

New insights into cystinuria: 40 new mutations, genotype–phenotype correlation, and digenic inheritance causing partial phenotype

M Font-Llitjós*, M Jiménez-Vidal*, L Bisceglia*, M Di Perna, L de Sanctis, F Rousaud, L Zelante, M Palacín, V Nunes

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See end of article for authors' affiliations

Correspondence to: Dr Virginia Nunes, Centre de Genética Mèdica i Molecular (IRO-IDIBELL), Hospital Duran i Reynals, Gran Via Km 2.7, s/n, L'Hospitalet de Llobregat, Barcelona E-08907, Spain; vnunes@iro.es

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* These authors contributed equally to the study

Objective: To clarify the genotype–phenotype correlation and elucidate the role of digenic inheritance in cystinuria.

Methods: 164 probands from the International Cystinuria Consortium were screened for mutations in *SLC3A1* (type A) and *SLC7A9* (type B) and classified on the basis of urine excretion of cystine and dibasic amino acids by obligate heterozygotes into 37 type I (silent heterozygotes), 46 type non-I (hyperexcretor heterozygotes), 14 mixed, and 67 untyped probands.

Results: Mutations were identified in 97% of the probands, representing 282 alleles (86.8%). Forty new mutations were identified: 24 in *SLC3A1* and 16 in *SLC7A9*. Type A heterozygotes showed phenotype I, but mutation DupE5-E9 showed phenotype non-I in some heterozygotes. Type B heterozygotes showed phenotype non-I, with the exception of 10 type B mutations which showed phenotype I in some heterozygotes. Thus most type I probands carried type A mutations and all type non-I probands carried type B mutations. Types B and A mutations contributed to mixed type, BB being the most representative genotype. Two mixed cystinuria families transmitted mutations in both genes: double compound heterozygotes (type AB) had greater aminoaciduria than single heterozygotes in their family.

Conclusions: Digenic inheritance is an exception (two of 164 families), with a limited contribution to the aminoaciduria values (partial phenotype) in cystinuria. Further mutational analysis could focus on one of the two genes (*SLC3A1* preferentially for type I and *SLC7A9* for type non-I probands), while for mixed probands analysis of both genes might be required, with priority given to *SLC7A9*.

Cystinuria is an autosomal inherited metabolic disorder characterised by impaired transport of cystine and dibasic amino acids in the proximal renal tubule and the gastrointestinal tract. A high cystine concentration in the urinary tract most often causes the formation of recurring renal cystine stones because of the low solubility of this amino acid. The overall estimated prevalence of the disease is 1/7000 neonates, ranging from 1/2500 neonates in Libyan Jews to 1/100 000 in the Swedish population.¹

Cystinuria is classified into three types according to the urine phenotype in heterozygotes: type I (MIM220100), type non-I (MIM600918), and mixed. In type I cystinuria, heterozygotes have a normal pattern of amino acid excretion in the urine (phenotype I), which implies that the disease is transmitted by an autosomal recessive trait. In non-type I cystinuria, heterozygotes have a variable degree of hyperexcretion of cystine and dibasic amino acids (phenotype non-I), indicating that the disease is transmitted in a dominant mode with incomplete penetrance.² Patients with mixed type cystinuria, who inherit phenotype I and non-I alleles from either parent, have also been described.³

Two genes responsible for cystinuria have been identified. Mutations in *SLC3A1*, located on chromosome 2p16.3–21 and encoding the b^{0,+} transporter related protein rBAT, have up to now been shown to cause only type I cystinuria,^{4–7} and not type non-I.^{8–9} The gene causing type non-I cystinuria was assigned by linkage analysis to 19q12–13.1,^{9–10} confined to a 1.3 Mb region¹¹ and identified as *SLC7A9*,² the protein product of which (b^{0,+}AT) heterodimerises with rBAT (*SLC3A1*) in kidney brush border membranes.¹² The rBAT/b^{0,+}AT heteromeric complex is responsible for the b^{0,+} amino acid transport

system. This transporter belongs to the heteromeric amino acid transporter (HAT) family, which is formed by a heavy subunit (rBAT or 4F2hc) linked by a disulphide bridge to a range of light subunits (b^{0,+}AT in the case of rBAT).¹³ At present, 79 *SLC3A1* and 50 *SLC7A9* mutations—including nonsense, missense, splice site, and frameshift mutations and large rearrangements in both genes—have been described.^{1–2, 6, 8, 14–27}

Despite the classification of the disease described above, we found that some *SLC7A9* heterozygotes presented a urinary excretion phenotype near the control range, resembling *SLC3A1* heterozygotes that are phenotypically silent.²¹ Since then, phenotype I heterozygotes carrying *SLC7A9* mutations have been reported, and two *SLC7A9* mutations (I44T and P261L) have been associated with this phenotype.^{24–28} These findings make the existing cystinuria classification imprecise because type I cystinuria could be caused by mutations in *SLC7A9* instead of *SLC3A1*. Consequently, a new scheme based only on genetic aspects has recently been proposed²⁸: type A, caused by two mutations in *SLC3A1* (rBAT); type B, caused by two mutations in *SLC7A9* (b^{0,+}AT); and the possibility of type AB, with one mutation on each of the above genes (that is, cystinuria with digenic inheritance). However, it remains unclear whether type AB implies a digenic inheritance of the disease. Given a similar frequency of mutations in *SLC7A9* and *SLC3A1*,²⁸ we would expect half

Abbreviations: DHPLC, denaturing high performance liquid chromatography; HAT, heteromeric amino acid transporter; RT-PCR, reverse transcriptase polymerase chain reaction; SSCP, single strand conformation polymorphism

to be from AB disease. The prevalence of type A disease is similar to that of type B disease, but type AB is extremely rare.²⁸ Two explanations could account for this low prevalence. Type AB patients may have a mild phenotype and escape detection. Alternatively, these individuals may represent type B or type A disease with a coincidental mutation in the other subunit gene. In this case, type AB carriers might be obligate heterozygotes with the possibility of digenic inheritance causing some effects at the phenotypical level.

