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Molecular Analysis of Cystinuria in Libyan Jews: Exclusion of the *SLC3A1* Gene and Mapping of a New Locus on 19q

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Summary

Cystinuria is a hereditary disorder of amino acid transport and is manifested by the development of kidney stones. In some patients the disease is caused by mutations in the *SLC3A1* gene, which is located on the short arm of chromosome 2 and encodes a renal/intestinal transporter for cystine and the dibasic amino acids. In Israel cystinuria is especially common among Jews of Libyan origin. After excluding *SLC3A1* as the disease-causing gene in Libyan Jewish patients, we performed a genomewide search that shows that the Libyan Jewish cystinuria gene maps to the long arm of chromosome 19. Significant linkage was obtained for seven chromosome 19 markers. A maximal LOD score of 9.22 was obtained with the marker *D19S882*. Multipoint data and recombination analysis placed the gene in an 8-cM interval between the markers *D19S409* and *D19S208*. Significant linkage disequilibrium was observed for alleles of four markers, and a specific haplotype comprising the markers *D19S225, D19S208, D19S220,* and *D19S422* was found in 11 of 17 carrier chromosomes, versus 1 of 58 Libyan Jewish noncarrier chromosomes.

Introduction

Cystinuria is an autosomal recessive disease characterized by the formation of kidney stones (McKusick 1992). The disease has been thought to be caused by a defect in a renal tubular amino acid–transport molecule, resulting in impaired reabsorption of cystine and the dibasic amino acids—lysine, arginine, and ornithine (Dent and Rose 1951). Cystine, which has a low solubility, can precipitate and form kidney stones, causing pain, obstruction, infection, and sometimes renal failure (Dahlberg et al. 1977). The disease is common worldwide, with 1:15,000 Americans affected (Levy et al. 1972). A very high frequency of the disease has been noted among Libyan Jews living in Israel, in whom the prevalence rate is 1:2,500 (Weinberger et al. 1974).

Three different types of cystinuria have been defined. Type I heterozygotes exhibit normal levels of urinary cystine. Type II and type III heterozygotes show high or moderate hyperexcretion of cystine, respectively. Type I and type II homozygotes exhibit a minimal increase of plasma cystine levels after oral loading, whereas type III homozygotes show a near-normal response (Rosenberg et al. 1966*a*).

Rosenberg et al. (1966*b*) have suggested that the three types are due to allelic variations of the same gene. In contrast, Goodyer et al. (1993) have noted that compound heterozygotes have significantly lower levels of cystine and the dibasic amino acids in their urine, as compared with homozygotes, and have proposed a mechanism of complementation between distinct loci, to explain these findings.

Recently, *SLC3A1,* a gene encoding a transporter for cystine and the dibasic amino acids, has been isolated (Bertran et al. 1993; Lee et al. 1993). Mutations in this gene, which maps to the short arm of chromosome 2, were found in a substantial number of cystinuria patients (Calonge et al. 1994; Miyamoto et al. 1995; Pras et al. 1995). Other studies have demonstrated genetic heterogeneity in cystinuria. Mutations in *SLC3A1* were found only in type I patients (Gasparini et al. 1995; Horsford et al. 1996), whereas in type III patients *SLC3A1* was excluded as the disease-causing gene (Calonge et al. 1995).

Previously we have shown linkage between a panel of 17 cystinuria families (8 of which were of Libyan Jewish origin) and markers on the short arm of chromosome 2 (Pras et al. 1994). Our results suggested locus homogeneity. Subsequently we have reported a study in which screening of the *SLC3A1* coding sequence did not reveal any mutations in a number of these Libyan Jewish cystinuria patients (Pras et al. 1995). In the present report

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we show data excluding *SLC3A1* as the disease gene in Libyan Jewish cystinuria patients, and we present the results of a genomewide search for the gene causing the disease in this population.

Families and Methods

Families and DNA Extraction

Families were recruited at the Sheba, Beilinson, and Hadassah Medical Centers in Israel. The institutional review board at each center approved the study, and participants gave informed consent.

