BRIEF REPORT

LINKAGE OF DOMINANT HEREDITARY SPHEROCYTOSIS TO THE GENE FOR THE ERYTHROCYTE MEMBRANE-SKELETON PROTEIN ANKYRIN

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HEREDITARY spherocytosis is a heterogeneous disorder characterized by hemolytic anemia, spheroidal red cells, and increased osmotic fragility of erythrocytes. The majority of the patients have an autosomal dominant pattern of inheritance. It is the most common hereditary hemolytic disorder in people of northern European ancestry, occurring at a prevalence of approximately 1 in 5000. Several lines of evidence suggest that it is a disorder of the red-cell membrane skeleton, although the precise molecular defect has not been identified.[1-3]

The erythrocyte membrane skeleton is composed of a network of proteins underlying the lipid bilayer, including spectrin, actin, protein 4.1, protein 4.2, and ankyrin. The membrane skeleton is attached to transmembrane proteins in the lipid bilayer by ankyrin, which binds to the cytoplasmic portion of the anion exchanger (protein 3), and by protein 4.1, which binds to glycophorin.[4,5] Most patients with hereditary spherocytosis have a partial deficiency of erythrocyte spectrin, and the clinical severity of the disorder correlates with the degree of spectrin deficiency.[6,7] Partial deficiency of ankyrin and spectrin has also been reported in two patients with atypical hereditary spherocytosis.[8] In addition, a defect in the capacity of spectrin to bind to protein 4.1 was detected in some kindreds with dominant hereditary spherocytosis,[9-11] and deficiency of protein 4.2 has been found in other patients with nondominant spherocytosis.[2,12,13]

The few genetic-linkage studies of hereditary spherocytosis have been inconclusive.[14,15] Possible linkage between hereditary spherocytosis and the locus for the immunoglobulin heavy chain, which is localized on chromosome 14, was reported in one study[15] but not another,[16] whereas an association between cases of hereditary spherocytosis and the deletion or translocation of the short arm of chromosome 8 has been found in five different kindreds.[15,17-21]

In the present study we analyzed a large kindred with typical dominant hereditary spherocytosis for genetic linkage between the disease and the genes for α spectrin, β spectrin, protein 4.1, and ankyrin by studying restriction-fragment–length polymorphisms (RFLPs). We were able to exclude close linkage in this family between hereditary spherocytosis and the genes for α spectrin, β spectrin, and protein 4.1. In contrast, dominant hereditary spherocytosis in this kindred was shown to be linked to the gene for ankyrin, with a lod score of +3.63, indicating a statistical probability of linkage between the two loci in this family of 4200 to 1.

METHODS

Kindred

The oldest known member of this white family had anemia that was resistant to iron therapy, and she died suddenly at the age of 62, before our study began. At the age of 40 she was given a diagnosis of spherocytosis and underwent splenectomy, with improvement of the anemia. Two of her nine children died of unknown causes during infancy, and two died as adults in accidents before the study began. Our analyses confirmed that all five living siblings had spherocytosis, and medical records confirmed spherocytosis in one dead sibling. Although two of the affected siblings had some degree of anemia, none of the siblings were severely affected, and two were entirely asymptomatic. Four eventually underwent splenectomy. We were able to study 23 of the 37 grandchildren and great-grandchildren of the proband and confirmed that 13 had mild spherocytosis. Three of the affected family members had recurrent leg ulcers, and two had undergone cholecystectomy for recurrent gallstones.

Osmotic fragility was determined in all the family members studied. The osmotic fragility of unincubated red cells from affected family members demonstrated initial lysis in 0.6 to 0.65 percent sodium chloride (normal, 0.5 percent), 50 percent lysis in 0.48 to 0.51 percent sodium chloride (normal, 0.40 to 0.42 percent), and complete lysis in 0.43 percent sodium chloride (normal, 0.35 percent). Spherocytes were apparent in the peripheral-blood smears. Clinical laboratory values for four affected family members who had not undergone splenectomy included hemoglobin levels of 6.2 to 7.4 mmol per liter (10 to 12 g per deciliter), hematocrits of 0.29 to 0.36, and 2 to 7 percent circulating reticulocytes. Values for four affected family members who had undergone splenectomy included hemoglobin levels of 6.8 to 8.6 mmol per liter (11 to 14 g per deciliter), hematocrits of 0.34 to 0.41, and less than 2 percent reticulocytes.

