane protein studies

Erythrocyte ghosts were prepared from blood anticoagulated in acid citrate/dextrose by the hypotonic lysis procedure of Dodge et al.,[14] with the modification that 5 mM phenylmethylsulphonyl fluoride (PMSF) was added at the lysis step. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed utilizing both the continuous buffer system of Fairbanks with 3.5 to 17% exponential acrylamide gradient,[15] and the discontinuous buffer system of Laemmli with 5 to 15% linear acrylamide gradient.[16] Blue Coomassie-stained gels were scanned by a laser densitometer, and the amount of major proteins (spectrin, protein 4.1, etc.) was expressed as the ratio of the surface area of the corresponding peaks to band 3 peak (spectrin/band 3 ratio, protein 4.1/band 3 ratio etc.). Spectrin extraction was carried out after 20h of membrane incubation (4°C); spectrin dimer percentage was assessed by non denaturing polyacrylamide gel electrophoresis with linear gradient from 2.5% to 7.5%. Limited tryptic digestion of spectrin (spectrin: trypsin ratio 100:1) was performed in patients showing an abnormal dimer increase. The amount of abnormal αI peptide (78, 74 or 65 kDa) was estimated by laser densitometric analysis of one-dimensional polyacrylamide gel electrophoresis (Laemmli buffer system with linear gradient from 7 to 15%) of tryptic digests, and was expressed as the 74/, 78/ or 65/α I ratio.

DNA analysis

DNA amplification was carried out according to Saiki et al. in the presence of Taq polymerase, using 0.5 mcg of genomic DNA.[17] The two 20-

bp amplimers were made according to Tse et al.[18] They were designed to anneal to target sequences in genomic DNA, allowing amplification of exon 2 or 4 of the α-sp gene. Analysis of exons X,Y and Z of β-spectrin cDNA was performed as described elsewhere.[12]

Amplified DNA fragments isolated after electrophoresis in low melting point agarose gel were recovered after phenol extraction and ethanol precipitation. Direct sequencing of amplified fragments was performed using Sequenase version 2.0 (USB).

Analysis of a low expression allele (α^{LE}) due to a substitution (C→T) at nt-12 of intron 45, invariably associated with αV/41 polymorphism, was performed by means of AvaII digestion of the amplified fragment spanning from intron 45 to exon 47.[13,19,20]

Figure 1. Geographical distribution of 20 families affected with hereditary elliptocytosis in Italy.
(●): family 4.1(-). (○): family αI/65; (□): family αI/74 and αI/78.

Results

Patients enrolled in this study were widely distributed throghout Italy (Figure 1). Indeed regions in both Northern and Southern Italy were represented. Examination of clinical history and evaluation of standard hematological data allowed us to identify three distinct groups of subjects: 40 asymptomatic carriers (AC), 19 patients with chronic moderate hemolysis (CMH) and 2 severe forms (S) of common HE. We found a very heterogeneous spectrum of cytoskeletal alterations: 18 (29%) subjects showed partial protein 4.1 deficiency, whereas 31 (51%) patients displayed an increased amount of spectrin dimers; we were not able to assess any alteration in 12 (20%) HE patients (Table 1).

All patients exhibiting protein 4.1 reduction showed a similar quantitative decrease (around 25%). Conversely, we found a remarkable heterogeneity regarding both spectrin dimer percentage and abnormal αI peptide levels in the subjects displaying alteration of spectrin tetramerization.

Furthermore, different biochemical and molecular alterations were present in this group of patients; limited tryptic digestion of spectrin showed three different abnormal αI peptides (78,74 or 65 kDa)(Table 1). In two cases we also observed a statistically significant reduction of the spectrin/band 3 ratio (Figure 2). There was a single molecular basis for αI/65 HE (duplication of codon 154 of α spectrin gene), whereas several mutations could lead to the αI/74 biochemical phenotype (Table 1). In particular, mutations of both α and β spectrin genes were responsible for αI/74 HE. Correlation between the clinical phenotypes and the heterogeneous spectrum of cytoskeletal alterations found is shown in Table 2.

