CTNS Mutations in an American-Based Population of Cystinosis Patients

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Summary

Nephropathic cystinosis is an autosomal recessive lysosomal storage disease characterized by renal failure at 10 years of age and other systemic complications. The gene for cystinosis, *CTNS*, has 12 exons. Its 2.6-kb mRNA codes for a 367–amino-acid putative cystine transporter with seven transmembrane domains. Previously reported mutations include a 65-kb "European" deletion involving marker *D17S829* and 11 small mutations. Mutation analysis of 108 American-based nephropathic cystinosis patients revealed that 48 patients (44%) were homozygous for the 65-kb deletion, 2 had a smaller major deletion, 11 were homozygous and 3 were heterozygous for 753G→A (W138X), and 24 had 21 other mutations. In 20 patients (19%), no mutations were found. Of 82 alleles bearing the 65-kb deletion, 38 derived from Germany, 28 from the British Isles, and 4 from Iceland. Eighteen new mutations were identified, including the first reported missense mutations, two in-frame deletions, and mutations in patients of African American, Mexican, and Indian ancestry. *CTNS* mutations are spread throughout the leader sequence, transmembrane, and nontransmembrane regions. According to a cystinosis clinical severity score, homozygotes for the 65-kb deletion and for W138X have average disease, whereas mutations involving the first amino acids prior to transmembrane domains are associated with mild disease. By northern blot analysis, *CTNS* was not expressed in patients homozygous for the 65-kb deletion but was expressed in all 15 other patients tested. These data demonstrate the origins of *CTNS* mutations in America and provide a basis for possible molecular diagnosis in this population.

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Introduction

Classical nephropathic or infantile cystinosis (MIM 219800) is a rare autosomal recessive disorder characterized by lysosomal storage of free, nonprotein cystine (Gahl 1986; Gahl et al. 1995). Cystinosis patients are normal at birth but develop renal tubular Fanconi syndrome at 6–12 mo of age, accompanied by failure to thrive, polyuria and polydipsia, dehydration, and hypophosphatemic rickets. Photophobia and hypothyroidism generally appear in the 1st decade of life. Glomerular damage results in renal failure at ~10 years of age (Gahl et al. 1995). After renal transplantation, progressive cystine storage can cause a distal vacuolar myopathy (Charnas et al. 1994), swallowing difficulty (Sonies et al. 1990), retinal blindness (Kaiser-Kupfer et al. 1986), diabetes mellitus (Fivush et al. 1987), and neurological deterioration (Ehrich et al. 1979; Fink et al. 1989). Milder variants of the classical disease include an intermediate form with late-onset renal disease and a benign form with corneal involvement but no renal impairment (Gahl et al. 1995). Oral therapy with the free thiol cysteamine lowers the cystine content of leukocytes (Thoene et al. 1976) and a variety of cell types, including muscle (Gahl et al. 1992). Cysteamine has proven efficacy in preventing renal deterioration, enhancing growth, and obviating the need for thyroid-hormone replacement (Gahl et al. 1987; Markello et al. 1993; Kimonis et al. 1995).

The basic defect in cystinosis is deficiency of a cystine carrier in the lysosomal membrane (Gahl et al. 1982*a*, 1982*b*; Jonas et al. 1982). The cystine-transport system is saturable and bidirectional (Gahl et al. 1983), and it displays a gene-dosage effect in obligate heterozygotes for cystinosis (Gahl et al. 1984). The therapeutic action of cysteamine involves its reaction with cystine to produce the free thiol amino acid cysteine and the mixed disulfide cysteine-cysteamine, both of which exit the cystinotic lysosome by a process not requiring the defective carrier system (Gahl et al. 1985; Pisoni et al. 1985).

The gene for cystinosis was first mapped to a 4-cM region of chromosome 17p13, by linkage analysis (Cystinosis Collaborative Research Group 1995). The critical

Table 1

Severity Score Parameters

Parameter	Severity Score		
	1	2	3
Age at onset (mo)	>15	9–15	<9
Leukocyte cystine (nmol half-cystine/mg protein)[a]	<5	5–15	>15
Fanconi syndrome index (μmol/kg/d)[b]	<500	500–2,000	>2,000
Age at renal failure (years)	>10	8–10	<8
Age at nonrenal complication (years)	None	⩾20	<20

[a] Not receiving cysteamine therapy. Normal value is <0.2 nmol half-cystine/mg protein.

[b] Normal value is 95 ± 45 μmol/kg/d (Charnas et al. 1991).

region was progressively narrowed (Jean et al. 1996; McDowell et al. 1996; Peters et al. 1997), and several cystinosis patients were found to have deletions in a specific microsatellite marker, *D17S829*, in the region. Physical mapping near this deletion led to isolation of the cystinosis gene, *CTNS* (Town et al. 1998; Genome Database, accession number 700761). The gene has 12 exons spanning 23 kb of genomic DNA. Its 2.6-kb mRNA codes for a 367–amino acid protein, cystinosin, with homology to a 55.5-kD *Caenorhabditis elegans* protein and to a yeast transmembrane protein, ERS1 (Town et al. 1998). Cystinosin has seven transmembrane domains, a GY dipeptide near the C terminus, and eight potential glycosylation sites. Eleven different mutations in exons 3, 4, and 6–9 have been described, with no correlation with clinical phenotype (Town et al. 1998). The most common mutation, involving *D17S829*, consists of a deletion extending from intron 10 upstream ⩾65 kb.

We report the *CTNS* mutations identified in an American-based population of 108 nephropathic cystinosis patients, with concomitant phenotype correlations employing a clinical severity score. We trace the possible origin of the 65-kb deletion, describe 18 previously unreported mutations, and delineate the mutations present in cystinosis fibroblasts available from the Human Genetic Mutant Cell Repository (HGMCR). Finally, we identify the extent of *CTNS* gene expression in a sample of cystinosis fibroblasts with various mutations.

Patients, Materials, and Methods

Patients

All patients or their parents gave informed consent to participate in Institutional Review Board–approved protocols for the study of cystinosis at the National Institutes of Health (NIH) Clinical Center or the University of Michigan Medical Center. Of 108 patient DNA samples, 102 were obtained from individuals who could potentially provide clinical information; 5 samples were from skin-biopsy fibroblasts stored at the NIH, and one patient was unreachable. The subjects of this investigation had classical nephropathic cystinosis, with onset of their disease in early childhood, and came from throughout the United States, Mexico, and Iceland.