Quantitation of the Spectrin and Ankyrin Content of the Red-Cell Membrane

Samples of venous blood were obtained from family members with spherocytosis, unaffected members, and normal control subjects. Erythrocyte membranes were prepared as described elsewhere[6] and subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis; the gels had a gradient of 3.5 to 17 percent polyacrylamide.[6] The relative amounts of spectrin (proteins 1 and 2) and ankyrin (protein 2.1) in the membranes were compared with those in the protein 3 region by the pyridine-dye elution method and were measured as the ratios of the absorbance at 605 nm of spectrin or ankyrin to that of protein 3.[6]

The number of copies of spectrin per erythrocyte was quantitated by radioimmunoassay as previously described.[6,7]

DNA Extraction and Analysis

Total genomic DNA was extracted from peripheral-blood leukocytes by a modification of the method of Blin and Stafford.[22]

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Then 5 to 10 μg of DNA was digested to completion with the appropriate restriction endonuclease under conditions recommended by the manufacturer, fractionated by electrophoresis in 0.8 percent agarose gels, and transferred to nylon membranes as described elsewhere.[23]

Probes

Four probes were used in the linkage analysis: The first was a genomic *Eco*RI DNA fragment of 13 kilobases (kb) (3021-E1), corresponding to the αI domain of α spectrin,[24,25] with a chromosomal location of 1q22–25.[26] The second was a 1.0-kb *Eco*RI complementary DNA (cDNA) fragment (pTM-1) encoding the C-terminal region of protein 4.1,[27] with a chromosomal location of 1p32→ter.[28] The third was a 2.8-kb (β28) or a 4.8-kb (β21) *Eco*RI cDNA fragment encoding approximately 40 percent or 70 percent, respectively, of the C-terminal region of the β-spectrin chain,[29-31] with a chromosomal location of 14q23–24.2.[32] The fourth probe was a 1.4-kb *Eco*RI cDNA fragment (ank1B) encoding the C-terminal region of ankyrin,[33] with a chromosomal location of 8p11.2.[21] Each probe detects a two-allele polymorphism. The probes were radiolabeled by nick translation; hybridization and autoradiography were performed according to standard procedures.[23]