Analysis of low expression α spectrin allele showed that 18 of our patients were heterozygous for this characteristic.

Discussion

A correct flow-chart for analyzing HE is based, first of all, upon spectrin dimer percentage evaluation. Subjects with 4.1 protein deficiency have a normal dimer percentage, and the correct

Table 1. Molecular defects in 61 (28 families) Italian HE patients.

No. of patients (families)	Membrane protein abnormalities	Molecular defects
18 (8)	Mild (20% to 30%) protein 4.1 deficit	" " "
19 (7)	65-Kd. peptide	154 TTG duplication in exon 4 of α Sp
1 (1)	74-Kd. peptide	28 CGT→TGT in exon 2 of α Sp
3 (1)	74-Kd. peptide	2018 GCC→GGC in exon X of β Sp
2 (1)	74-Kd. peptide	34 CGG→TGG in exon 2 of α Sp
2 (1)	74-Kd. peptide associated with ß-Sp of lower molecular weight	8 nt deletion in exon X of β Sp
4 (1)	78 Kd. peptide	codon 45 exon 2 of α Sp
12 (8)	No alterations found	" " "

defect could be directly demonstrated by means of Laemmli gel electrophoresis. This kind of HE was found in 18 cases (eight families; 29% of overall subjects). It is a very homogeneous biochemical defect (4.1 protein deficiency ranges between 20 and 25%), and this homogeneity reflects a very mild clinical phenotype: all subjects are asymptomatic carriers. We do not observe any difference regarding clinical or biochemical expression of HE due to protein 4.1 deficiency, either among different families or among affected members of the same family. Furthermore, blood smears in these cases demonstrated a very homogeneous distribution of elliptical-shaped red cells, whereas in the other cases we found a variable percentage of elliptical shaped cells. This defect was widely distributed throughout Italy (Figure 1). In particular, we did not identify any specific geographical area with a high incidence of protein 4.1 deficiency, as previously described in France.[22]

The majority of our HE subjects showed a defect at the tetramerization site (51% of all subjects). The dimer percentage varied, ranging from 10% to 85%, and appeared to be pretty much related to clinical phenotype.

Analysis of limited tryptic digests showed a qualitative and/or a quantitative abnormality in the first domain of α-spectrin, rarely in the second domain.[6] In 19 of our subjects HE was associated with the presence of the α I/65 peptide (Table 1). This abnormality is very common and originated in Central West Africa.[2] Analysis of geographic distribution would suggest a gradient from southern (higher incidence) to northern Italy (lower incidence)

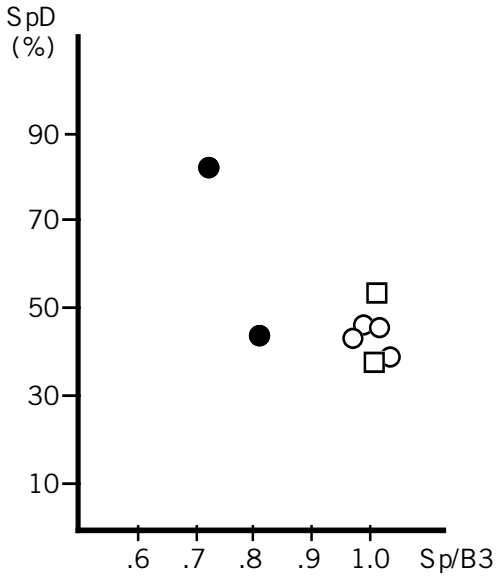

Figure 2. Correlation between spectrin dimer percentage and spectrin content (Sp/B3) in patients showing αI/74 hereditary ellptocytosis. (●): severe forms of common HE. (○): HE with moderate chronic hemolysis; (□): asymptomatic HE.

Table 2. Correlation between clinical and biochemical phenotype.