We focused our study on an exhaustive mutational analysis of a large cohort of patients with extensive genotype–phenotype correlations in order to clarify the cystinuria classification from a genetic point of view. Special emphasis was placed on *SLC7A9* alleles that segregate as a silent phenotype in obligate heterozygotes, on mixed cystinuria families, and on possible digenic cases. Here we report the following: 40 new cystinuria specific mutations (24 in *SLC3A1* and 16 in *SLC7A9*); 10 *SLC7A9* mutations that associate with phenotype I in heterozygotes; the first *SLC3A1* mutation (dupE5-E9) that associates with phenotype non-I in heterozygotes; and two families with mixed cystinuria which carry mutations in both genes and whose amino acid excretion phenotype in urine indicates a contribution of digenic inheritance, described for the first time in cystinuria.

METHODS

Patients: phenotypic and genetic classification

The institutional review board of the participating centres approved the study and participants gave their informed consent.

Amino acid excretion was determined using morning urine samples or in a 24 hour collection, as described,²⁹ and corrected per gram of creatinine excreted. All subjects who fulfilled the following criteria were included in the study:

- urine cystine excretion more than 1040 $\mu\text{mol/g}$ creatinine in a single urine sample or in a 24 hour collection, and at least one identified cystine stone (emitted or surgically removed);
- siblings of cystinuria patients, defined above, with the same genetic mutations, even in the absence of stone identification.

We studied 164 independent patients and their relatives of the International Cystinuria Consortium (ICC) database. These probands were from Italy (91), Spain (50), Israel (12), Belgium (4), Portugal (3), Switzerland (2), England (1),

and Germany (1). Information on genotype or phenotype for some of these patients is available elsewhere.^{2 14 15 20–22 28}

For genotype–phenotype correlation studies two classifications were established, one based on urinary amino acid excretion and the other on the genetic findings.

Classification based on urinary amino acid excretion

Patients were classified as type I, type non-I, mixed, and untyped on the basis of the urinary excretion values of cystine and dibasic amino acids (lysine, arginine, and ornithine) by the obligate heterozygotes. Thus heterozygotes were considered *phenotype non-I* when at least two of the indices (cystine, lysine, arginine, ornithine, or the sum of cystine and the three dibasic amino acids in the urine) were above the 95th centile in controls; they were considered *phenotype I* when these indices were within the control range.

Type I

Both parents showed a normal urine amino acid pattern (heterozygotes with excretion values within the control range—that is, phenotype I).

Type non-I

Urinary excretion of both parents was above controls (that is, phenotype non-I heterozygotes). In a few cases, the probands had a urinary amino acid profile similar to the parent transmitting the disease and within the upper range of the non-I heterozygote phenotype. In these cases, when the other parent showed a normal urinary excretion phenotype, the probands were considered to be heterozygotes with non-I cystinuria.

Mixed

Urinary excretion was above the control values in one of the parents (phenotype non-I) and normal in the other (phenotype I).

Untyped

Urinary excretion values for one or both parents were not available or were of doubtful classification (that is, inconsistent classification of members of the same family).²¹

Using these criteria, the 164 studied probands were classified as 37 type I, 46 type non-I, 14 mixed, and 67 untyped (table 1).

Table 1 Cystinuria type and genetic frequencies of the probands

Genotype	Cystinuria type		Non-I carriers*	Mixed	Untyped	Total probands (%)	
	I	Non-I					
AA	29†			2	25†	56 (34.1)	126 (76.8)
AA(B)				1		1 (0.6)	
BB	1	34‡		7	23†	65 (39.6)	
B+			3			3 (1.8)	
BB(A)				1		1 (0.6)	
A?	5			1	5	11 (6.7)	33 (20.1)
B?	2	7		2§	11	22 (13.3)	
??		2			3	5 (3.0)	
Total probands (%)	37 (22.6)	43 (26.2)	3 (1.8)	14 (8.5)	67 (40.9)	164 (100)	
Total alleles	74	89	28	134			
Explained alleles (%)	67 (90.5)	78 (87.6)	25 (89.3)	112 (83.6)			

*Heterozygote probands with cystine lithiasis.

†Includes one family with two mutations in the same chromosome in addition to the mutation found in the other chromosome.

‡Includes two families with two mutations in the same chromosome in addition to the mutation found in the other chromosome.

§Includes two families with two mutations found in one chromosome and no mutation found in the other chromosome.

For patients AA(B) and BB(A), two alleles causing the disease and two explained alleles in each case have been taking into account in the calculations.

A, allele *SLC3A1* mutated; B, allele *SLC7A9* mutated; +, normal allele; ?, unknown allele.

Table 2 New *SLC3A1* and *SLC7A9* mutations

Type	Nucleotide change	Amino acid change/predicted consequence	Exon or intron	Detection method	
<i>SLC3A1</i> new mutations					
Missense	c.266T→C	L89P	Exon 1	SSCP	
	c.368T→G	M123R	Exon 1	DHPLC	
	c.371A→G	Y124C	Exon 1	DHPLC	
	c.388T→C	S130P	Exon 1	DHPLC/SSCP	
	c.410A→G	D137G	Exon 1	DHPLC	
	c.446T→A	L149Q	Exon 2	DHPLC	
	c.566C→T	T189M	Exon 2	SSCP	
	c.1043A→C	H348P	Exon 6	DHPLC	
	c.1230C→A	N410K	Exon 7	DHPLC	
	c.1322C→G	P441R	Exon 7	SSCP	
	c.1364C→T	S455L	Exon 8	DHPLC	
	c.1366C→T	R456C	Exon 8	DHPLC/SSCP	
	c.1367G→A	R456H	Exon 8	SSCP	
	c.1520C→T*	S507L	Exon 9	SSCP	
	c.1702G→A	G568S	Exon 10	DHPLC	
	c.1998C→G	C666W	Exon 10	DHPLC	
	c.1865T→G*	L622X	Exon 10	SSCP	
	Nonsense	c.1035G→A	Aberrant splicing†	Exon 6	SSCP
		c.1012-23C→G	D338_Y378delfsX5	Intron 5	SSCP and RT-PCR
	Silent	c.664delT	W222fsX3	Exon 3	SSCP
Splice	c.1221dupT	P408fsX18	Exon 7	SSCP	
	c.1699_1700delAG	R567fsX8	Exon 10	SSCP	
Frameshift	c.1966_1968delCTT	L656del	Exon 10	SSCP	
In frame deletion	c.892_1332del	E298_M444del	Intron 4-7	PCR	
<i>SLC7A9</i> new mutations					
Missense	c.184G→A	V62M	Exon 3	DHPLC	
	c.562G→A	V188M	Exon 5	DHPLC	
	c.695A→G	Y232C	Exon 6	SSCP	
	c.847C→T	L283F	Exon 8	DHPLC	
	c.947C→T	A316V	Exon 9	DHPLC	
	c.171C→T	Aberrant splicing‡	Exon 3	SSCP	
	c.235+3_+293del	L30fsX10	Intron 3	RT-PCR	
	c.586C→T	L196_G202delfsX5	Exon 5	SSCP and RT-PCR	
	c.604+2T→C	L160X	Intron 5	SSCP	
	c.605-3C→A	I203fsX27	Intron5	DHPLC, SSCP	
Silent	c.1399+1G→T	K447fsX22§	Intron12	DHPLC	
	c.1400-3C→G	K447fsX22§	Intron12	DHPLC	
	c.151delT	S51fsX38	Exon 3	DHPLC	
	c.1265_1266delTG	L424fsX62	Exon 12	DHPLC	
Large rearrangements	c.1224+4166_1399+119dup(4972)	K386fsX78	Exon 12	Multiplex	
	c.1224+4166_1399+119del(4972)	V409fsX9	Exon 12	Multiplex	