Severity was determined in accordance with five parameters (table 1), each assigned a score of 1 (mild), 2 (average), or 3 (severe). The final severity score was the mean of all applicable parameter scores, which were obtained by history and chart review. Age at onset was the age at the first clear appearance of symptoms. The leukocyte cystine value refers to a level obtained prior to initiation of cysteamine therapy. For a sample of 66 patients, the mean leukocyte cystine value, in nanomoles half-cystine/milligram protein, was similar in three different age groups: 10.1 for 33 patients <5 years of age; 8.9 for 16 patients 5–15 years of age; and 12.2 for 17 patients >15 years of age. The normal value is <0.2 nmol half-cystine/mg protein. The Fanconi syndrome index offers an objective measure of the severity of Fanconi syndrome, which is not influenced by cysteamine therapy (Thoene et al. 1976: Gahl et al. 1987), by quantitating the daily urinary excretion of 21 amino acids (Charnas et al. 1991). Age at renal failure (i.e., dialysis or transplantation) and nonrenal complications (i.e., myopathy, swallowing difficulty, retinal blindness, CNS deterioration, and diabetes mellitus requiring insulin therapy) applied only to patients who had not received previous cysteamine therapy. For 102 individuals for whom a clinical history was available, a total of 277 parameters were applicable, for a mean of 2.7 parameters/patient. At least two parameters were required in order to assign a se-

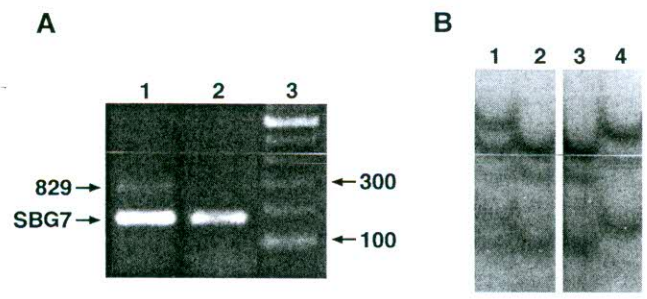

Figure 1 *A*, Gel-electrophoresis pattern for markers *D17S829* (266 bp; denoted by "829"), within the common 65-kb deletion region, and SBG7 (168 bp), outside the deletion region. Samples were from a nondeletion patient (lane 1) and a deletion patient (lane 2). Lane 3 shows the size markers. *B*, SSCP analysis of exon 7. Lanes 2 and 3 show the normal band pattern. Lane 4 contains DNA from a patient subsequently shown by sequencing to be homozygous for 753G→A (W138X). DNA from a patient heterozygous for this mutation was run in lane 1. All the lanes are from the same gel; intervening lanes were removed.

verity score; 97 of 102 patients fulfilled this criterion. All severity scores were determined without knowledge of the patient's mutation.

Cells

Cystinosis fibroblast strains (GM 8, 18, 46, 90, 304, 489, 706, 760, 2066, 2894, and 6966) were purchased from the HGMCR or were obtained by skin biopsy after informed consent. They were cultured as described elsewhere (Tietze et al. 1989). Leukocytes were prepared in accordance with procedures published previously (Thoene et al. 1976; Steinherz et al. 1982). DNA was extracted from whole blood, leukocytes, or fibroblasts, by use of standard techniques (Sambrook et al. 1989).

PCR Detection of Homozygous D17S829 *Deletions*

By use of genomic DNA from leukocytes or fibroblasts as a template, a 266-bp fragment of *D17S829* in intron 3 of *CTNS* was amplified by PCR. The 25-μl reaction volume contained 1 × PCR buffer (GIBCO-BRL), 1.5 mM $MgCl_2$, 200 μM dGTP, 200 μM dATP, 200 μM dTTP, 200 μM dCTP, 0.2 μM primers, 2.5 U *Taq* DNA polymerase, and 500 ng template DNA. After an initial denaturation step at 94°C for 4 min, PCR was conducted for 30 cycles, each with a denaturation step at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s. An elongation step at 72°C for 10 min finalized the procedure. The PCR products were electrophoresed in 1% agarose and were stained with ethidium bromide.

Table 2

Types of Mutations in Cystinosis Patients with Clinical Data

Mutation Status	No. of Patients
D17S829 deletion (homozygous)	50
W138X:	
Homozygous	11
Heterozygous	3
Other mutations:[a]	
Two alleles identified	18
One allele identified	6
No mutations identified	20
Total	108

[a] Includes 17 mutations not identified previously.

As a positive control, DNA templates were demonstrated, by PCR amplification (fig. 1*A*), to contain a marker, SBG7, which is contained on YAC 767F9 of chromosome 17 (McDowell et al. 1996) but which is outside of the 65-kb deletion area since SBG7 was amplified in all cystinosis patients. The primers for this marker were 5′-TTG CTG GGC TTA ATA CCT AGG T-3′ and 5′-ATT GCT TTT GCT GTA TTC TAT AG-3′. DNA templates that were not PCR amplified for *D17S829* were also analyzed for the extent of the deletion, by PCR amplification using four more primer pairs, that is, exons 11 and 3 of *CTNS* and expressed sequence tag (EST) markers AA313583 and T85505 (Town et al. 1998). Primers for T85505 were 5′-ATC CTG TTT CAA ATG ATG ACC C-3′ and 5′-GTG AGA