Eight of the Libyan Jewish families included in this study have been reported elsewhere (Pras et al. 1994). Since then we have added to this panel an additional family (family 303) and two more affected family members (individuals 176-04 and 158-03). Previously the diagnosis of cystinuria was confirmed by thin-layer chromatography. For the present study we employed quantitative amino acid analysis using a high-performance liquid-chromatography analyzer. This analysis enabled us to classify family members as affected (homozygotes), carrier (heterozygotes), or noncarrier (normals). Mean urine cystine levels for affected individuals, carriers, and noncarriers were 1816 ± 648, 330 ± 278, and 36 ± 15 μmol/g creatinine, respectively (normal range 5–62). Urine cystine levels in all living parents shown in figure 1 were above the normal range.

This resulted in a change of the diagnosis in three individuals; individual 157-07 changed from unaffected to affected (urine cystine level 930 μmol/g creatinine). Individuals 174-04 and 157-11 were found to be carriers (urine cystine levels 336 and 254 μmol/g creatinine, respectively) and therefore were classified as unaffected.

Fifty-six individuals were included in this panel. On the basis of these quantitative urine cystine levels, 23 individuals were classified as affected, 18 as carriers, and 6 as noncarriers. Quantitative urine results were unavailable for nine sibs included in this panel. These individuals were classified as unaffected on the basis of both the lack of symptoms and thin-layer urine chromatography. Twenty-one of the 23 homozygotes and 2 of the heterozygotes suffered from kidney stones.

Twenty milliliters of heparinized blood were drawn from each family member, and the lymphocytes were immortalized with the Epstein-Barr virus. DNA was extracted according to standard techniques (Davis et al. 1986).

Sequencing

Transcripts of the *SLC3A1* cDNA from six Libyan patients were amplified and sequenced as described elsewhere (Pras et al. 1995). For sequencing of the 200 bp 5′ to the initiation codon, we first amplified this segment, using the primers 5′ GGTGATCTCTGCCCGCCTCGGC 3′ (−241 to −219, relative to the adenosine in the initiation codon) and 5′ CGATGGAGTCTCTCTTGCTT 3′ (+34 to +14) (Pras et al. 1996). The amplification product was purified (Ultrafree-MC; Millipore) and sequenced with a commercial kit (Perkin-Elmer).

Screening of P1 Clones for $(AC)_n$ Repeats

A clone, P1-DMPC/HFF1/1619 C7, which contains the entire *SLC3A1* gene (Pras et al. 1996), was screened for $(AC)_n$ repeats by use of degenerate primers, as described elsewhere (Hawkins et al. 1994). This clone was found to contain one $(AC)_n$ repeat, which was amplified with the primers Q11 (5′ CCCTTTTTTCTGTTCCC 3′) and Q12 (5′ CCTCAGATTACTCAGTAGAGT 3′).

For determining the location of the $(AC)_n$ repeat, clone P1-DMPC/HFF1/1619 C7 was digested with *Eco*RI, blotted onto nylon membranes, and probed first with the ^{32}P end-labeled Q11 and then with Q12. For further confirmation of the repeat location, amplified introns from the *SLC3A1* gene were blotted onto nylon membranes and were probed with Q11 and Q12.

Genotyping

A genomewide search was performed with dinucleotide repeats, evenly spaced throughout the genome, obtained from the Généthon map (Gyapay et al. 1994). Markers described in this study include *D19S410, D19S409, D19S882, D19S225, D19S208, D19S220, D19S422, D19S223, D2S177, D2S119, D2S391, D2S288,* and the new intragenic marker that we have developed, *Q11/Q12*. PCR reactions were carried out in a 10-μl volume containing 50 ng of genomic DNA, 13.4 ng of each unlabeled primer, 0.2 mM dNTPs, and 0.08 μg of ^{32}P-labeled primer, in 1.5 mM $MgCl_2$ PCR buffer, with 1.2 U of *Taq* polymerase (Perkin-Elmer). After an initial denaturation of 5 min at 94°C, 30 cycles were performed (94°C for 30 s, 55°C for 30 s, and 72°C for 40 s), followed by a final extension time of 5 min at 72°C. For the markers *D2S177, D2S119, D2S391, D2S288,* and *Q11/Q12,* we used an annealing temperature of 57°C instead of 55°C. Samples were mixed with an equal volume of loading buffer, denatured at 94°C for 5 min, and electrophoresed on a 6% denaturing polyacrylamide gel.