In addition to the 40 listed new mutations, a deletion of the 3' end of *SLC3A1* has been identified in one chromosome of one patient, but the limits of this deletion have not been studied.

When RT-PCR is indicated in the detection method, the effect of the mutation has been determined at the mRNA.

*These two mutations are located in the same chromosome.

†Mutation eliminates an SF2/ASF exonic splicing enhancer motif.

‡Mutation may create an alternative donor splice site 64 bp upstream of the consensus donor site, resulting in the deletion of 22 amino acid residues (N58_G79del22).

§The same protein product prediction but different genomic change.

bp, base pair; RT-PCR, reverse transcriptase polymerase chain reaction.

Classification based on the genetic findings

Patients were classified as type A, type B, type AB, and type ?? on the basis of the genetic findings.

Type A

Probands have mutations in *SLC3A1* (A? for one mutated allele identified and AA for two mutated alleles identified).

Type B

Probands have mutations in *SLC7A9* (B? for one mutated allele identified and, BB and B+ for two mutated alleles identified).

Type AB

Individuals have one mutation in *SLC3A1* and one in *SLC7A9*. Proband with more than two mutated alleles were classified as AA(B) or BB(A), depending on the distribution of mutations in the two genes.²⁸

Type ??

No mutations are found in the probands.

Mutational analysis

Probands and their relatives have been analysed for mutations in *SLC3A1*, *SLC7A9*, or in both genes. Mutations in this cohort of patients have been reported previously.^{2 6 8 14 20-22} Genomic DNA or total RNA was isolated from peripheral blood lymphocytes following standard protocols. Patients with type I or type non-I cystinuria were initially screened for mutations in *SLC3A1* and *SLC7A9*, respectively. Patients not fully genotyped were also analysed for the other cystinuria gene. Untyped patients were analysed for both genes as well. Mutational screening of the whole coding region, all intron/exon boundaries, and some intron sequences (on average 50 base pairs (bp) on each side of the exons) was carried out by polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR) (when RNA samples were available), single strand conformation polymorphism

Table 3 Type of mutations distributed by allele in families with mixed cystinuria

Genotype	Families	Alleles	
		Phenotype I	Phenotype non-I
		Explained	Explained
AA	2	2	2
AA(B)*	1	1	1
BB	7	7	7
BB(A)†	1	1	1
A?	1	1	0
B?	2	0	2
Total	14	12	13

The urine phenotype of the obligate heterozygotes was used to classify the phenotype associated to each allele.
 *The proband carries three mutations, two mutations A (one in each *SLC3A1* allele) and one mutation B in one of the *SLC7A9* alleles (see fig 2B).
 †The proband carries three mutations, two mutations B (one in each *SLC7A9* allele) and one mutation A in one of the *SLC3A1* alleles (see fig 2A).

(SSCP), or Southern blot as described elsewhere,^{14, 21} and by denaturing high performance liquid chromatography (DHPLC) or semiquantitative multiplex PCR as described below. Direct sequencing was also carried out in all the individuals analysed to confirm the results obtained, to identify the mutated position in the gene, and to screen for mutations not detectable by these techniques. Southern blotting or semiquantitative multiplex PCR was used in patients with no mutations or only one mutation found with the other techniques, including direct sequencing. All the mutations described were checked on 100 control chromosomes to discard polymorphisms. Mutations listed or newly described here follow the nomenclature of the Human Genome Variation Society (www.genomic.unimelb.edu.au/mdi/mutnomen/). All new mutations have been introduced in the HGMD database (www.hgmd.org/).

DHPLC analysis

Samples for DHPLC analysis were amplified in a final reaction volume of 50 µl, using 100 ng genomic DNA, 10× PCR buffer with 15 mM MgCl₂ (Applied Biosystems, Foster City, California, USA), 200 µM dNTPs, 0.30 µM primer, and 1.5 units AmpliTaq Gold™ (Applied Biosystems). PCR cycling conditions consisted of an initial denaturation step at 95°C for 12 minutes followed by 35 cycles at 94°C for 30 seconds; the annealing temperature range was 55–57°C for 30 seconds, followed by 72°C for 30 seconds, and ending with a final elongation step at 72°C for eight minutes. DHPLC analysis was carried out on an automated DHPLC instrument (Transgenomic Inc, Santa Clara, California, USA).

Heteroduplex formation was induced by mixing an aliquot of unpurified PCR amplicon from patients and wild type sample in a 1:1 ratio. The mixed samples were heated to 95°C for five minutes and cooled slowly over 25 minutes to 35°C. Usually, 7–10 µl of reannealed DNA duplexes were injected

Table 5 Distribution of alleles per genotype

Probands	Alleles	
	Type A	Type B
AA	112	–
AA(B)*	2	–
BB	–	130
B+	–	3
BB(A)*	–	2
A?	11	–
B?	–	22
Total	125	157
% of total alleles	38.5	48.3
% of identified alleles	44.3	55.7

*In probands with mutations in both genes only the two mutated allele in the same gene have been used for calculations.

onto the column and then run at predetermined temperatures on the WAVE® system. The resolution temperature (Tr) for each of the 12 PCR amplicons of genomic DNA from *SLC3A1* and 13 from *SLC7A9* analysed was predicted by using WaveMaker software (Transgenomic). The primers used for PCR amplification and DHPLC conditions are reported in table 1s in Supplementary Material, available online at the JMG website (www.jmg.com/supplemental). Fragments that showed variation in retention time or chromatogram shape from that of an unaffected control amplicon were then sequenced following the Big Dye Terminator protocol (PE-ABI, Foster City, California, USA) and analysed on an ABI 3100 DNA sequencer (Applied Biosystems).