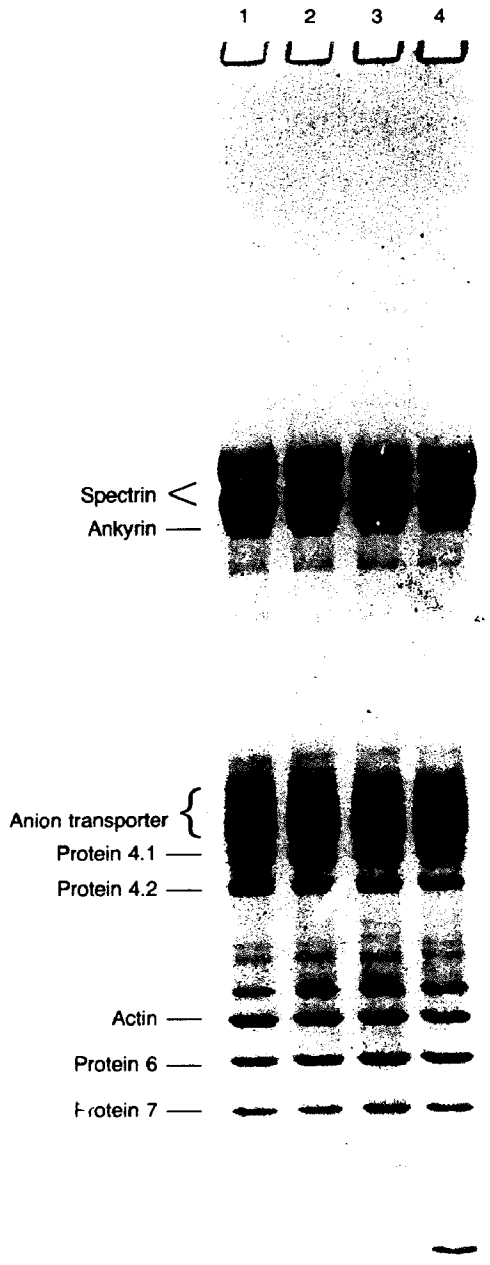

Figure 1. Sodium Dodecyl Sulfate–Polyacrylamide-Gel Electrophoresis of Erythrocyte Membrane Proteins.

Approximately 25 μg of membrane proteins was applied to a polyacrylamide slab gel with an exponential gradient of 3.5 to 17 percent, subjected to electrophoresis for three hours at 240 V, and stained with Coomassie blue. Lane 1 represents samples from a normal control subject; lane 2, family member II-8, who had hereditary spherocytosis but had not undergone splenectomy; lane 3, family member III-12, who had hereditary spherocytosis but had not undergone splenectomy; and lane 4, family member III-13, who had hereditary spherocytosis and had undergone splenectomy. The results of spectrin and ankyrin fractionation are shown in Table 1. The pedigree is shown in Figure 2.

Table 1. Ratio of Spectrin and Ankyrin to Band 3.

Subject	Splenectomy	Spectrin	Ankyrin
Control	No	1.00	0.196
Family member*			
II-8	No	0.90	0.184
III-12	No	0.89	0.192
III-13	Yes	0.82	0.170

*The pedigree is shown in Figure 2.

Linkage Analysis

The lod score[34] was calculated with the LINKAGE computer program.[35] The lod score reflects the significance of the linkage detected between a polymorphic locus and a disease locus. It is calculated by dividing the probability of observing coinheritance of two loci assumed to be genetically linked by the probability of observing coinheritance of the two loci even though they are not genetically linked. In this study, because the four markers (probes) represented candidate genes themselves, any evidence of recombination between the hereditary spherocytosis locus and the RFLP detected by the candidate gene probe eliminated that gene as the possible site of the molecular defect of hereditary spherocytosis. The gene frequency of allele 1 for the ankyrin *Nco*I RFLP was 0.4 in the population analyzed, and a penetrance of 100 percent was assumed.

Results

Clinical and Biochemical Features

The clinical course of and laboratory values for the members of this kindred fulfilled the criteria for the diagnosis of classical, dominant hereditary spherocytosis.

Erythrocyte membrane proteins were electrophoretically separated on polyacrylamide gels containing sodium dodecyl sulfate and stained with Coomassie blue (Fig. 1). Quantitation of membrane proteins by dye elution in three affected members of the family showed a small deficiency of both proteins, although the degree of spectrin deficiency was slightly greater than that of ankyrin deficiency (Table 1). The quantitation of spectrin by radioimmunoassay in several affected family members showed a mild deficiency of spectrin, with levels ranging from 77 to 82 percent of those in normal controls (Family F of Agre et al.[7]).

Linkage Analysis

The pedigree of the family is shown in Figure 2. Coinheritance of the allelic marker and hereditary spherocytosis was not observed with the RFLPs detected by the probes for α spectrin, β spectrin, and