Linkage Analysis

Linkage was calculated with the LINKAGE (version 5.1) package of computer programs (Lathrop et al. 1984), under the assumptions of a recessive model of inheritance, 100% penetrance in both sexes, and a gene frequency of .02. Recombination fractions were transformed into map distances by Kosambi's (1944) formula. Allele frequencies for the markers were calculated on the basis of 20 unrelated control individuals of Libyan Jewish origin and on the basis of the noncarrier

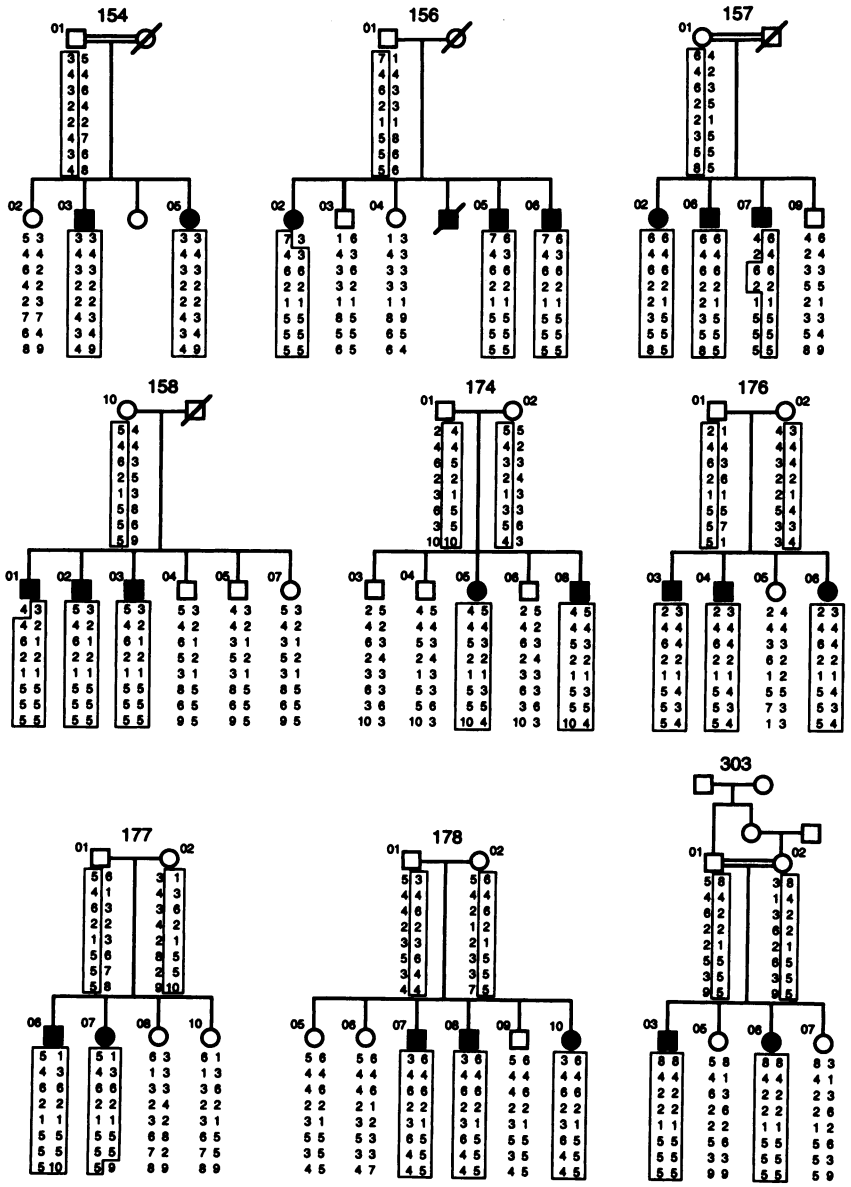

Figure 1 Typings for eight markers on chromosome 19q (from top to bottom): *D19S410, D19S409, D19S882, D19S225, D19S208, D19S220, D19S422,* and *D19S223*. Carrier chromosomes in the parents and in affected individuals are presented in boxes.

chromosomes derived from the Libyan Jewish cystinuria families.