Semiquantitative multiplex PCR analysis in *SLC7A9*

We undertook multiplex analysis for *SLC7A9* in 13 patients from independent families in which no mutations or only one mutation had been found with the other techniques. This

Table 4 Distribution of alleles per phenotype

Probands	Alleles					
	Phenotype I			Phenotype non-I		
	Explained	Total	%	Explained	Total	%
I	67	74	90.5	–	–	–
non-I	–	–	–	78	89	87.6
Mixed	12	14	85.7	13	14	92.9
Total	79	88	89.7	91	103	88.3

Table 6 Urine excretion of cystine and dibasic amino acids in heterozygotes bearing the *SLC3A1* mutation dupE5-E9

dupE5-E9 heterozygote	Phenotype	Urine amino acid concentrations (μmol/g creatinine)				
		Cystine	Lysine	Arginine	Ornithine	Sum
F14-C119	non-I	57	481	318*	94*	950*
F77-SR	non-I	218*	1090*	33	30	1371*
F79-A145	non-I	167*	1595*	43	100*	1905*
F79-A148	non-I	998*	1762*	12	133*	2905*
F106-D18	I	53	150	<1	<1	253
F106-D20	untyped	NA	345	<1	35	-
Type A heterozygote (n)†	I (47)	70 (7)	171 (18)	24 (4)	26 (4)	300 (23)
[5th, 95th centile]		[21, 160]	[28, 359]	[0, 51]	[5, 62]	[100, 555]
(+/-) (n)†	Control (83)	52 (3)	181 (16)	17 (3)	28 (3)	277 (19)
[5th, 95th centile]		[23, 109]	[35, 499]	[0, 48]	[7, 70]	[93, 620]

Type A heterozygotes are those carrying mutations in *SLC3A1* other than dupE5-E9. Controls are members of the fully genotyped ICC cystinuria families not carrying the identified type A or type B mutations.

*Values above the 95th centile in controls.

†These values (mean (SEM)) have been reported previously.²⁹

I, family; n, number of individuals; NA, not analysed; <1, below the level of detection.

method was used previously for the analysis of large rearrangements in *SLC3A1*.²⁰ Primer pairs for each of the 13 exons of *SLC7A9* were used (table 2s in Supplementary Material). One primer of each pair was labelled with FAM fluorescence (Invitrogen, San Diego, California, USA). As an external double dose control, a primer pair amplifying exon 5 from *DSCR1* (Down syndrome critical region gene 1) in chromosome 21 was added, together with the other set of 13 primer pairs. In a final volume of 20 μl, we mixed 100 ng DNA, 0.2 mM dNTPs, 1.5 U Taq (Roche Diagnostics, Mannheim, Germany), 0.01% gelatine, PCR buffer with 1.5 mM MgCl₂, and a mix of primers. The reaction started with an initial denaturation of five minutes at 94°C, followed by 23 cycles of 94°C for 25 seconds, 58°C for 25 seconds, and 72°C 25 seconds. A final extension of seven minutes at 72°C was used. The PCR products were loaded on a 48-well acrylamide gel together with an external fluorescent size standard (TAMRA-Gs500) and run in an ABI PRISM 377 DNA sequencer (Applied Biosystems). The results were processed by GENESCANTM software (fig 1s in Supplementary Material). To confirm the duplication of exon 12 in family 73, we amplified a 518 bp fragment (only in the carriers) with 5 pmol of primers F11 (5'-ATGATTGAAATTGGAGGAGGG-3') and R10d (5'-CCTAATAAGCAGTCTACAAGA-3'), following standard methods, for 36 cycles of 94°C 30 seconds, 58°C 30 seconds, 74°C 30 seconds, plus five minutes at 74°C. To confirm the deletion in family 94, we amplified a 251 bp fragment using 5 pmol of primer F13 (5'-CAGACCATACTGTAAAGGACC-3') and R13 (5'-CGTGGGCATGTCTCCTCT-3'), following standard methods, for 36 cycles, 10 cycles of 94°C 30 seconds, 60°C 30 seconds, 74°C 30 seconds, 26 cycles of 90°C 30 seconds, 59°C 30 seconds, 74°C 30 seconds, plus seven minutes at 74°C.

RESULTS

Identification of 40 new cystinuria specific mutations in *SLC3A1* and *SLC7A9*

Mutational analysis was carried out exhaustively in 164 families that transmit cystinuria and which are included in the ICC database. We identified 24 new mutations in *SLC3A1* and 16 in *SLC7A9* (table 2). None of these mutations was identified in 100 control chromosomes tested (data not shown). They included five frameshift mutations (three in *SLC3A1* and two in *SLC7A9*) and one nonsense mutation in *SLC3A1* which produce early stop codons; seven splice mutations (one in *SLC3A1* and six in *SLC7A9*); 22 changes that affect single amino acid residues (16 and five missense mutations in *SLC3A1* and *SLC7A9*, respectively, and one

deletion of a single amino acid residue in *SLC3A1*); two silent changes affecting the coding region of the gene (one in *SLC3A1* and one in *SLC7A9*); and three large rearrangements (one in *SLC3A1* and two in *SLC7A9*).

The two large *SLC7A9* rearrangements were detected in two patients by semiquantitative multiplex PCR analysis: a 5 kb deletion (c.1224+4166_c.1399+119del) and a 5 kb duplication (c.1224+4166_c.1399+119dup), both affecting exon 12, originated by the crossing over of two 195 bp sequences which differ by one nucleotide and are separated by 4778 bp (fig 2s in Supplementary Material). The abnormal multiplex exon pattern in these two patients is shown in fig 3s in Supplementary Material. These two mutations were confirmed by PCR primers which specifically amplify the mutated allele in both cases (fig 2s in Supplementary Material). The protein product expected from the 5 kb duplication would have a frameshift from residue K386 with the addition of 78 missense residues before the first stop codon (p.K386fsX78). The 5 kb deletion affects intron 11 and the first 100 bp of the 175 bp of exon 12, and most probably results in the skipping of exon 12, because the donor splice site is deleted and there are no potential donor sites around. The protein product of this deletion would have a frameshift from residue V409, adding nine missense residues before the first stop codon (p.V409fsX9), and lacking the last transmembrane domains XI and XII. However, RNA was not available from these patients to confirm the predicted changes.