	AC	CMH	S
4.1 (-)	18	—	—
αI/65	11	8	—
αI/74	2	4	2
αI/78	3	1	—
NA	6	6	—

Clinical phenotype: asymptomatic carriers (AC), chronic moderate hemolysis (CMH) and severe form (S) of common HE.
NA = detectable alteration

(Figure 1). It is very homogeneous at the genetic level: in our cases we always found a duplication of Leu at codon 154.[2,9,11] This abnormality does however appear to be heterogeneous at the clinical level: 11 subjects were asymptomatic, whereas 8 had mild chronic hemolysis. This heterogeneity is also expressed in the same family. This aspect could be related to the presence in trans of a defect leading to a reduction of α spectrin propensity to associate with the corresponding β chain, thereby favoring attachment of the elliptocytogenic α spectrin allele. Wilmotte et al. demonstrated that α^{LE} allele acts in this way.[19,20] Analysis of this low expression allele demonstrate that HE αI/65 is also homogeneous at the clinical level; indeed all αI/65 patients showing mild chronic hemolysis had α^{LE} in trans, justifying the worsened clinical finding.

The αI/74 variant is very heterogeneous both at the clinical and the molecular level.[3-6,21] Our results showed that 2 cases were asymptomatic, 4 had mild hemolysis and two had severe hemolytic expression of HE. Analysis of molecular defects showed that either α or β spectrin variants could be associated with this abnormality (Table 1).

One family showed new β-chain shortening due to an 8-nucleotide deletion in exon X of the β spectrin gene; this novel change creates a frameshift that places a nonsense codon in frame at position 2076 and 2077.[23]

In another family the αI/74 variant was associated with a point mutation in exon X of the β-spectrin gene at position 2018 (GCC-GGC);[12] this mutation causes an Ala-Gly substitution in repeat 17, which severely disrupts the structure of the self association site of the erythrocyte spectrin heterodimer. In this case severe HE is related to a homozygous HE mutation. This variant appears to be selectively distributed in Sardinia (Galanello R. et al., unpublished data) and up to now had not been found in continental regions.

In two families αI/74 was associated with an α spectrin variant. In one case, a severe form of HE, we found a *de novo* mutation at codon 28 of the α spectrin gene,[10] which is a hot spot area; in the other case, a mutation was found at the codon 34 level that defined a new spectrin variant named spectrin Genova.[13] More recently, we described a HE associated with spectrin variant αI/78. Direct sequencing of the second exon of the α spectrin gene showed a mutation at codon 45 (Perrotta S. et al., unpublished data).

The remarkable clinical heterogeneity of HE patients displaying an αI/74 biochemical phenotype cannot be linked merely to the spectrin dimer percentage. As a matter of fact, Figure 2 correlates clinical observations with both dimer percentage and spectrin content; analysis of this figure shows that clinical findings depend on both total spectrin content and the amount of unassembled spectrin in the red cells. Our data confirm the observations of Liu et al., who demonstrated a good correlation between the loss of integrity of the erythrocyte skeletal network and the net amount of tetrameric spectrin crosslinks, as calculated from the spectrin content and the percentage of spectrin tetramers in the membrane extracts.[24]

Biochemical discrepancies involving dimer percentage and in particular the amount of spectrin abnormal peptide among HE αI/74 members of the same family were always due to the presence of the α^{LE} in trans. However, α^{LE} cannot be the reason for the clinical differences observed among αI/74 patients carrying different mutations. The position of the mutation leading to the αI/74 phenotype or the nature of amino acid change could probably explain these differences.

Analysis of the geographical distribution of elliptocytosis due to defects at the tetrameriza-

tion site demonstrate that unlike all known variant α and β chains, which are sporadic, αI/65 appears to be common. A distribution gradient was found from southern to northern Italy, and such incidence suggests that the αI/65 allele provides resistance to the malaria *plasmodium falciparum*. This hypothesis agrees with the data of Shulman et al. which showed that αI/65 elliptical erythrocytes could not sustain normal parasite growth.[25]

In conclusion, examination of 61 Italian cases of HE revealed that the subgroup related to alteration of the junctional complex is homogeneously asymptomatic, whereas forms due to disruption of tetramerization site are very heterogenous, and clinical severity appears to be related to spectrin dimers and especially to spectrin content. These two parameters in turn are related to the degree of disruption of head-to-head contact between α and β chains and to the presence of a low expression α allele in trans.

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