Multipoint LOD scores were calculated by use of the HOMOZ program, which permits multipoint linkage analysis for both consanguineous and nonconsanguineous nuclear families, for a dense map of highly polymorphic markers (Kruglyak et al. 1995). The marker order and distance, which either were taken from published sources (Gyapay et. al 1994; Dib et al. 1996) or were estimated from observed recombination events in our family data, are as follows: *19qter–D19S410–8* cM–*D19S409–0.01* cM–*D19S882–5* cM–*D19S225–3* cM–*D19S208–4*cM–*D19S220–1* cM–*D19S422–1* cM–*D19S223–pter* and *2pter–D2S177–6.2* cM–*D2S119–0.5* cM–*Q11/Q12–3.8* cM–*D2S391–0.8* cM–*D2S288–qter.*

Linkage Disequilibrium and Haplotype Analysis

Parental alleles associated with disease susceptibility were identified in each pedigree. In family 303, which is composed of an uncle-niece marriage, the cystinuria gene and the flanking chromosomal region were derived from a single ancestral chromosome. Therefore, the disease-associated allele was counted only once. Control allele distributions were determined from 20 unaffected control individuals of Libyan Jewish origin and from the

noncarrier chromosomes in the Libyan Jewish cystinuria families. The significance of allelic associations at a single locus was determined by the χ^2 statistic for multiallelic loci (Hill 1975; Weir and Cockerham 1978), as described elsewhere (Aksentijevich et al. 1993). The Bonferroni procedure was used to adjust for multiple comparisons (Weir 1990). Haplotypes were inferred so as to minimize recombinants.

Results

Exclusion of the SLC3A1 *Region on Chromosome 2*

Sequencing of the whole coding region and 200 bp 5′ to the initiation codon in patients from different families did not reveal any mutations. Since mutations could exist in the introns or in parts of the promotor that were not screened, we developed a short microsatellite marker designated "*Q11/Q12*," from within intron 9 of the *SLC3A1* gene, and tested for linkage in these families. At least four recombinant events were detected by use of this intragenic marker (data not shown), and LOD-score calculations excluded 1.7 cM from each side of this marker. Since the *SLC3A1* gene spans ~45,000 bp, these findings virtually exclude it as the cause of cystinuria in these families. Additional LOD-score calculations for the markers *D2S177, D2S119, D2S391,* and *D2S288* are presented in table 1. When these unlinked Libyan Jewish families were removed from the original panel (Pras et al. 1994), *SLC3A1* was placed centromeric to *D2S119,* rather than telomeric to it, as we had reported elsewhere. Multipoint LOD scores in the Libyan Jewish families, calculated with the five markers as fixed points, yielded a result of <-10 throughout the entire *D2S177-D2S391* interval.

Previously, three of the Libyan Jewish families had shown apparent linkage to chromosome 2. In one of these families, the addition of an affected sib reversed the chromosome 2 linkage. In the other two families, normal urinary cystine levels were found in sibs who should have been carriers on the basis of their chromosome 2 haplotypes. Moreover, quantitative urinalysis in these two families demonstrated a non–type I disease (mean urine cystine level in obligatory heterozygotes from these two families was 304 ± 101 μmol/g creatinine), and no mutations in the *SLC3A1* coding or promoter regions were found in affected members from these families.

Table 1

Two-Point LOD Score, between Cystinuria in Libyan Jewish Families and Chromosome 2p Markers

	LOD Score at θ =						
Locus	.00	.05	.10	.15	.2	.25	.30
D2S177	−∞	−4.76	−2.25	−1.06	−.43	−.11	.03
D2S119	−∞	−1.26	.23	.75	.87	.79	.61
Q11/Q12	−∞	−.69	−.02	.24	.32	.31	.43
D2S391	−∞	−7.04	−3.74	−2.13	−1.21	−.66	−.35
D2S288	−∞	−2.64	−1.43	−.84	−.50	−.29	−.16

Linkage to Chromosome 19

After testing >75 microsatellites, linkage was detected with chromosome 19q markers. Seven markers from the long arm of chromosome 19 showed LOD scores >3.00. One marker, *D19S409,* gave a positive LOD score of <3.00 but was uninformative for most of the families. Pairwise LOD scores between cystinuria and the chromosome 19 markers are presented in table 2. A maximal two-point LOD score of 9.22 was obtained with the marker *D19S882* at θ = .00.