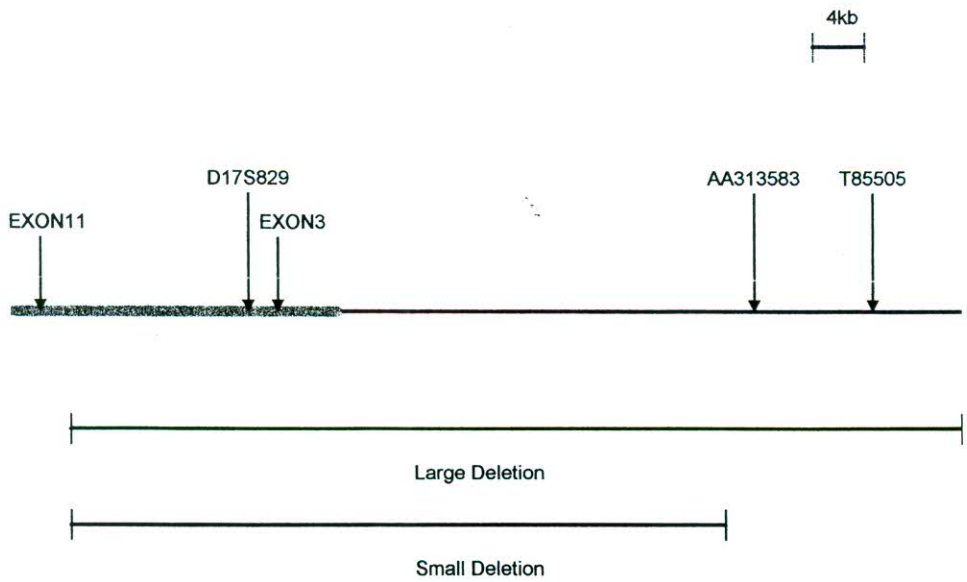

Figure 2 Gross map of the *CTNS* gene and upstream region. Five markers, *D17S829*, AA313583, T85505, and the PCR products of exons 3 and 11, helped characterize deletions in cystinosis patients. The common 65-kb "large" deletion encompasses the first four markers, with the 3′ breakpoint before exon 11. A smaller deletion, found in two patients and in fibroblast strain GM8, involves *D17S829* and exon 3 but not exon 11 or markers AA313583 and T85505. The maximum size of the smaller deletion is shown.

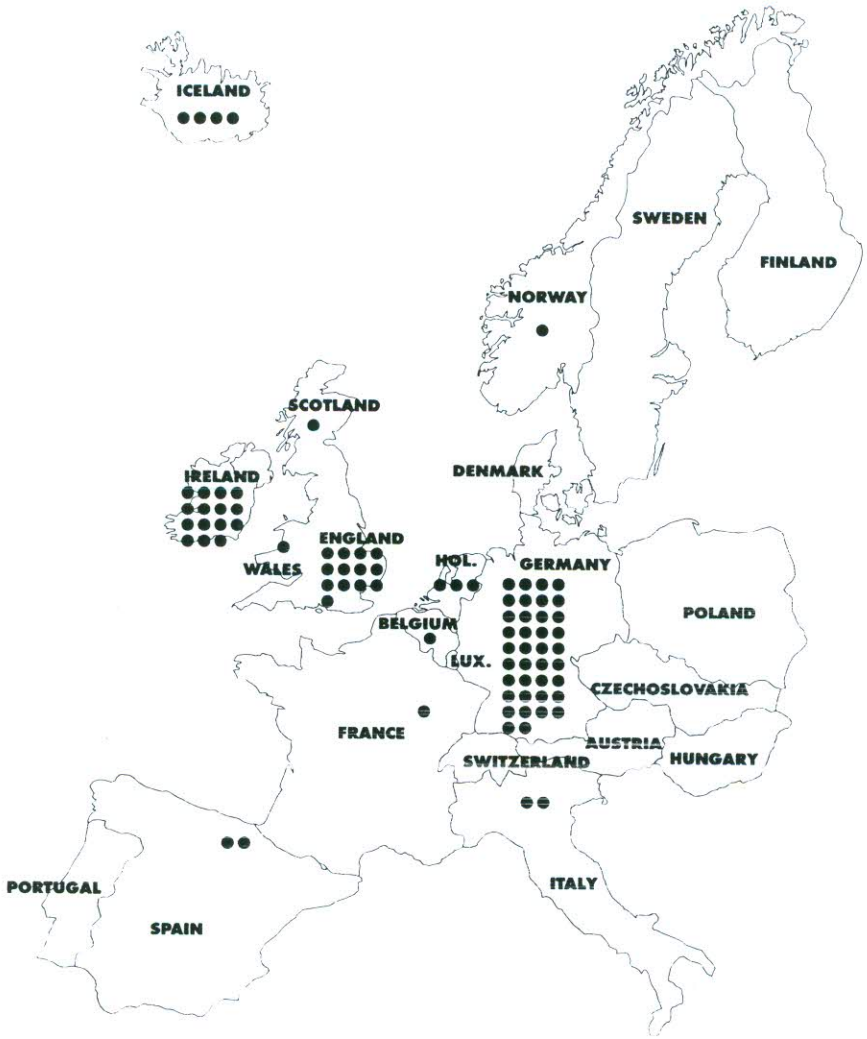

Figure 3 Distribution of 65-kb–deletion alleles. Eighty-two alleles of 48 *CTNS* deletion patients were assigned a most likely country of origin, on the basis of family history. Each blackened circle represents one allele.

TAA ATA CGG CTC TCG-3′. Primers for AA313583 were 5′-TCT TGC TGC ATG TAC TCA GC-3′ and 5′-TGG GAT ACA GAG TTC TCT TTG-3′.

SSCP Analysis

Exons 3–12 of *CTNS* were amplified from genomic DNA (500 ng) in a volume of 25 μl, by use of a radioactive PCR mixture consisting of 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dGTP, 200 μM dATP, 200 μM dTTP, 200 μM dCTP, 1 μCi α[^{32}P]-dCTP, 0.2 μM primers, and 2.5 U *Taq* DNA polymerase. After PCR amplification for 30 cycles, as stated above, 3 μl of PCR product was treated with 10 μl of SSCP stop solution and was denatured at 94°C for 4 min. The samples then were fractionated by electrophoresis at 4°C for 6–8 h on a 6% nondenaturing precast gel (Stratagene), with 1 × Tris borate–EDTA (TBE), and were subjected to autoradiography. An example of the results of SSCP analysis of exon 7 is shown in figure 1*B*.