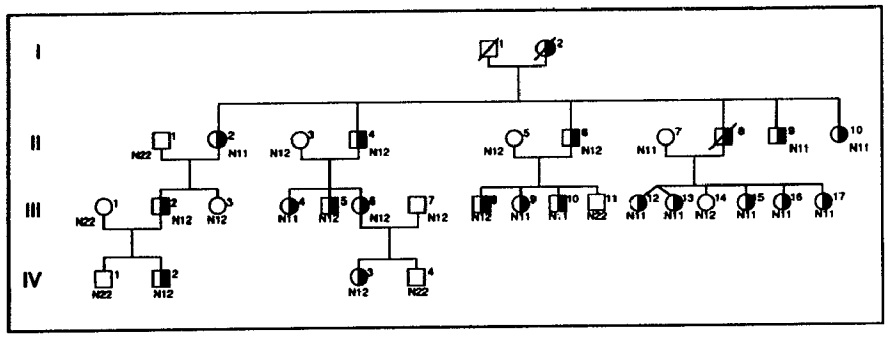

Figure 2. Pedigree of the Family with Dominant Hereditary Spherocytosis, Showing Linkage with the Ankyrin Gene.

The pattern of inheritance of the *Nco*I RFLP (N) detected by the ankyrin gene probe (ank1B) is shown below each symbol: N11 indicates homozygosity for allele 1 (3.8 kb); N22, homozygosity for allele 2 (3.6 kb); and N12, double heterozygosity for alleles 1 and 2. Semisolid symbols denote affected family members, circles female family members, and squares male family members. Slashes denote deceased family members.

protein 4.1. These results indicated that there is no close linkage between hereditary spherocytosis and the genes for α spectrin, β spectrin, or protein 4.1 in the kindred studied. Preliminary results regarding the α-spectrin and β-spectrin RFLPs in this family have been reported previously.[30,36] The patterns of inheritance of the spectrin and the protein 4.1 RFLPs that exclude linkage to hereditary spherocytosis in this family are summarized in Table 2. The results exclude linkage of hereditary spherocytosis in this family to within 1 cM of the α-spectrin and protein 4.1 genes and to within 6 cM of the β-spectrin gene.

The cDNA for human ankyrin has recently been cloned.[33,37] We tested the ability of ankyrin cDNA to detect RFLPs in normal human DNA. The test probe was ank1B, which encodes the C-terminal region of ankyrin.[33] DNA from four unrelated subjects of different ethnic origins was digested separately with 27 different restriction endonuclease enzymes, fractionated by agarose-gel electrophoresis, transferred to filters, and probed with ^{32}P-labeled ank1B cDNA. Differences between subjects consistent with two-allele RFLPs were observed with four different enzymes (Table 3). The enzyme *Nco*I yields polymorphic DNA fragments of 3.8 kb (allele 1) and 3.6 kb (allele 2), as shown in Figure 3. Both alleles of the *Nco*I RFLP detected by the ankyrin probe alleles were present and segregated in an autosomal codominant fashion in the family under study, as illustrated in Figure 2. The pattern of inheritance of the ankyrin *Nco*I RFLP in the various family members shows clear cosegregation of allele 1 with hereditary spherocytosis in all members studied. There is no evidence of recombination between the locus for hereditary spherocytosis and ankyrin. The lod score was +3.63 at a recombination fraction of zero (confidence interval, 0 to 0.14). Generally, a lod score above +3 is considered statistical evidence of linkage.[34] The value of +3.63 indicates that the odds in favor of hereditary spherocytosis and ankyrin being linked, in this family, are greater than 4200 to 1. The *Msp*I RFLP was also informative in this family, but the results did not significantly affect the lod score.

Table 2. Inheritance Patterns of RFLPs for Spectrin and Protein 4.1 Genes.

Family Member*	α Spectrin†	β Spectrin†	Protein 4.1†
II-1	22	12	22
II-2	12	12	12
II-3	12	12	22
II-4	12	22	12
II-5	11	22	22
II-6	12	12	12
II-9	12	22	12
II-10	12	12	12
III-1	12	12	NT
III-2	22‡	12	22‡
III-3	22‡	22‡	22‡
III-4	12	12	12‡
III-5	22	12	22‡
III-6	12	12	12‡
III-7	22	NT	NT
III-8	11‡	22	NT
III-9	12	12‡	12
III-10	12	22	12
III-11	11‡	22‡	NT
IV-1	22	22‡	22
		lod scores	
θ value			
0.0	−11.60	−14.78	−6.65
0.01	−1.97	−4.53	−1.97
0.06	−0.58	−2.08	−0.58
0.1	−0.31	−1.40	−0.26
0.2	−0.01	−0.57	0.01
0.3	0.04	−0.21	0.05
0.4	0.02	−0.05	0.02

*The pedigree is shown in Figure 2.