Multipoint linkage results for the nine families, with eight markers used as fixed points simultaneously, are presented in figure 2. The maximal LOD score of 11.52 occurred at the location of *D19S225,* and the 1-LOD-unit support interval was within the 8 cM between *D19S882* and *D19S208.*

Figure 1 shows typing results for the Libyan Jewish cystinuria families, with eight chromosome 19 markers. Two affected individuals, 157-07 and 177-07, were recombinant for *D19S223.* Only one of them, 157-07, was recombinant for *D19S220* and *D19S208* (*D19S422* was not fully informative for this family). This recombinant defines the telomeric boundary of the interval. Three recombinants (individuals 156-02, 157-07, and 158-01) are seen with the marker *D19S410,* and one recombinant (157-07) is seen with *D19S409,* defining the centromeric boundary of the interval, which spans 8 cM. No recombination events were detected with the markers *D19S225* and *D19S882,* both of which were fully informative for family 157.

For nine of the unaffected sibs in these families, we have performed quantitative urinary amino acid analysis, which clearly distinguishes between carriers and noncarriers. We found a complete correlation between the phenotype and the genotype in these unaffected sibs. Individuals 156-04, 157-09, 176-05, 174-03, 174-06, and 177-08 had normal urinary cystine levels and a corresponding noncarrier haplotype. Individuals 158-07, 174-04, and 178-09 had elevated urinary cystine levels and a matching carrier haplotype. Individual 174-04 inherited a carrier chromosome from the father and a recombinant chromosome from the mother. Alleles from *D19S410* and *D19S409* are from the maternal carrier chromosome, but alleles from *D19S225, D19S208, D19S220, D19S422,* and *D19S223* were inherited from the maternal noncarrier chromosome. For *D19S882,* the maternal chromosome is uninformative. In view of the carrier phenotype, these findings provide

Table 2

Two-Point LOD Score, between Cystinuria and Chromosome 19 Markers in Libyan Jewish Families

Locus	LOD Score at θ = .00	.05	.10	.15	.2	.25	.30	Maximum θ	Maximum LOD Score
D19S410	−∞	4.52	4.42	3.93	3.27	2.55	1.82	.07	4.56
D19S409	−∞	2.04	1.90	1.65	1.35	1.05	.77	.05	2.04
D19S882	9.22	8.58	7.57	6.44	5.25	4.04	2.87	.00	9.22
D19S225	7.38	6.56	5.70	4.83	3.94	3.05	2.18	.00	7.38
D19S208	−∞	4.41	3.97	3.38	2.72	2.06	1.42	.03	4.47
D19S220	−∞	6.48	5.80	4.97	4.08	3.17	2.27	.02	6.67
D19S422	8.57	7.62	6.60	5.53	4.45	3.38	2.35	.00	8.57
D19S223	−∞	5.94	5.50	4.78	3.94	3.06	2.17	.04	5.95

further evidence that the gene is telomeric to *D19S409*. For the other unaffected sibs, we were unable to obtain quantitative urine results.

Two markers, *D19S422* and *D19S882*, which are located 14 cM apart, gave high LOD scores at θ = .00, whereas several markers between them yielded a negative LOD score at θ = .00. This results from the fact that *D19S422* was uninformative for the maternal allele in family 157 (fig. 1) and therefore concealed a recombination event, which was detected by other markers in the region.

We have determined the allele distributions for chromosome 19 markers among Libyan Jewish cystinuria carrier chromosomes, for noncarrier chromosomes derived from 20 normal individuals of Libyan Jewish origin, and for the noncarrier chromosomes in the Libyan Jewish cystinuria families (table 3). When the Bonferroni procedure was used to adjust for multiple comparisons, a *P* value of .006 was statistically significant. Significant linkage disequilibrium was observed at *D19S882*, *D19S225*, *D19S208*, and *D19S422*. For the locus *D19S225*, all cystinuria chromosomes carried allele 2, whereas only 27 of 56 control chromosomes carried that allele. This result suggests that the gene causing cystinuria in these families lies close to the marker *D19S225*, and it is consistent with the multipoint data. Linkage disequilibrium reflects recombinants that have occurred in the carrier chromosomes in past generations. The degree of significance for a given marker depends on the allele distribution in carrier and noncarrier chromosomes. Linkage expressed by LOD score depends mainly on the informativeness of a marker and on recombinants that occurred in the last generation. Therefore, markers that yield the highest LOD scores do not necessarily disclose the most significant linkage-disequilibrium results.