Base-Excision Sequence Scanning (BESS) T-Scan Mutation Detection

Primers of exons 3–12 were 5′ end labeled by use of γ[^{32}P]-ATP and T4 polynucleotide kinase (Epicenter), as recommended by the company. After end labeling the primers, exons 3–12 were amplified from genomic DNA (500 ng), in a 25-μl reaction with 5 pmol of radioactive primer, 5 pmol of nonradioactive primer, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dGTP, 200 μM dATP, 200 μM dTTP, 200 μM dCTP, 16 μM dUTP, and 2.5 U *Taq* DNA polymerase. PCR conditions were identical to those for SSCP. Eight microliters of PCR product was

Table 3

Individual Mutations in American-Based Cystinosis Patients and Fibroblasts

Case	Ethnicity[a]	Severity Score	mRNA[b]	Exon(s)	Base Change	AA Change	Stop Codon
Two mutations identified:							
1	Eng/Eng	2.7	ND	3	357delGACT[c,d]	T7F	13
				3	357delGACT[c]	T7F	13
2	Ind/Ind	1.3	+	3	357delGACT[c,d]	T7F	13
				3	357delGACT[c]	T7F	13
3	Ger/Ger	2.3	ND	3	357delGACT[c,d]	T7F	13
				3	357delGACT[c]	T7F	13
4	Hol-Eng/Hun	2.5	ND	3	357delGACT[c]	T7F	13
				10	1033insCG	V233A	253
5	ND	ND	+	5	537del21bp[d]	del67–73[e]	...
				5	537del21bp	del67–73[e]	...
6	Eng-Wal/Ger	2.0	+	5	537del21bp[d]	del67–73[e]	...
				5	537del21bp	del67–73[e]	...
7	Nor-BrI/Ger-BrI	1.3	+	5	537del21bp[d]	del67–73[e]	...
				5	537del21bp	del67–73[e]	...
8	Ger-BrI/Ger	2.0	ND	6	651delTCAC[d]	H105P	116
				6	651delTCAC	H105P	116
9	Fra/Eng	1.7	ND	7	721C→T[c,d]	Q128X	128
				7	721C→T[c]	Q128X	128
10	Fra/Swe	2.0	+	8	845G→A[d]	G169D	...
				8	845G→A	G169D	...
11	Aus-Swi/Den-Ire	2.0	+	8	883T→C	W182R	...
				12	1367TCGTCTTC→A	I343K	364
12	Ger/BrI	2.5	ND	8	900delG[c,d]	5′ Splice	...
				8	900delG[c]	5′ Splice	...
13	Por/Por	1.7	ND	9	950delACG[d]	D205del	...
				9	950delACG	D205del	...
14	Dom/Dom	2.0	ND	9	985insA[d]	T216N	227
				9	985insA	T216N	227
15	Ita/Ita	1.5	ND	9	952G→A	D205N	...
				11	1232G→A	S298N	...
16	Ger-Ire/ND	1.5	+	10	1035insC	V233R	296
				11	1261insG	S310Q	364
17	Afr/Afr	1.3	ND	11	1209C→G	Y290X	290
				11	1209C→G	Y290X	290
18	Kur-Afg/Eng-Fra	1.0	+	11	1261G→A	G308R	...
				1–10	65-kb deletion[f]	...	...
One mutation identified:							
19	Mex/Mex	2.7	ND	3	357delGACT[c]	T7F	13
20	Eng-Ger/Eng	2.0	+	10	1080delC	F247L	252
21	Pol-Ger/Eng	1.3	ND	10	1035insC	V233R	296
22	ND	ND	+	11	1253A→G	D305G	...
23	Ire-Swe/Ire-Fra	2.3	ND	11	1261G→A	G308R	...
24	Ita/Ita	1.7	ND	12	1354G→A	G339R	...

(*continued*)

subjected to excision/cleavage with 0.5 μl of BESS T-scan excision-enzyme mix and 1 × excision-enzyme buffer (Epicenter), in a final volume of 10 μl. After incubation of the mixture for 30 min at 37°C, 5 μl of stop/loading buffer (Epicenter) was added, and the reaction mixture was heated at 75°C for 5 min. The radiolabeled cleavage products then were loaded onto a 5.5% Long Ranger Precast Gel (Stratagene), with 1 × TBE, and were subjected to autoradiography for 48 h.

Direct DNA Sequencing

Sequencing was performed by use of a PCR-product sequencing kit, from Amersham Life Science, Inc., and 5.5% Long Ranger Precast Gels, in accordance with the manufacturer's recommendations, or by automated sequencing at the University of Michigan core sequencing facility.

Table 3 (continued)

Case	Ethnicity[a]	Severity Score	mRNA[b]	Exon(s)	Base Change	AA Change	Stop Codon
Fibroblasts:							
GM8				1–10	65-kb deletion[g]	...	...
				1–10	65-kb deletion	...	...
GM46				5	545delTCCTT	I69R	73
				5	545delTCCTT	I69R	73
GM706				1–10	65-kb deletion	...	...
				1–10	65-kb deletion	...	...
GM760				7	753G→A[c]	W138X	138
				7	753G→A	W138X	138
GM2894[h]				1–10	65-kb deletion	...	...
				1–10	65-kb deletion	...	...

[a] Eng = England; Ind = Indian subcontinent; Ger = Germany; Hol = Holland; Hun = Hungary; BrI = British Isles, that is, a mix of England, Scotland, Wales, and Ireland; Wal = Wales; Nor = Norway; Fra = France; Swe = Sweden; Aus = Austria; Swi = Switzerland; Den = Denmark; Ire = Ireland; Por = Portugal; Dom = Dominican Republic; Ita = Italy; Afr = African American; Kur = Kurd; Afg = Afghanistan; Mex = Mexico; Pol = Poland; and ND = not determined.

[b] A plus sign (+) indicates present on northern, and a minus sign (−) indicates absent. ND = not determined.

[c] Previously reported by Town et al. (1998).

[d] Patient could be hemizygous for this mutation and could carry the 65-kb deletion.

[e] Amino acids ITILELP deleted.

[f] Genotype analysis indicated that the English/French mother carried the 65-kb deletion.

[g] This cell strain was negative for *D17S829* and exon 3 but was positive for AA313583, T85505, and exon 11 markers.

[h] The donor of this SV40-transformed fibroblast strain was also an NIH patient and is included in the group of 50 deletion patients listed in table 2 (Oshima et al. 1977).

Subcloning and DNA Sequencing

After PCR amplification, PCR products were subcloned into a pNoTA/T7 shuttle vector (5 Prime→3 Prime). Four to six clones were selected and sequenced by use of the universal −20 M13/pUC DNA-sequencing primer or the −24 M13/pUC reverse–DNA-sequencing primer and a T7 Sequenase Quick-Denature Plasmid Sequencing kit (Amersham Life Science), in accordance with the manufacterer's recommendations.

