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Genomewide Search for Dehydrated Hereditary Stomatocytosis (Hereditary Xerocytosis): Mapping of Locus to Chromosome 16 (16q23-qter)

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Summary

Dehydrated hereditary stomatocytosis, also known as "hereditary xerocytosis," is caused by a red blood cell–membrane defect characterized by stomatocytic morphology, increased mean corpuscular hemoglobin concentration, decreased osmotic fragility, increased permeability to the univalent cations Na^+ and K^+, and an increased proportion of phosphatidylcholine in the membrane. The clinical presentation is heterogeneous, ranging from mild to moderate hemolytic anemia associated with scleral icterus, splenomegaly, and cholelithiasis. Iron overload may develop later in life. The disease is transmitted as an autosomal dominant trait. We recruited a large three-generation Irish family affected with DHS and comprising 23 members, of whom 14 were affected and 9 were healthy. Two additional, small families also were included in the study. The DNA samples from the family members were used in a genomewide search to identify, by linkage analysis, the DHS locus. After the exclusion of a portion of the human genome, we obtained conclusive evidence for linkage of DHS to microsatellite markers on the long arm of chromosome 16 (16q23-q24). A maximum two-point LOD score of 6.62 at recombination fraction .00 was obtained with marker D16S520. There are no recombination events defining the telomeric limit of the region, which therefore is quite large. No candidate genes map to this area.

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Introduction

Most hemolytic disorders are associated with a change in the morphological appearance of red blood cells. Stomatocytes are red blood cells that show a slitlike central zone of pallor, on dried smears. In the set of hemolytic anemias known as the "hereditary stomatocytoses," this shape abnormality is associated with abnormally high membrane permeability to the monovalent cations Na and K, which compromises the osmotic stability of the erythrocyte and which is an important element of the pathophysiology (Stewart 1993; Lux and Palek 1995). The hereditary stomatocytoses were among the first human diseases for which abnormal membrane transport was recognized to be crucial. Of these conditions, dehydrated hereditary stomatocytosis (DHS; MIM 194380), also known as "hereditary xerocytosis," is the most common variant.

Like all the other hereditary stomatocytoses, DHS is dominantly inherited. Approximately 30 families with DHS have been described in the literature (Miller et al. 1971; Glader et al. 1974; Nolan 1984; Wiley 1984; Vives-Corrons et al. 1995). The patients show anemia, moderate jaundice, splenomegaly, gallstones, and a very marked tendency to develop thrombosis in adult life, if a splenectomy has been performed (Stewart et al. 1996). An association between DHS and severe intrauterine ascites has been reported (Entezami et al. 1996; Grootenboer et al. 1997). It seems likely that DHS is in fact identical to hereditary hyperphosphatidylcholine hemolytic anemia (HPCHA), as has been described by Jaffe and Gottfried (1968) (also see Clark et al. 1993).

A number of other variants among the stomatocytoses exist. They can be classified according to the severity of the cation leak and the state of hydration of the cells, which reflects the relative permeability of Na to K. Cases in which the K-permeability increase outweighs Na, as well as those in which the reverse is true, show dehydration. The most striking variant is the very rare over-

hydrated hereditary stomatocytosis (OHS)(Lock et al. 1961), a form of anemia more severe than DHS, in which the permeability defect is much more severe and in which the 32-kD integral membrane protein, known as "stomatin" or "erythrocyte membrane protein 7.2b," is absent from the membrane (Lande et al. 1982), although the gene is apparently normal (Wang et al. 1992). First recognized in the red cell, this protein, the function of which remains obscure, is distributed widely in nature (Huang et al. 1995; Barnes et al. 1996).

The molecular lesion underlying DHS has never been identified. To map the DHS locus, a large three-generation Irish kindred comprising 14 affected and 9 unaffected subjects was recruited, and a genomewide search was performed. In addition, two small families were used to confirm or exclude positive loci. This strategy allowed identification of the disease-gene locus.