Two of the splice mutations were localised outside the splice consensus sequences, and were checked to produce aberrant splicing by RT-PCR:

- 586C→T in *SLC7A9* is a silent exonic mutation that creates alternative donor site 20 bp upstream of the consensus donor site. The resultant RNA (r.585_604del) is expected to produce a frameshift from residue L196 of b⁰AT (p.L196_G202delfsX5) (data not shown);
- c.1012-23C→G in *SLC3A1* localises in intron 5 and produces the skipping of exon 6 (r.1012_1136del), which is expected to result in a frameshift from residue D338 of rBAT (p.D338_Y378delfsX5) (data not shown).

The rest of the new splice mutations affect the consensus donor or acceptor splice site sequences, thereby indicating aberrant splicing.

The two silent mutations located in exonic regions (that is, no amino acid change) are predicted to create splicing defects, but it was not possible to check this by RT-PCR:

Table 7 Urine excretion of cystine and dibasic amino acids in heterozygotes carrying the type B mutations (*SLC7A9*) associated with phenotype I

	Phenotype	n	Urine amino acid concentrations (μmol/g creatinine)				
			Cystine	Lysine	Arginine	Ornithine	Sum
<i>Common mutations</i>							
A182T/+†	I	6	82 (16)	151 (52)	31 (7)	21 (6)	285 (57)
	Non-I	5	373 (135)*	1865 (1022)*	151 (60)*	138 (50)*	2527 (1090)*
T123M/+	I	2	29, 229*	271, 140	8, 25	19, 10	327, 404
	Non-I	2	NA	2596*, 734*	42, 17	176*, 53	NA
V170M/+	I	4	112 (16)*	254 (54)	6 (2)	18 (5)	391 (64)
	Non-I	12	441 (87)*	1471 (391)*	46 (14)	129 (28)*	2087 (475)*
c.614dupA/+‡	I	1	260*	122	19	25	426
	Non-I	18	607 (95)*	2249 (260)*	90 (14)*	220 (31)*	3165 (342)*
G105R/+	I	1	26	456	35	13	530
	Non-I	31	524 (75)*	1685 (181)*	69 (13)*	184 (32)*	2461 (249)*
R333W/+	Non-I	19	634 (139)*	2371 (330)*	137 (74)*	163 (28)*	3304 (482)*
<i>Uncommon mutations</i>							
G63R/+	I	2	60, 59	330, 322	11, 14	41, 38	442, 433
	Non-I	1	202*	504*	25	19	750*
A126T/+	I	1	140*	455	ND	8	603
	Non-I	2	308*, 338*	1144*, 1373*	ND, 102*	0, 237*	1452*, 2050*
G195R/+	I	1	116*	428	ND	19	563
	Non-I	1	439*	1948*	63*	247*	2697*
Y232C/+	I	1	108	125	11	42	286
W69X/+	I	1	51	249	55*	22	377
Heterozygotes B+ [5th, 95th centile]	Non-I	127	534 (37) [138, 1348]	1930 (113) [498, 4492]	76 (13) [0, 337]	198 (20) [21, 522]	2758 (154) [807, 6081]

Values are mean (SEM) μmol/g creatinine or individual values. R333W is a common mutation with phenotype non-I in all the heterozygotes studied. Urine excretion of amino acids for heterozygotes with one mutated *SLC7A9* allele (B+) with phenotype non-I is also shown. Urine amino acid levels in controls are shown in table 6. *Individual or mean values greater than 95th centile in controls (see table 6). †A182T is not associated with mutation A354T in these alleles. ‡c.614dupA is the present nomenclature for mutation c.800–801insA. NA, not analysed; ND, not detectable.

- c.171C→T in *SLC7A9* may create an alternative donor splice site 64 bp upstream of the consensus donor site;
- c.1035G→A in *SLC3A1* localizes in a putative exonic splicing enhancer (ESE) for SF2/ASF with a score prediction of 2.36, while the mutation reduces the ESE prediction to 0.44 (that is, below threshold = 1.96), suggesting aberrant splicing.³⁰

In the ICC cohort of patients M467T is the most common *SLC3A1* mutation, accounting for 26.4% of the total type A alleles, and together with T216M (6.4%), DupE5-E9 (4.0%), c.163delC (4.0%), R270X (4.0%), and c.765+1G→T (3.2%) represents 48.0% of total type A alleles (table 3s in

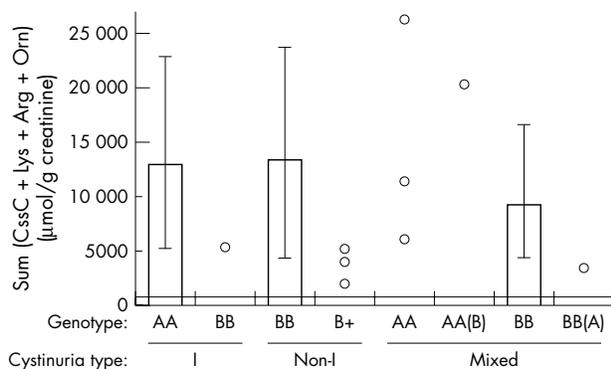


Figure 1 Sum of the urine of cystine and dibasic amino acids in patients with cystinuria classified by genotype and phenotype. Average (histograms) or individual data (dots), expressed as μmol/g creatinine, correspond to the sum of the urine levels of cystine and dibasic amino acids shown in table 8. Vertical lines represent the 5th and 95th centile of the values of the corresponding group. Horizontal line = 95th centile in the control group (detailed in table 6).

Supplementary Material). For *SLC7A9* the most common alleles are: G105R (27.4%), R333W (12.1%), V170M (11.5%) (17 alleles in the Libyan-Jewish population and one in the Italian population), c.614dupA (the Human Genome Variation Society nomenclature for mutation c.800–801insA) (7.0%) (eight alleles from Spain, two from Italy, and one from England), A182T (5.1%), T123M (3.8%) and c.605–3C→A (2.5%), which account for 69.4% of total type B alleles. Other *SLC3A1* or *SLC7A9* mutations were identified in three or fewer independent alleles.