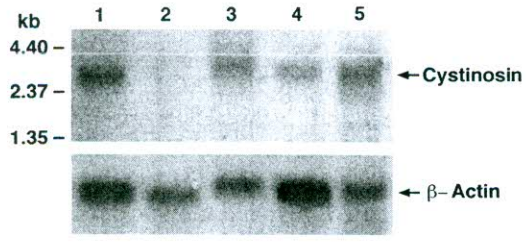

Figure 4 Representative northern blot of patient RNA probed with *CTNS* cDNA. RNA was from controls (lanes 1 and 5), a homozygous 65-kb–deletion patient (lane 2), a patient (case 18) who is compound heterozygous for the 65-kb deletion and G308R (lane 3), and a patient (case 7) who is homozygous for an in-frame deletion of amino acids 67–73 (lane 4). The cytinosin mRNA is ~2.6 kb in size. β-Actin mRNA helped to assess RNA loading; lane 1 served as a control for lane 4, while lane 5 served as a control for lanes 2 and 3.

Northern Blot Analysis

Total RNA (20 μg), isolated from cultured fibroblasts by use of Trizol reagent (Life Technologies), was separated on a 1.2% agarose/3% formaldehyde gel and was blotted onto a Nytran nylon membrane (Schleicher & Schuell) in the presence of 20 × SSC. The blot was prehydridized and then hybridized with ExpressHyb solution (Clontech), at 68°C. The probe was human *CTNS* cDNA, random-primer labeled with α[^{32}P]-dCTP (DuPont/New England Nuclear) and prepared by PCR amplification of exons 3–12 after reverse transcription of total RNA. After hybridization, the blot was washed several times with 2 × SSC/0.05% SDS, at room temperature, and once with 0.1 × SSC/0.1% SDS, at 50°C, and was exposed to Kodak X-Omat AR film, with an intensifying screen, for 24 h at −70°C.

Genotype Analysis

Marker *D17S829* was used for genotype analysis of case 18 and her parents. PCR products were randomly labeled with α[^{32}P]-dCTP (DuPont/New England Nuclear). After PCR amplification, the products were denatured and the polymorphic alleles analyzed on 6% denaturing gels.

Table 4

***CTNS* Polymorphisms and Regional Distribution of Small Mutations**

Domain	Total No. of Amino Acids	Mutation Number	Mutation Names
Leader sequence	22	3	357delGATC; 371delT; 397delTG
Transmembrane	152	11	753G→A; 845G→A; 857delAC; 883T→C; 985insA; 1080delC; 1253A→G; 1261G→A; 1261insG; 1354G→A; 1367TCGTCTTC→A
Nontransmembrane	193	15	479+1G→T; 537del21bp; 545delTCCTT; 622G→T; 651delTCAC; 721C→T; 900delG; 901-1G→C; 908del9bp; 950delACG; 952G→A; 1033insCG; 1035insC; 1209C→G; 1232G→A
Polymorphisms	...	5	669-5T→C; 843A→G, 1020+9A→G; 1214A→G[a]; 1299C→T

[a] This base change alters amino acid 292, from Lys to Arg, and could be pathologic; the other base changes are silent.

Results

DNA from 108 unrelated patients was analyzed by three methods. First, it was tested for PCR-based amplification of *D17S829* (fig. 1*A*). Fifty patients (46%) were homozygously deleted for this marker (table 2). DNA from the remaining patients was subjected to SSCP analysis, followed by sequencing of abnormally migrating exons, and then was analyzed by Bess T scanning to detect still unidentified mutations. These two methods revealed mutations in 38 additional individuals (35%). No mutations were found in the remaining 20 patients (19%).

D17S829 *Deletions*

Of 50 patients deleted in *D17S829*, 48 also were deleted in EST markers AA313583 and T85505 and in exon 3 (Town et al. 1998) but were positive for an exon 11 marker (see fig. 2). These 48 patients were considered to have the common 65-kb European deletion. Two patients had a smaller deletion involving *D17S829* and exon 3 but were positive for AA313583, T85505, and exon 11 (fig. 2). Both were of mixed European heritage and had severity scores of 2.0.

The homozygous, 65-kb–deletion patients were analyzed for their ethnicity and disease severity. Of 96 alleles in 48 patients, 82 were assigned a most likely nation of origin, on the basis of family history. Thirty-eight (46%) of the alleles were from Germany, 15 (18%) from Ireland, and 13 (16%) from England, but the mutation was apparent in alleles derived from Iceland, Italy, and Spain (fig. 3). The Spanish alleles were ascribed to an American with Hispanic parents. For the 46 patients with the large deletion and at least two clinical parameters to assess, the mean ± SD severity score was 1.98 ± 0.40 (standard error 0.06), with a normal distribution around the mean.

Our techniques did not rule out heterozygosity for the 65-kb deletion. Hence, some of the mutations noted below as homozygous actually may be hemizygous, with the second allele carrying the large deletion.

W138X Mutations

Fourteen patients exhibited a G→A substitution at nucleotide 753 of exon 7, giving rise to a Trp → nonsense mutation in codon 138 (table 2). Eleven were homozygous for this mutation; their countries of origin included Germany, France, and the British Isles. The severity scores were either 2.0 or 2.3, with a mean ± SD of 2.13 ± 0.16. The three patients heterozygous for W138X, in addition to being from Germany, France, and the British Isles, had Scandinavian and Romanian heritage. In these individuals, whose severity scores were 1.5, 2.0, and 2.3, no definitive second mutation could be identified.

Other Mutations

Twenty-four patients had mutations other than W138X and the 65-kb deletion (table 3). Three mutations have been reported elsewhere (Town et al. 1998). First, a homozygous GACT deletion resulting in a T7F substitution and truncation at amino acid 13 was present in three patients of English, Italian, and German heritage (cases 1–3, table 3). The parents of case 2 were first cousins. Heterozygous 357delGACT mutations were also found in case 4 and in case 19, whose parents were Mexican. Second, a homozygous C→T substitution in nucleotide 721, which resulted in Q128X, was identified in case 9 (table 3), a 29-year-old man with diabetes mellitus. Finally, a homozygous 900delG mutation, which altered the 5′ splice site of intron 8, appeared in case 12, a 17-year-old girl with a serum creatinine of 1.3 mg/dl who had been treated with cysteamine from 15 mo of age.