Patients and Methods

Patients

We studied a large three-generation Irish kindred with DHS, as well as two additional, small families. The three pedigrees are shown in figure 1. In the large pedigree (family A), the hemoglobin level typically was 10–12 g/dl, the mean cell volume was 105–115 fl, and the reticulocyte count was 5%–10%. Median osmotic fragility was reduced to 0.33%–0.37% NaCl (normal 0.4%–0.45%). Cell water was reduced to 62.5%–62.9%, which is consistent with the "dehydrated" description. Intracellular Na levels were increased to 13–15 mmol cells/liter (normal 5–11 mmol cells/liter), and K levels typically were reduced to 82–86 mmol cells/liter (normal 88–105 mmol cells/liter). The ouabain-plus-bumetanide–insensitive (OBI) leak and the ouabain-sensitive (OS) K-pump influxes were increased 2–3 × normal, reflecting the increased passive leak and compensatory Na/K-pump acceleration characteristic of these conditions (Stewart 1993) and comparable with other cases (Miller et al. 1971; Glader et al. 1974; Wiley 1984). The bumetanide-sensitive (BS) (NaK_2Cl cotransport) fluxes were at the upper limit of the normal range, which is consistent with dehydrated cells (Stewart 1988). The influxes of Na also showed an increased OBI rate, which is consistent with the findings for K. The membrane showed no deficiency of the band 7.2b membrane protein, by Coomassie staining of two-dimensional analyses or by western blotting with a band 7.2b–specific antibody (M. M. Ho and G. Stewart, unpublished data). Thin-layer chromatography of the membrane lipids showed an increased molar proportion of PC, which was more marked in the individuals (III-8 and III-9) who had not undergone a splenectomy and which is consistent with the link between DHS and HPCHA (Clark et al. 1993), although the phosphatidylcholine proportion in this family was not as high as that found in the family described by Jaffe and Gottfried (1968). For family A, two affected members (II-2 and III-11) had been diagnosed as suffering from Crohn disease, and a third (III-14) had abdominal pain and therefore was suspected of also having this condition. In family B, a small Irish family, the propositus, individual II-2, presented with mild chronic hemolytic anemia; his mother had an identical condition, and his deceased grandmother was reported to have had a history of intermittent jaundice. The propositus had a hematologically normal brother. The propositus had gallstones and splenomegaly but no other major pathology; his mother had a history of Dupytren contracture and depression but otherwise was well. No member had undergone a splenectomy. A blood film showed the presence of stomatocytes. Tests of red-cell glycolytic enzymes were normal. Measurements of intracellular Na and K showed abnormal values, and the fluxes of Na and K were similar to those in family A. For family C, a French family, hematologic data demonstrated a well-compensated hemolytic status (table 1). Diagnosis of DHS was established firmly by osmotic-gradient ektacytometry, which showed a leftward shift of the osmotic-gradient ektacytometric curve (Johnson and Ravindrannah 1996).

Genomewide Search

For the genomewide search, the ABI PRISM Linkage Mapping set (Perkin Elmer) was used. This set comprises >375 markers and defines a 10-cM–resolution human index map (Gyapay et al. 1994). PCR reactions using fluorescently labeled primers were run under the conditions suggested by the supplier. An aliquot of PCR reaction was run in an ABI PRISM 373 or 377 DNA sequencer, and the results were processed by GENESCAN software. Allele assignation was performed by use of the GENOTYPER software. To saturate the chromosome 16 positive region, additional pairs of fluorescently labeled primers were synthesized specifically for the following markers: D16S511, D16S3037, D16S520, D16S498, D16S413, D16S3026, and D16S3121 (Dib et al. 1996). All living individuals of each family were genotyped and, thus, contributed to the following linkage calculations.

Linkage Analysis

Statistical analysis was performed on the basis of an autosomal dominant disease with complete penetrance. The disease-gene frequency was set to .0012, and all marker alleles were considered to be equally frequent.