The new mutations, together with those previously reported by the ICC (table 3s in Supplementary Material) and by others,^{15–19 23–27} make a total of 103 and 66 mutations identified in *SLC3A1* and *SLC7A9*, respectively: 28 frameshift (14 in *SLC3A1* and 14 in *SLC7A9*), seven nonsense (five in *SLC3A1* and two in *SLC7A9*), 18 splice (seven in *SLC3A1* and 11 in *SLC7A9*), 89 missense (59 in *SLC3A1* and 30 in *SLC7A9*), two silent (one in *SLC3A1* and one in *SLC7A9*), five deletions or insertions of a single amino acid residue (one in *SLC3A1* and four in *SLC7A9*), 18 large rearrangements (14 in *SLC3A1* and four in *SLC7A9*), and two cystinuria specific sequence variants in the promoter region of *SLC3A1*.

Distribution of cystinuria alleles per phenotype and genotype

The 164 cystinuria probands studied were classified on the basis of the urinary excretion of cystine and dibasic amino acids in obligate heterozygotes (urine phenotype) (see Methods) and also following the genetic classification proposed by Dello Strologo *et al*²⁸ (type A, mutations in *SLC3A1*; type B, mutations in *SLC7A9*) (table 1). Potentially, these probands represent 325 cystinuria independent alleles (three probands were classified as non-I cystinuria heterozygotes, see below). In all, 282 alleles were characterised (that is, 86.8%). Thus 13.2% of the potential alleles were not

Table 8 Urine amino acid levels in patients classified by genotype and type of cystinuria

Genotype	Cystinuria type	n	Urine amino acid concentrations (μmol/g creatinine)			
			Cystine	Lysine	Arginine	Ornithine
AA	I	34	1655 [647, 3404]	6577 [2651, 11635]	3144 [226, 8373]	1743 [587, 3441]
AA	Mixed	3	782, 2117, 5556	3308, 5715, 11418	1234, 2817, 7025	718, 1636, 1918
AA(B)	Mixed	1†	2569	9836	2952	5172
BB	I	1	2692	2280	111	304
BB	Non-I	37	1623 [498, 3304]	6510 [1717, 14670]	3447 [499, 6151]	2198 [295, 4766]
B+	Non-I carriers	3	257*, 441*, 801	1638*, 2450, 3873	23*, 123*, 145*	43*, 274*, 285*
BB	Mixed	11	1824 [431, 3181]	4581 [1573, 8723]	1540 [211, 3506]	1329 [470, 2449]
BB(A)	Mixed	1	425	3270	489	603

The mean of the amino acid levels for each group is indicated, with the exception of categories with less than four patients, where individual data are shown. When applicable, the 5th and 95th centile limits are shown in square brackets.

*Data below the 5th centile of values in individuals BB with type non-I cystinuria.

†A sister of this patient has the same genotype and she is in dialysis, and therefore urine amino acid levels are not available. Genotypes are as described in the legend to table 1.

n, number of patients.

identified. The alleles explained were 90.5% in type I probands, 87.6% in non-I, 89.3% in mixed, and 83.6% in untyped. In 126 probands (76.8%) we identified two mutations, in 33 (20.1%) one mutation, and in five (3.0%) no mutations. Of the fully explained probands, 56 (34.1%) and 68 (41.5%) carry two mutations in *SLC3A1* (probands AA) and *SLC7A9* (probands BB and B+) respectively.

Table 3 describes the type of mutation identified in each allele of the 14 mixed cystinuria probands, which potentially represent 14 phenotype I and 14 phenotype non-I alleles. This

table shows that all the possible genotypes in cystinuria are found in mixed families, BB being the most representative genotype.

Data in tables 1 and 3 allow calculation of the number of explained alleles classified by phenotype and genotype (tables 4 and 5): 89.7% and 88.3% of the phenotype I and non-I alleles have been explained, respectively. Alleles identified as type A and type B were 125 and 157, respectively. This represents 44.3% (type A) and 55.7% (type B) of the alleles identified.