†The following RFLPs were analyzed: α-spectrin *Pvu*II, allele 1 (8.0 kb) and allele 2 (6.5 kb)[25]; β-spectrin *Hind*III,[30] allele 1 (17 kb) and allele 2 (14 kb); and protein 4.1 *Hind*III, allele 1 (3.5 kb) and allele 2 (3.0 kb). The number 11 indicates homozygosity for allele 1; 22, homozygosity for allele 2; and 12, double heterozygosity for alleles 1 and 2. NT denotes not tested.

‡Values are results that, considered alone or in combination with the other similarly marked values in the same column, exclude linkage to the listed gene.

Discussion

In this study, we found a definite genetic linkage between hereditary spherocytosis and the gene for ankyrin in a large kindred with the clinical picture of typical dominant hereditary spherocytosis.

There are few reports of ankyrin abnormalities in hereditary spherocytosis. Abnormal accumulation of ankyrin in the red-cell membrane due to a truncated ankyrin chain has been reported in a murine model of recessive spherocytosis,[38,39] and partial ankyrin deficiency has been shown in two patients with severe atypical spherocytosis.[8] In these two patients, the precise pattern of inheritance of the defect could not be established. The recent cloning of ankyrin cDNA[33,37]

Table 3. RFLPs Detected by the Ankyrin cDNA Probe ank1B.

Enzyme	Polymorphic Fragments	
	Allele 1	Allele 2
	kilobases	
*Bsp*MI	1.6	1.5
*Msp*I	1.6	1.4
*Nco*I	3.8	3.6
*Rsa*I	1.2	1.1

has permitted the localization of the gene to chromosome 8[21,37] and the demonstration of deletion of the ankyrin gene in cases of hereditary spherocytosis associated with interstitial deletions of the short arm of chromosome 8.[21] Cases of hereditary spherocytosis associated with partial ankyrin deficiency[8] or with deletion of one ankyrin gene[21] are unusually severe clinically. In contrast, the family in this report, with linkage of hereditary spherocytosis to the ankyrin gene, had the more typical clinical picture usually associated with dominantly inherited hereditary spherocytosis.

Hereditary spherocytosis is heterogeneous with regard to clinical presentation and biochemical findings.[1-3] Therefore, it is unlikely that one specific molecular defect is responsible for all or most cases of hereditary spherocytosis. Evidence of heterogeneity in the molecular defect of hereditary spherocytosis is provided by the findings of abnormalities of α spectrin[40,41] or protein 4.2 [12,13] in several kindreds with recessively inherited spherocytosis and of functionally abnormal β-spectrin chains in kindreds with a dominant form of hereditary spherocytosis.[9-11] In the family with hereditary spherocytosis that we studied, we were able to show that the primary molecular defect is linked to the ankyrin gene. Study of additional families will be necessary to determine whether defects of the ankyrin gene are a common cause of hereditary spherocytosis.