Seventeen new mutations were identified in patient DNA (table 3). Three patients (cases 5–7) were homozygous for a 21-bp deletion beginning at nucleotide 537 and eliminating seven amino acids, ITILELP. The pa-

tients had different severity scores (e.g., 1.3 and 2.0) and came from the British Isles and Germany.

Five patients displayed new, private, homozygous mutations (table 3). Case 8 (651delTCAC) received cysteamine therapy from 1 year of age and had a serum creatinine of 0.9 mg/dl at 13 years of age. Her mother is German/Scottish/Irish, and her father is German. Case 10 (845G→A) had a serum creatinine of 1.1 mg/dl, at 8 years of age. Her father is French Canadian, and her mother is Swedish and American Indian. Case 13 (950delACG), whose parents were Portuguese first cousins, is now 20 years of age and had not received cysteamine prior to her renal allograft at 14 years of age. Case 14 (985insA) is a 32-year-old man whose parents are first cousins from the Dominican Republic. He never had significant cysteamine therapy, but he had received a renal allograft at 11 years of age and had developed muscle wasting, hypophonia, and difficulty swallowing in his early 20s. Case 17 (1209C→G) is a 26-year-old African American who had received a kidney allograft at 12 years of age, was diagnosed with cystinosis at 13 years of age, and, despite poor compliance with cysteamine therapy, has avoided the late complications of cystinosis.

In five compound heterozygotes, both mutations were identified. Case 4 carries the 357delGACT and 1033insCG mutations. This 11-year-old boy entered chronic renal failure at 9 years of age, despite cysteamine therapy from 8 mo of age. Case 11, a 25-year-old woman, carries a W182R mutation and an 8-bp deletion/A substitution. Case 15, 28 years of age, has milder cystinosis, and both he and his twin brother also have Klinefelter syndrome (Oberfield et al. 1981). The patient did not enter renal failure until 15 years of age, despite absence of cysteamine therapy, and he has had muscle wasting and swallowing difficulty since 23 years of age. He carries the unique mutations D205N and S298N. Case 16 carries two new mutations, 1035insC and 1261insG. Because of good cysteamine therapy, her serum creatinine was 0.8 mg/dl at 8 years of age and currently is 1.6 mg/dl, at 12 years of age. Case 18 had very mild disease. Diagnosed because her older sister was affected, she was treated with cysteamine from 6 mo of age, and her current serum creatinine, at 9 years of age, is 0.5 mg/dl. Her height is 128 cm (25th centile). The patient's father, who is Kurdish and Afghan, contributed the G308R mutation, whereas her mother, who is English and French, contributed the typical 65-kb European deletion.

Three new mutations, 1080delC, 1253A→G, and 1354G→A, were found on single alleles in cases 20, 22, and 24, respectively. Case 21, a 17-year-old girl with Polish, German, and English ancestry, is heterozygous for 1035insC, a mutation that also is present in case 16 (table 3). Case 23 is heterozygous for 1261G→A, the same mutation that case 18 carries on one allele.

Our techniques failed to identify mutations in 20 patients. Five apparent polymorphisms were found. Three of these, which include one in an intron (669−5T→C) and two silent mutations (843A→G and 1299C→T), were present in patients who also manifested two pathologic mutations. The 1020+9A→G change is speculated to be nonpathologic because no consensus sequence has been described for the +9 position of the intervening sequences. One base change, 1214A→G, maintained the amino acid charge (K292R) and may represent a true mutation, since it was not seen in normal patients or in cystinosis patients in whom two other mutations were identified.

HGMCR Fibroblast Mutations

Homozygous mutations were found in 5 of 11 fibroblast strains obtained from the HGMCR (table 3). Three (GM8, GM706, and GM2894) were deleted in *D17S829*, one (GM760) was homozygous for W138X, and one (GM46) was homozygous for a new mutation, 545delTCCTT (I69R), which causes termination at codon 73. Two of the *D17S829* deletions encompassed the typical 65-kb span, but GM8 was positive for markers AA313583 and T85505 and for exon 11.

Northern Blot Analysis

RNA was available from 20 patients and two cell strains, for northern blot analysis. Five patients homozygous for the typical 65-kb European deletion showed no *CTNS* expression in fibroblasts (fig. 4), whereas various amounts of *CTNS* mRNA were apparent in all other samples studied (table 3 and fig. 4). These included 2 patients homozygous for W138X (data not shown), 10 patients with recognized *CTNS* mutations, and 3 patients in whom no mutation was found. *CTNS* mRNA was present in cell strain GM2066 but not in GM8.

Discussion

Since the U.S. population is genetically heterogeneous, it can illustrate the different mutations causing nephropathic cystinosis, and we were able to identify mutations in 81% of our patients. Some mutations correlated with ethnicity and some did not. Forty-eight (44%) of 108 American-based patients were homozygous for the 65-kb European deletion, compared with 23 (33%) of 70 patients in a French/British report (Town et al. 1998). In both studies, only patients derived from European countries were found to carry the 65-kb deletion, which eliminates all *CTNS* mRNA (fig. 4) and causes typical nephropathic cystinosis. Nearly half of the 65-kb–deletion alleles in our patients appear to have arisen in

Germany (fig. 3), although many derived from countries surrounding Germany, and 28 (34%) of 82 arose from the British Isles. The finding of two apparently unrelated patients with homozygous deletions who are from Iceland, which has been genetically isolated since its early settlement, suggests that the distribution of the 65-kb deletion represents a founder effect. Since Germanic tribes settled the British Isles in the late seventh century and since the Vikings traveled from Norway, Scotland, and Ireland to Iceland in 874 A.D., we speculate that the 65-kb deletion may have occurred initially in Germany, prior to 700 A.D.; however, this hypothesis requires verification by haplotype analysis across the *CTNS* gene.

In addition to the 48 patients homozygous for the 65-kb deletion, many of our patients may have one copy of the deletion. Once the upstream deletion breakpoint has been determined, a PCR-based test of heterozygosity for the deletion can be developed; only the allele with the 65-kb deletion will amplify when flanking primers are used. We currently are pursuing this.

Two patients and one HGMCR cell strain (GM8) had deletions that were smaller than the typical 65-kb deletion but that were still several kilobases in size. These mutations may be identical to the smaller major deletion reported by Town et al. (1998).