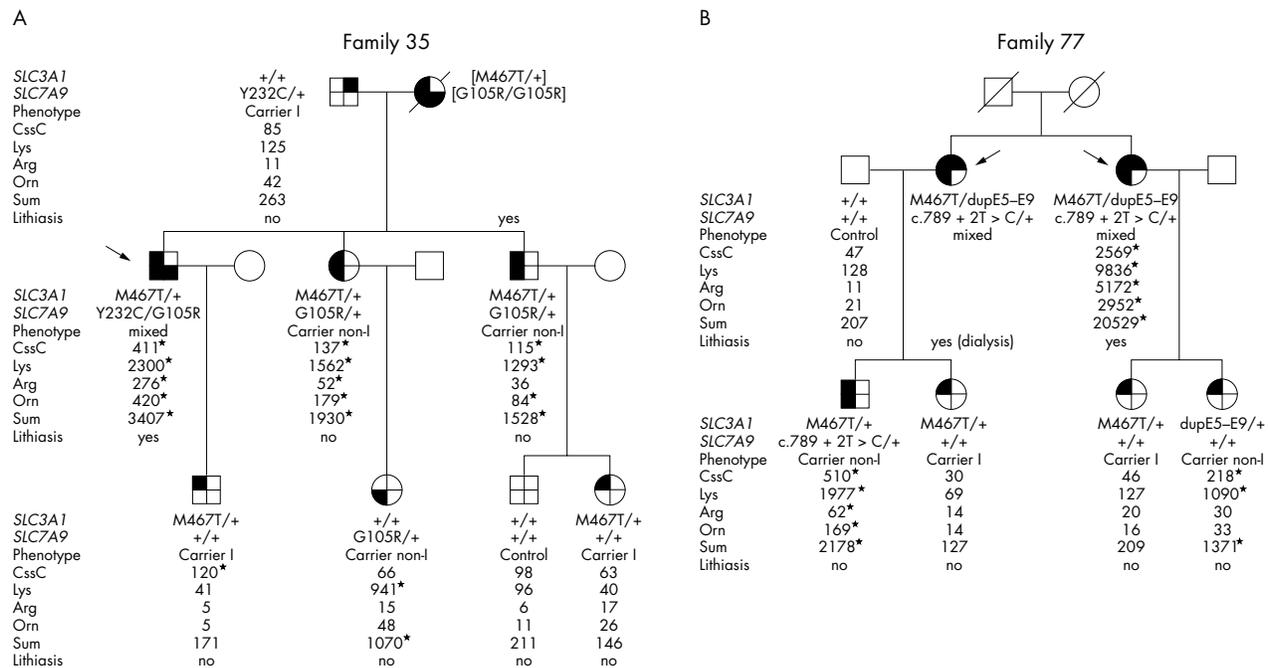


Figure 2 Pedigrees of families with patients that carry mutations in *SLC3A1* and *SLC7A9*. (A) Pedigree of patient BB(A) (family 35). The probands (arrow) have two mutated alleles in *SLC7A9* (G105R/Y232C) (type B mutations) and one in *SLC3A1* (M467T/+) (type A mutation), and present cystine calculi (lithiasis). Double heterozygotes carrying G105R/+ and M467T/+ had greater amino acid excretion in the urine than single carriers (G105R/+ or M467T/+), indicating an impact of digenic inheritance on the urine phenotype. (B) Pedigree of patients AA(B) (family 77). The two patients (arrows) within the family carry two mutated alleles in *SLC3A1* (M467T/dupE5-E9) (type A mutations) and one mutation in *SLC7A9* (c.789+2T→C) (type B mutation) and have cystine calculi (lithiasis). One of the patients is on dialysis as indicated. The other had amino acid excretion values in urine that were above the 89th centile values in patients AA. Again, this indicates that digenic inheritance contributes to the urine phenotype. None of the double carriers in either family had lithiasis. Urine amino acid excretion values for cystine (CysC), lysine (Lys), arginine (Arg), ornithine (Orn), and the sum of the four amino acids are expressed in μmol/g creatinine. To facilitate interpretation, symbols are divided in four sectors. The upper and lower ones represent *SLC3A1* and *SLC7A9* alleles respectively. When mutated the sectors are black. *Values above the 95th centile of corresponding amino acid urine values in controls (table 6).

Genotype–phenotype correlation

Most type A alleles segregate with cystinuria type I, but type A mutations explained phenotype non-I in obligate heterozygotes in three mixed cystinuria families (tables 1 and 3). To our knowledge this is the first description of *SLC3A1* mutations with phenotype non-I in heterozygotes. Type B alleles segregated mainly with phenotype non-I (tables 1 and 3). However, in agreement with previous reports,^{24–28} *SLC7A9* mutations also segregated with phenotype I in heterozygotes. Thus one type I proband carries two type B mutations (BB), and two other type I probands carry one type B mutation (B?) (table 1). Moreover, the phenotype I allele in eight mixed cystinuria probands was explained by type B mutations (seven BB and one BB(A) probands; table 3).

SLC3A1 dupE5-E9 show phenotype non-I in most of the heterozygotes

Recently, Schmidt *et al.*²⁷ described a cystinuria specific *SLC3A1* rearrangement (c.(891+1524_1618–1600)dup; that is, duplication of exons 5 to 9; dupE5-E9) that results in the in frame duplication of amino acid residues E298 to D539 of the rBAT protein (p.E298_D539dup) as demonstrated by our own RNA studies (not shown) and by Schmidt *et al.*²⁷ Five probands of the ICC database carry this duplication. Four are compound heterozygotes with *SLC3A1* mutations (M467T, Spanish family 79; R452W, Spanish family 14; c.765+1G→T; Spanish family 92; c.1749delA, Belgian family 106). Two patients in the fifth family (Italian family 77) are compound heterozygotes with *SLC3A1* mutations dupE5-E9 and M467T, and also carry the *SLC7A9* mutation c.789+2T→C in one allele (fig 2B). Amino acid levels in urine for six single dupE5-E9 carriers of families were available (table 6). Interestingly, four of these carriers (F14-C119, F77-SR, F79-A145, and F79-A148) showed excretion of cystine and dibasic amino acids within the range of phenotype non-I heterozygotes. In contrast, carrier F106-D18 showed amino acid levels in urine within the control range like most of *SLC3A1* heterozygotes. Cystine in urine was not analysed in the other carrier in this family but the urine excretion of the other amino acids was within the control range, indicating phenotype I. To our knowledge dupE5-E9 is the first *SLC3A1* mutation that shows phenotype non-I in heterozygotes.

SLC7A9 mutations that show phenotype I in heterozygotes

SLC7A9 mutations with phenotype I in heterozygotes are shown in table 7. A182T, G105R, V170M, and c.614dupA are common *SLC7A9* mutations (table 3s in Supplementary Material). Six of the 11 A182T carriers showed phenotype I as the average urine levels of cystine, lysine, and the sum of the four amino acids were below the fifth centile of values in heterozygotes B+ with phenotype non-I (table 7). In contrast, the other five A182T carriers had amino acid levels in the urine above the 95th centile in controls (phenotype non-I) and within the range of values for B+ heterozygotes with phenotype non-I. The proportion of heterozygotes with phenotype I carrying the other common *SLC7A9* mutations was variable: T123M (two of four), V170M (four of 16), c.614dupA (one of 19), and G105R (one of 32). In contrast, carriers of the common and severe mutation R333W²¹ showed phenotype non-I in all cases. Moreover we describe carriers of six uncommon *SLC7A9* mutations that also show phenotype I (G63R, A126T, G195R, Y232C, and W69X) (table 7). Interestingly, in some of the *SLC7A9* heterozygotes with phenotype I, urine levels of cystine but not of dibasic amino acids were above than 95th centile of control values and within the lower range of values of B+ heterozygotes (phenotype non-I). All phenotype I alleles with common or uncommon *SLC7A9* mutations belong to mixed (and

untyped) families except for family F112, which transmit type I cystinuria (the proband is a G105R/T123M compound heterozygote).

Urine amino acid excretion in patients with cystinuria classified by genotype and phenotype

Table 8 shows the excretion of cystine and dibasic amino acids in urine of patients in relation to their allele genotype (A or B) and their cystinuria type. Three groups of patients were numerous enough for statistical comparisons: patients AA with type I (n = 34), patients BB with type non-I (n = 37), and patients BB with mixed type (n = 11). The average urine levels of cystine and the three dibasic amino acids, as well as the 5th and 95th centile of these values, were very similar for patients AA with type I and BB with type non-I. This is shown graphically for the sum of the urine excretion of the four amino acids in fig 1. The average and the 95th centile of urine excretion of lysine, arginine, and ornithine, and the sum of the four amino acids were lower in patients BB with mixed type than in patients AA with type I and patients BB with type non-I. Similarly, patients BB with type I cystinuria showed urine excretion values for dibasic amino acids within the lower limit of excretion values in patients BB with type non-I and AA with type I. In contrast, urine cystine levels were very similar in all these groups. This probably reflects the fact that most of the cystinuria patients reached the limit of solubility of cystine in urine (300 mg/l or 1.2 mM),³¹ and this resulted in an underestimation of this amino acid in the urine when the sample was not previously diluted to dissolve cystine microcrystals. Indeed, the 95th centile of cystine levels in urine in these four groups of patients (up to ~3400 μmol/g creatinine) represents 3.4 mM, assuming 1 g creatinine/litre of urine.