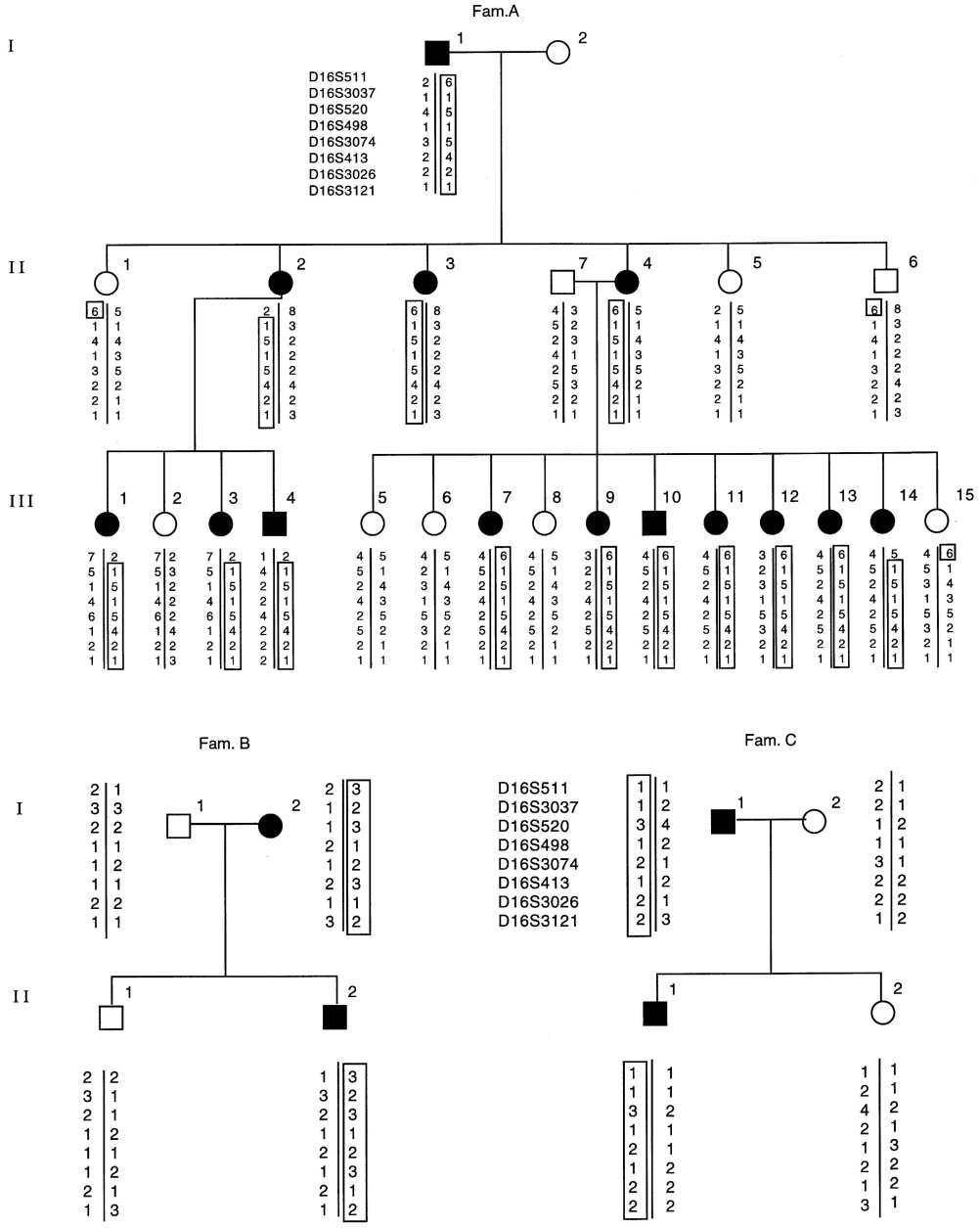

Figure 1 Pedigrees of the three families studied. Boxes indicate the disease haplotypes.

Two-point linkage analysis was performed by use of the MLINK program, version 5.1, from the LINKAGE software package (Ott 1992). Values for the maximum LOD score (Z_{max}) were calculated by use of the ILINK program from the same software package. The approximate 95% confidence limits for the maximum recombination fraction (θ_{max}) at Z_{max} were calculated by the 1-LOD–down method (Ott 1992). Alleles were down-coded without loss of informativity, to reduce computation time.