We found that W138X, a nonsense mutation associated with residual *CTNS* RNA expression, was present in 25 (12%) of 216 patient alleles examined and in 2 (9%) of 22 HGMCR fibroblast alleles examined. Patients homozygous for this mutation had nephropathic cystinosis of slightly worse than average severity and were from Germany, France, or the British Isles.

Our identification of 18 new mutations in *CTNS* (table 3) brings to 29 the total number of small *CTNS* mutations reported. Whereas the 11 previously reported small mutations (six deletions, three nonsense mutations, and two splice-site mutations) result in truncated proteins, we found two deletions and seven missense mutations that did not alter the frame of the message. Significant *CTNS* expression was associated with the homozygous missense mutation G169D and with hemizogosity for G308R. All seven missense mutations gave rise to amino acid changes either inside a transmembrane domain or in the first amino acid prior to a transmembrane domain. Similarly, in the *NKCC2* gene responsible for Bartter syndrome, 8 of the 10 missense mutations identified were inside or within a few amino acids of transmembrane domains (Vargas-Poussou et al. 1998). For *CTNS*, Q128X is at the border of transmembrane domain 1, Δ205 and D205N are at the border of transmembrane domain 3, and S298N is at the border of transmembrane domain 6. When one patient who was compound heterozygous for D205N and S298N was included, the severity scores associated with these mutations were within the range 1.5–1.7 (table 3), which indicates that these mutations may cause relatively mild disease. Of the seven *CTNS* missense mutations, four (G169D, D205N, D305G, and G308R) involve amino acids conserved from yeast to *C. elegans* to humans, and two (S298N and G339R) affect amino acids conserved between *C. elegans* and humans. The conservation of these amino acids, as well as the charge difference associated with the base changes, indicates that the alterations are mutations rather than simply polymorphisms.

Two in-frame deletions, Δ205 and 537del21bp, were associated with severity scores of 1.7 and 1.3–2.0, respectively. The relatively mild disease may be due to frame preservation. Moreover, although Δ205 alters a conserved aspartic acid, it occurs just before a transmembrane domain, which may mitigate its phenotypic impact. The 537del21bp mutation inteferes with a potential N-linked glycosylation site, but the N66 residue may not be routinely glycosylated; the homozygous mutation does allow for some *CTNS* expression (fig. 4). We also caution that severity scores based on only two or three parameters are most useful when a large group of patients with the same mutation is assessed.

The *CTNS* mutations are distributed relatively evenly across exons 3–12 (table 3) and across the different structural domains (table 4). Three distinct mutations arose in the 22–amino acid leader sequence, compared with 11 in the seven transmembrane regions comprising 152 amino acids and 15 in the eight nontransmembrane regions comprising 193 amino acids. The C-terminal lysosomal targeting signal was not mutated in any patient. Similar severity scores were associated with mutations in the different regions.

A few comments on our patients' mutations are warranted. First, we found 357delGACT in the homozygous state among three nationalities (English, German, and Indian), which confirms the report of Town et al. (1998) that this mutation arose on different continents. In addition, the 1261G→A (G308R) mutation was associated, in the heterozygous state, with a Kurdish/Afghan parent (case 18) and an Irish/Swedish/French family (case 23). Hence, 357delGACT and G308R appear to be recurrent mutations. Moreover, 357delGACT was associated with widely variable disease severities (cases 1–3, table 3), which indicates that ancillary genes can influence the severity of cystinosis.

Second, two small regions of *CTNS* each display multiple mutations, namely, 950delACG/950G→A and 1261G→A/1261insG. These regions may prove to be somewhat mutation prone. Third, the large number of *CTNS* deletions—that is, 10 in addition to the 2 major deletions—supports the contention that this area of chromosome 17p is susceptible to deletions. Charcot-Marie-Tooth disease type 1A, hereditary neuropathy with liability to pressure palsies, Smith-Magenis syndrome, and Miller-Dieker syndrome are all caused by

deletions or duplications between 17p13 and 17p11.2 (Stratton et al. 1984; Smith et al. 1986; Reiter et al. 1996). Sequence data for *CMT1A, HNPP,* and *SMS* have implicated unequal crossing over between homologous repeats as the mechanism for these rearrangements (Reiter et al. 1996), although altered activity of a "mariner" transposon–like element at 17p11.2 also could contribute (Reiter et al. 1996). Interestingly, 17p13 is the site of a large olfactory receptor–gene cluster produced by multiple intrachromosomal duplications (Glusman et al. 1996). When the exact breakpoints of the large *CTNS* deletion have been determined, the mechanism for the high frequency of deletions in this area may be revealed.

Oral cysteamine will remain the mainstay of cystinosis therapy, and both postnatal and prenatal diagnosis will continue to depend heavily on leukocyte cystine measurements; however, molecular diagnosis of nephropathic cystinosis now will take its place in cases involving difficulty in the preparation of leukocytes for assay, equivocal results, or heterozygote detection.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genome Database, http://gdbwww.gdb.org/ (for *CTNS* [700761])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for nephropathic cystinosis [MIM 219800])

References

Charnas LR, Bernardini I, Rader D, Hoeg JM, Gahl WA (1991) Clinical and laboratory findings in the oculocerebrorenal syndrome of Lowe, with special reference to growth and renal function. N Engl J Med 324:1318–1325

Charnas LR, Luciano CA, Dalakas M, Gilliat RW, Bernardini I, Ishak K, Swik VA, et al (1994) Distal vacuolar myopathy in nephropathic cystinosis. Ann Neurol 35:181–188

Cystinosis Collaborative Research Group (1995) Linkage of the gene for cystinosis to markers on the short arm of chromosome 17. Nat Genet 10:246–248

Ehrich JHH, Stoeppler L, Offner G, Brodehl J (1979) Evidence for cerebral involvement in nephropathic cystinosis. Neuropaediatrie 10:128–137

Fink JK, Brouwers P, Barton N, Malekzadeh MH, Sato S, Hill S, Cohen WE, et al (1989) Neurologic complications in long-standing nephropathic cystinosis. Arch Neurol 46:543–548

Fivush B, Green OC, Porter CC, Balfe JW, O'Regan S, Gahl WA (1987) Pancreatic endocrine insufficiency in posttransplant cystinosis. Am J Dis Child 141:1087–1089