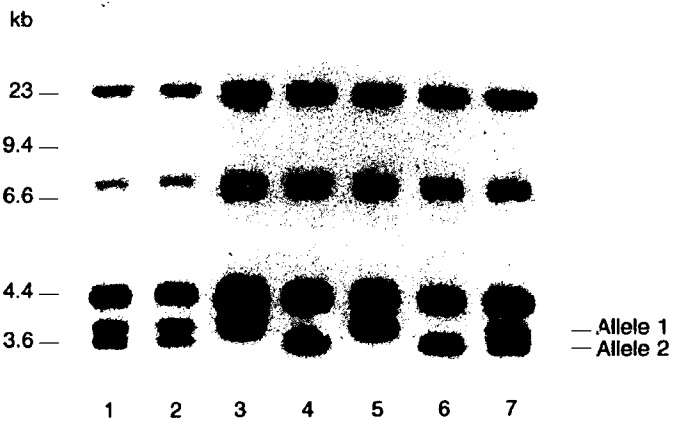

Figure 3. Autoradiograph Prepared by Hybridizing ^{32}P-Labeled Ankyrin cDNA with Total Cellular DNA Previously Digested with *Nco*I and Separated by Agarose-Gel Electrophoresis.

The DNA samples were obtained from the following family members shown in Figure 2: lane 1, III-8; lane 2, III-7; lane 3, II-7; lane 4, II-1; lane 5, III-15; lane 6, III-11; and lane 7, IV-2. The two RFLP alleles (1 and 2) are indicated.

The deficiency of spectrin observed in this kindred must be due to a defect in the structure or function of ankyrin. In support of this conclusion, previous reports of studies in mice[7,8] and humans[21] suggest that diminished incorporation of ankyrin into the red-cell membrane leads to decreased assembly of spectrin in the membrane skeleton.

The primary defect of the ankyrin gene in this family remains to be identified. A variety of primary gene defects could result in abnormal structure or synthesis of ankyrin. The finding of nearly normal levels of ankyrin in the membranes of affected family members suggests that a structural abnormality is the most likely, perhaps in a region of the ankyrin molecule involved in its binding to the β-spectrin chain or the anion exchanger. This possibility is currently under investigation.

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CASE RECORDS
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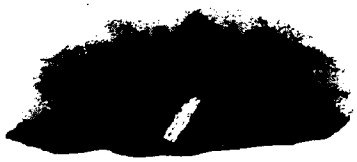

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CASE 41-1990

PRESENTATION OF CASE

A 66-year-old woman was admitted to the hospital because of progressive deterioration of renal function.

There was a 10-year history of hypertension, which was under satisfactory control until recently. Five years before admission a stroke occurred, with right hemiparesis and impaired speech. A daily dose of aspirin was begun, and the deficits slowly resolved, with residual minimal stiffness and clumsiness of the right hand and occasional mild slurring of speech. Four years before entry upper gastrointestinal bleeding occurred from a duodenal ulcer and was managed with cimetidine and the omission of aspirin.

Four months before admission the creatinine was 80 μmol per liter (0.9 mg per 100 ml). The patient felt reasonably well until one month before entry, when edema appeared over the face and upper and lower extremities, with urinary frequency. Additional laboratory studies disclosed ++++ proteinuria; the creatinine was 170 μmol per liter (1.9 mg per 100 ml). Examination revealed anasarca; a stool specimen gave a trace-positive test for occult blood. Microscopical examination of the urinary sediment disclosed red cells and questionable red-cell casts. In a 24-hour specimen of urine the protein was 5 g. A serum immunoelectrophoresis, antistreptolysin-O titer, measurement of C3, C4, and CH50, and tests for antinuclear antibodies and anti–native DNA antibodies were negative. An upper gastrointestinal series was reported to show evidence of gastritis, including antritis. Furosemide was begun, and the edema diminished. Two weeks before entry the patient experienced impaired vision, and an ophthalmologist found peripheral retinal hemorrhages consistent with the hyperviscosity syndrome or hypertensive retinopathy. One week before admission she began to have increasing dyspnea, lethargy, weakness, intermittent chilliness and warm sensations, and anorexia. Several days later nausea and vomiting appeared. One day before entry repeated laboratory studies revealed that the creatinine was 340 μmol per liter (3.9 mg per 100 ml) and the lactic dehydrogenase (LDH) 1658 U per liter. The patient was admitted to this hospital.

The patient was married and a homemaker. A breast biopsy was performed at the age of 35 years;