Results

After analysis of data from 150 markers, which is ~50% of the human genome, evidence for linkage was obtained with marker D16S520 from the long arm of chromosome 16, whereas negative results were obtained for the remaining markers analyzed. Positive but less significant LOD scores were obtained with another marker (D16S511) of the panel. Six additional markers from the region (D16S3037, D16S498, D16S3074,

Table 1

Hematologic and Ion-Flux Diagnostic Data

Family and Subject	Sex	Age (years)	Hematologic Data[a]			Intracellular Electrolytes (mmol cells/liter)		Cell Water (%)	K Influx at 5 mM K_0 (mmol cells/liter/h)			Na Influx (mmol cells/liter/h)		
												At 15 mM Na_0		At 150 mM Na_0
			Hb (g/dl)	MCV (fl)	Retic (%)	Na_i	K_i		OS Na/K Pump	BS Na/K Cotransport	OBI Leak	BS Na/K Cotransport	OBI Leak	OBI Leak
A:														
II-2	F	56	11.9	116	6.4	12.7	86.4	62.5	5.1	1.5	.21	.83	.63	5.69
III-6[b]	F	28	12.1	108	7.5	14.2	83.5	62.5	4.2	1.3	.18	.70	.70	4.92
III-8	F	25	12.2	94	13.8	10.8	85.1	62.9	5.4	.9	.20	.78	.62	6.00
III-9	M	24	14.4	104	12	15.5	92.2	…	4.78	.891	.156	.68	.95	5.57
B:														
II-2	M	32	14.6	10.3	8.1	19.5	84.6	…	4.46	1.09	.22	.70	1.50	7.12
C:														
I-1	M	58	15.7	98.1	1.8									
II-1	M	30	11.2	111	2.8									
Normal			11.5–16	80–96	<2	5.9–11.3	88–105	62.2–67.2	.8–2.0	0–1	.06–.10	.15–.31	0–.49	1.7

Note.—Na_i and K_i were measured by flame photometry on washed cells. K influx was measured in isotonic NaCl media, as described by Stewart and Ellory (1985). Na influx was measured either in KCl media with 15 mM Na_0 or in 150 mM NaCl media with no added K, as indicated. Ouabain and bumetanide were used at .1 mM.

[a] Hb = hemoglobin level, MCV = mean cell volume, and Retic = reticulocyte count.

[b] Subject had undergone a splenectomy.

D16S413, D16S3026, and D16S3121) then were typed in all individuals of the families. The order and distances for the above-mentioned markers, as deduced from published maps (Dib et al. 1996), are cen–D16S511–9 cM–D16S3037–4 cM–D16S520–1 cM–D16S498–1 cM–D16S3074–2 cM–D16S413–3 cM–D16S3026–2 cM–D16S3121–tel. Pairwise LOD scores for the eight markers are shown in table 2, and the linkage map for these markers is shown in figure 2.

The highest LOD score obtained was with D16S520 (Z_{max} = 6.62 at recombination fraction [θ] .00), but, in addition, no recombinants were detected with D16S3037 (Z_{max} = 1.50 at θ = .00), D16S498 (Z_{max} = 4.81 at θ = .00), D16S3074 (Z_{max} = 3.31 at θ = .00), D16S413 (Z_{max} = 4.81 at θ = .00), D16S3026 (Z_{max} = 3.61 at θ = .00), and D16S3121 (Z_{max} = 1.80 at θ = .00). Several recombinations were detected with marker D16S511, which helped define the centromeric limit for the DHS-gene location, whereas no recombinations were detected on the telomeric side (fig. 1). Thus, the region for the DHS locus is ~20 cM, spanning from D16S511 to 16qter.