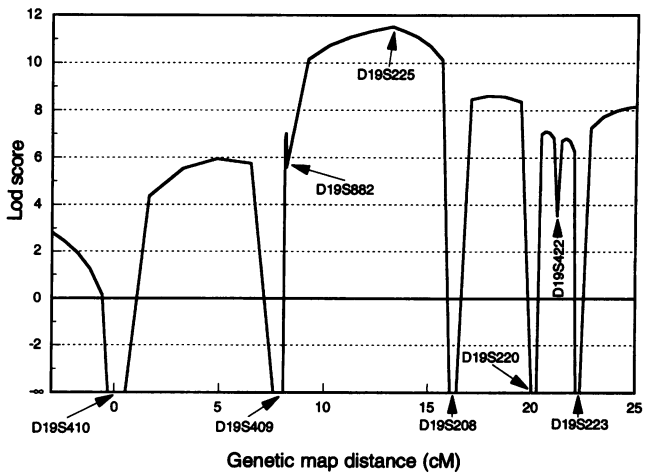

Figure 2 Multipoint linkage analysis between the Libyan Jewish cystinuria gene and markers on chromosome 19, for nine families. *D19S410* was arbitrarily assigned position 0; and multipoint LOD scores were calculated at each marker loci and at four equally spaced points within each intermarker interval. The maximal LOD score was obtained at the *D19S225* locus.

Examination of haplotypes of all affected individuals showed that the haplotype comprising the markers *D19S225*, *D19S208*, *D19S220*, and *D19S422* (alleles 2-1-5-5) was detected in 11 of 17 cystinuria chromosomes and in only 1 of 58 control chromosomes ($P < .000001$). The haplotype comprising the markers *D19S208*, *D19S225*, and *D19S882* (alleles 1-2-6) was found in 8 of 17 carrier chromosomes but in only 2 of 58 noncarrier chromosomes ($P < .00003$). These results provide strong evidence of a common ancestry among these families.

Discussion

Elsewhere we have reported linkage with homogeneity, between cystinuria and three markers on the short arm of chromosome 2, in a panel containing eight Libyan Jewish and nine non-Libyan families (Pras et al. 1994). The admixture test performed in that study failed

Table 3

Allelic Association between Libyan Jewish Cystinuria and Chromosome 19 Markers

Locus	Allele Number (bp)	Carrier Chromosomes	Noncarrier Chromosomes	χ^2	*P* (df)
D19S410	3 (168)	5/17 (30%)	14/54 (26%)	7.70	.36 (7)
D19S409	4 (169)	14/17 (82%)	28/54 (52%)	9.31	.05 (4)
D19S882	6 (271)	10/17 (59%)	6/57 (11%)	22.67	.001 (6)[a]
D19S225	2 (178)	17/17 (100%)	19/56 (34%)	22.77	.0004 (5)[a]
D19S208	1 (171)	13/17 (76%)	16/52 (31%)	12.74	.0017 (2)[a]
D19S220	5 (275)	11/17 (65%)	18/57 (32%)	11.33	.183 (8)
D19S422	5 (189)	13/17 (76%)	10/56 (18%)	26.29	.0002 (6)[a]
D19S223	5 (238)	9/17 (53%)	9/54 (17%)	15.17	.09 (9)

[a] Statistically significant at the .05 level, adjusted for multiple comparisons by the Bonferroni procedure.

to reveal evidence of heterogeneity. Subsequently, evidence has accumulated suggesting that mutations in the *SLC3A1* gene located on chromosome 2 cause type I disease (Gasparini et al. 1995; Horsford et al. 1996) and that at least one other gene is involved in type II disease and type III disease (Calonge et al. 1995).