Gahl WA (1986) Cystinosis coming of age. Adv Pediatr 33: 95–126

Gahl WA, Bashan N, Tietze F, Bernardini I, Schulman JD (1982*a*) Cystine transport is defective in isolated leukocyte lysosomes from patients with cystinosis. Science 217: 1263–1265

Gahl WA, Bashan N, Tietze F, Schulman JD (1984) Lysosomal cystine counter-transport in heterozygotes for cystinosis. Am J Hum Genet 36:277–282

Gahl WA, Charnas L, Markello TC, Bernardini I, Ishak KG, Dalakas M (1992) Parenchymal organ cystine depletion with long term cysteamine therapy. Biochem Med Metab Biol 48: 275–285

Gahl WA, Reed GF, Thoene JG, Schulman JD, Rizzo WB, Jonas AJ, Denman DW, et al (1987) Cysteamine therapy for children with nephropathic cystinosis. N Engl J Med 316: 971–977

Gahl WA, Schneider JA, Aula P (1995) Lysosomal transport disorders: cystinosis and sialic acid storage disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 7th ed. McGraw Hill, New York, pp 3763–3797

Gahl WA, Tietze F, Bashan N, Bernardini I, Schulman JD (1983) Characteristics of cystine counter-transport in normal and cystinotic lysosome-rich leucocyte granular fractions. Biochem J 216:393–400

Gahl WA, Tietze F, Bashan N, Steinherz R, Schulman JD (1982*b*) Defective cystine exodus from isolated lysosome-rich fractions of cystinotic leucocytes. J Biol Chem 257: 9570–9575

Gahl WA, Tietze F, Butler JDeB, Schulman JD (1985) Cysteamine depletes lysosomal cystine by the mechanism of disulfide interchange. Biochem J 228:545–550

Glusman G, Clifton S, Roe B, Lancet D (1996) Sequence analysis in the olfactory receptor gene cluster on human chromosome 17: recombinatorial events affecting receptor diversity. Genomics 37:147–160

Jean G, Fuchshuber A, Town MM, Gribouval O, Schneider JA, Broyer M, van't Hoff W, et al (1996) High-resolution mapping of the gene for cystinosis, using combined biochemical and linkage analysis. Am J Hum Genet 58:535–543

Jonas AJ, Smith ML, Schneider JA (1982) ATP-dependent lysosomal cystine efflux is defective in cystinosis. J Biol Chem 257:13185–13188

Kaiser-Kupfer MI, Caruso RC, Minckler DS, Gahl WA (1986) Long-term ocular manifestations in nephropathic cystinosis post-renal transplantation. Arch Ophthalmol 104:706–711

Kimonis VE, Troendle J, Rose SR, Yang ML, Markello TC, Gahl WA (1995) Effects of early cysteamine therapy on thyroid function and growth in nephropathic cystinosis. J Clin Endocrinol Metab 80:3257–3261

Markello TC, Bernardini IM, Gahl WA (1993) Improved renal function in children with cystinosis treated with cysteamine. N Engl J Med 328:1157–1162

McDowell G, Isogai T, Tanigami A, Hazelwood S, Ledbetter

D, Polymeropoulos MH, Lichter-Konecki U, et al (1996) Fine mapping of the cystinosis gene using an integrated genetic and physical map of a region within human chromosome band 17p13. Biochem Mol Med 58:135–141

Oberfield SE, Levine LS, Wellner D, Novogroder M, Laino P, New MJ (1981) Ascorbic acid treatment in nephropathic cystinosis in identical twins. Dev Pharmacol Ther 2:80–90

Oshima RG, Pellett OL, Robb JA, Schneider JA (1977) Transformation of human cystinotic fibroblasts by SV40: characteristics of transformed cells with limited and unlimited growth potential. J Cell Physiol 93:129–136

Peters U, Senger G, Rählmann M, Du Chesne I, Stec I, Köhler MR, Weissenbach J, et al (1997) Nephropathic cystinosis (CTNS-LSB): construction of a YAC contig comprising the refined critical region on chromosome 17p13. Eur J Hum Genet 5:9–14

Pisoni RL, Thoene JG, Christensen HN (1985) Detection and characterization of carrier-mediated cationic amino acid transport in lysosomes of normal and cystinotic human fibroblasts. J Biol Chem 260:4791–4798

Reiter L, Murakami T, Koeuth T, Pentao L, Muzny DM, Gibbs RA, Lupski JR (1996) A recombination hotspot responsible for two inherited peripheral neuropathies is located near a mariner transposon-like element. Nat Genet 12:288–297

Sambrook J, Fritsch EF, Maniatis T (eds) (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Smith ACM, McGavran L, Robinson J, Waldstein G, McFarlane J, Zonana J, Reiss J, et al (1986) Interstitial deletion of (17)(p11.2p11.2) in nine patients. Am J Med Genet 24: 393–414

Sonies BC, Ekman EF, Andersson HC, Adamson MD, Kaler SG, Markello TC, Gahl WA (1990) Swallowing dysfunction in nephropathic cystinosis. N Engl J Med 323:565–570

Steinherz R, Tietze F, Raiford D, Gahl WA, Schulman JD (1982) Patterns of amino acid efflux from isolated normal and cystinotic human leucocyte lysosomes. J Biol Chem 257: 6041–6049

Stratton RF, Dobyns WB, Airhart SD, Ledbetter DH (1984) New chromosomal syndrome: Miller-Dieker syndrome and monosomy 17p13. Hum Genet 67:193–200

Thoene JG, Oshima RG, Crawhall JC, Olson DL, Schneider JA (1976) Cystinosis: intracellular cystine depletion by aminothiols in vitro and in vivo. J Clin Invest 58:180–189

Tietze F, Seppala R, Renlund M, Hopwood JJ, Harper GS, Thomas GH, Gahl WA (1989) Defective lysosomal egress of free sialic acid (N-acetylneuraminic acid) in fibroblasts of patients with infantile free sialic acid storage disease. J Biol Chem 264:15316–15322

Town M, Jean G, Cherqui S, Attard M, Forestier L, Whitmore SA, Callen DF, et al (1998) A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. Nat Genet 18:319–324

Vargas-Poussou R, Feldmann D, Vollmer M, Konrad M, Kelly L, van den Heuvel LPWJ, Tebourbi L, et al (1998) Novel molecular variants of the Na-K-2CL cotransporter gene are responsible for antenatal Bartter syndrome. Am J Hum Genet 62:1332–1340