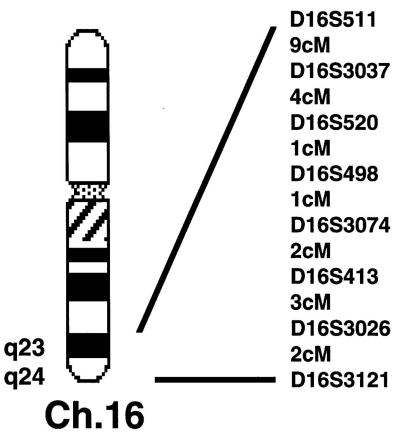

Figure 2 Linkage map of microsatellite markers from chromosome 16q23-q24. Genetic distances are indicated in centimorgans (cM).

Discussion

Our genomewide search has led to the identification of the DHS locus on the long arm of chromosome 16 (16q23-qter). The absence of recombination events on the telomeric side makes the candidate region quite large, ~20 cM. Additional analysis, with new markers, of an expanded set of families should lead to further refinement of the DHS locus. The disease clearly does not map to the gene for stomatin—the band 7.2b membrane protein that is absent from the membrane in OHS—which is located at 9q34 (Gallagher et al. 1993), confirming a previous study of an American pedigree (Gallagher and Forget 1995). The reason for the absence of stomatin in OHS remains unclear, since the gene for stomatin apparently is normal (Wang et al. 1992). This variant of the disease possibly is caused by a mutation at the same locus as that for DHS.

The red blood cells of the patients were characterized by some very interesting features that could help in the identification of some good candidate genes. In particular, the red blood cells presented increased intracellular Na levels and correspondingly reduced K levels. Tracer-flux studies showed increased leak and pump rates. The OBI leak and OS K-pump influxes were increased 2–3 × normal, reflecting the increased passive leak and compensatory Na/K-pump acceleration characteristic of this condition. Thus, genes involved in the transport of Na and/or K ions could be considered as good candidate genes for the disease.

A dominantly inherited condition characterized by a membrane leak suggests the existence of a "leaky-channel" dominant mutation involving some unknown channel (Caldwell and Schaller 1992). In addition, the phospholipid abnormality still may be primary, and the mutant gene may be some kind of lipid-handling or lipid-

Table 2

Pairwise LOD Scores between DHS and Chromosome 16 Markers

	LOD Score at θ =								
Marker	.00	.01	.05	.10	.20	.30	.40	Z_{max}	θ_{max}
D16S511	∞	−5.45	−1.56	−.14	.80	.92	.62	.93	.271
D16S3037	1.50	1.47	1.37	1.23	.95	.64	.33	1.50	.000
D16S520	6.62	6.51	6.06	5.48	4.25	2.89	1.43	6.62	.000
D16S498	4.81	4.73	4.41	4.00	3.12	2.17	1.14	4.81	.000
D16S3074	3.31	3.25	3.00	2.68	2.00	1.29	.56	3.31	.000
D16S413	4.81	4.72	4.37	3.91	2.93	1.87	.75	4.81	.000
D16S3026	3.61	3.55	3.32	3.02	2.37	1.67	.88	3.61	.000
D16S3121	1.80	1.77	1.63	1.45	1.08	.71	.35	1.80	.000

metabolizing protein. Shohet et al. (1971) showed that, in a similar family with an elevated proportion of phosphatidylcholine in the membrane, there was a block in the transfer of labeled acyl chains from phosphatidylcholine to phosphatidylethanolamine and phosphatidylserine. Among the several genes mapped to 16q23-qter, none have any obvious relationship to DHS, but the study of possible candidate expressed-sequence tags (ESTs) already mapped to this area is in progress. Unfortunately, the region defined here is very large, and it is only partially covered by a YAC contig. As soon as the physical map of the region is defined, it will be useful in the cloning of the DHS gene. cDNA selection and exon-trapping techniques could be used to isolate expressed sequences and, together with already-cloned ESTs, to construct a transcriptional map of the region. The identification of the DHS gene will lead to the understanding of the molecular basis of a human membrane disease involving abnormal cation permeability, abnormal phospholipid content, and a tendency for the development of thrombosis.

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Electronic-Database Information

Accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for DHS [MIM 194380])

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