We have found several lines of evidence excluding *SLC3A1* as the cause of cystinuria in Libyan Jewish patients. First, SSCP analysis (Pras et al. 1995) and sequencing failed to reveal *SLC3A1* mutations in these individuals. Second, a polymorphism from within the *SLC3A1* gene excluded 1.7 cM from each side of this marker. Third, multipoint analysis in the Libyan Jewish families excluded the disease locus from the entire *D2S177-D2S391* interval. Quantitative urinary amino acid analysis in these families demonstrated non–type I disease (final determination as to whether these families have type II disease or type III disease will depend on oral loading tests).

Taken together, these data strongly support the stratification of our original sample into Libyan and non-Libyan subsets. When Morton's test (Ott 1991) is applied to these two subgroups, there is significant evidence for heterogeneity at the chromosome 2 locus (data not shown). Nevertheless, even after we had expanded the Libyan subset of families and had introduced the phenotype corrections, the admixture test (which does not assume a particular stratification scheme) did not detect locus heterogeneity.

A genomewide search that we performed after excluding the *SLC3A1* region on chromosome 2 shows that a second susceptibility gene for cystinuria maps to the long arm of chromosome 19. Pairwise LOD scores >3.00 were achieved for seven markers. Multipoint results yielded a result $>+11$. Analysis of recombinants and multipoint data placed the gene in an 8-cM interval, between *D19S409* and *D19S208*. Haplotype data in all nine families is consistent with linkage to chromosome 19.

We have found both significant linkage disequilibrium with alleles from four of the markers and a specific haplotype associated with the carrier chromosome. These findings suggest that a single mutation that was introduced by a common ancestor segregates within these families. The Jewish community in Libya has lived in relative isolation and has had a high degree of inbreeding, factors that tend to obscure recombination and thereby extend linkage disequilibrium over more generations. Given the size of the region over which linkage disequilibrium can be seen, we would speculate either that the mutation has been introduced into this population fairly recently or that there has been a recent bottleneck.

Important advances have been made in recent years in understanding the physiology of cystine transport. Studies on isolated renal tubules have suggested that two separate systems are involved in cystine uptake. A high-affinity system, which is shared with lysine, arginine, and ornithine, has been localized to the proximal straight tubule and is responsible for the absorption of 10% of L-cystine. A distinct low-affinity, unshared system, located in the proximal convoluted tubule, is responsible for 90% of L-cystine reabsorption (Segal et al. 1977). Of these two transport systems only the high-affinity system interacts with the dibasic amino acids and, therefore, is likely to be involved in classical cystinuria (McNamara et al. 1981).

The *SLC3A1* gene product has been localized, in the kidney, to the proximal straight tubule and is probably one component of the high-affinity system (Furriols et al. 1993; Pickels et al. 1993). Studies of *SLC3A1* suggest that the functional transporter might be composed of at least two subunits (Mosckovitz et al. 1994). Recently, Wang and Tate (1995) have shown, both in rats and in

rabbits, that a 50-kD protein is bound to the SLC3A1 protein on the cell membrane by disulfide bonds. The gene sequence and the chromosomal location of this 50-kD protein are still unknown. Libyan Jewish cystinuria patients exhibit a urinary increase both in cystine and in the dibasic amino acids and therefore are likely to have a defect in the high-affinity system. Mutations in these patients may exist in the 50-kD protein or in other, yet unidentified components of the high-affinity system.

On chromosome 19, the 8-cM region that contains the Libyan Jewish cystinuria gene is fully covered by a YAC contig (Garcia et al. 1995). *SCN1B*, a gene encoding the sodium-channel beta-1 subunit, has been mapped to this interval (Makita et al. 1994). The sodium dependence of cystine reabsorption has been controversial for years. Although initially the high-affinity system was believed to be sodium dependent, recent studies both in the rat and in humans suggest it to be sodium independent (McNamara et al. 1992; Mora et al. 1996). Thus, from a physiological point of view, it is unlikely that *SCN1B* is the Libyan Jewish cystinuria gene. No other candidate genes have been mapped to this region.

In light of the data presented in this report, it would be interesting to check the type III families described by Calonge et al. (1995), for linkage to chromosome 19. Possibly yet a third locus encodes susceptibility in those families.

Acknowledgments

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