Three patients with cystine urolithiasis were classified as heterozygotes for *SLC7A9* mutations (B+) on the basis of their urine amino acid concentrations (table 8, fig 1), which were similar to that of the parent transmitting the mutation (data not shown) and within the range of excretion of heterozygotes B+ with phenotype non-I (table 7). Moreover, these three patients excreted less arginine and ornithine than the fifth centile of patients BB with mixed cystinuria (table 8).

Only three AA patients with mixed cystinuria were identified in the ICC database (table 8, fig 1). The two patients with higher amino acid excretion values are siblings (family 14) and compound heterozygotes (dupE5-E9/R452W), and one of them had very substantial aminoaciduria. The expected severe defect of mutations R452W (that is, a non-conservative amino acid substitution) and dupE5-E9 (the only *SLC3A1* mutation that associated with phenotype non-I in heterozygotes) might contribute to the severe urine phenotype in this patient. The third patient (family 79), who had the lowest amino acid excretion values in urine (within the lower range of patients AA (type I)), is a compound heterozygote (dupE5-E9/M467T). Patient homozygotes for M467T or compound heterozygotes carrying M467T showed lower amino acid excretion values in urine (mean (SEM), μmol/g creatinine, n = 20: 1414 (136) for cystine, 5753 (669) for lysine, 1718 (404) for arginine, 1227 (160) for ornithine, and 9859 (1222) for the sum of the four amino acids) than AA patients without this mutation (n = 33: 2376 (291) for cystine, 9173 (835) for lysine, 3655 (378) for arginine, 3526 (505) for ornithine, and 18 730 (1517) for the sum of the four amino acids). Indeed, three patients homozygous for M467T had amino acid concentrations in urine (sum of the four amino acids: 5613, 6433, and 8113 μmol/g creatinine⁶) that were among the lowest in patients AA. Thus the mild phenotype of M467T might contribute to the low amino acid excretion values in the compound heterozygote dupE5-E9/M467T.

Digenic inheritance causing a partial phenotype in two cystinuria families

Patient BB(A) (family 35; fig 2A) is a compound heterozygote of *SLC7A9* mutations (Y232C/G105R) that also carries the *SLC3A1* mutation M467T. Mutations Y232C and M467T showed phenotype I, whereas G105R showed phenotype non-I in the heterozygotes of this family. Patient BB(A) was then classified as presenting mixed cystinuria. This patient had very low urine amino acid levels, which were similar to the fifth centile of these values in patients BB (type non-I). Two members of this family are double carriers (AB) (G105R/+ for *SLC7A9* and M467T/+ for *SLC3A1*). Digenic cystinuria without urolithiasis is suggested in these two double carriers, as they had higher urinary excretion values than the single heterozygotes (G105R/+ or M467T/+) within the family. Patient AA(B) (family 77; figs 1 and 2B) had very high amino acid concentrations in the urine and at the time of this study her sister was on dialysis. Both carry *SLC3A1* mutations dupE5-E9 and M467T and the *SLC7A9* mutation c.789+2T→C. M467T showed phenotype I, whereas dupE5-E9 showed phenotype non-I in the heterozygotes identified in this family. Patients AA(B) were then classified as having mixed cystinuria. The type B mutation seems to contribute to the severe phenotype in these patients. Thus one of these patients (M467T/dupE5-E9 and c.789+2T→C/+) excreted larger amounts of amino acids than patient M467T/dupE5-E9 (family 79) (compare patient AA(B) and patient AA (mixed) with the lowest aminoaciduria in table 8 and fig 1). In contrast, the mild mutation M467T did not seem to aggravate the urine phenotype of the double carrier in family 77 (M467T/+ and c.2789+2T→C/+). Thus the aminoaciduria in this double carrier was lower than that of two single c.782+2T→C heterozygotes (family 58), which had the greatest aminoaciduria (the sum of the urine levels of the four amino acids was 8112 and 8307 mmol/g creatinine) among the heterozygotes B+ in our database.

DISCUSSION

We describe 40 new cystinuria mutations (24 in *SLC3A1* and 16 in *SLC7A9*) which bring the total number of cystinuria specific mutations identified to 103 in *SLC3A1* and 66 in *SLC7A9*. The mutations include missense, nonsense, splicing defects, frameshift, deletions and insertions of a single amino acid residue, and large rearrangements. To our knowledge, the present study represents the largest percentage of cystinuria alleles explained in any large cohort of patients reported (86.8%; 282 of 325 alleles). In a recent study,²⁷ only 64.7% of the alleles were explained (75 of 116 alleles). In the ICC database, the coverage of identified alleles is similar for probands with different cystinuria types (on average 89.3%). The percentage of explained alleles in untyped patients was lower (83.6%), which might be attributed to the presence of *SLC7A9* carriers with cystine urolithiasis.

The greater number of characterised alleles allows a general summary of the cystinuria mutations in the ICC database, which includes patients of mainly Italian, Spanish, or Libyan-Jewish origin. Type A (*SLC3A1*) and type B (*SLC7A9*) mutations accounted for 44.3% and 55.7% of the explained alleles, respectively. To our knowledge, these are the first relative frequencies of type A and type B alleles to be reported, but they must be viewed with caution. The ICC database is not a registry, as we did not collect all possible cases.²⁸ The ICC was very active in collecting phenotype non-I cystinuria cases in order to identify the *SLC7A9* gene,^{2 10 11} and the prevalence of type B mutations might be overestimated. M467T and G105R were the most common type A and type B mutations (26.4% and 27.4% of each type of alleles, respectively). This is consistent with reports on other smaller cohorts of patients with cystinuria.^{16 23 25} The five most

common type A and type B mutations accounted for 44.8% and 63.1% of the identified alleles of each type (A or B), respectively. Moreover, 55 *SLC3A1* and 45 *SLC7A9* mutations covered the 125 type A and the 157 type B explained alleles, respectively, showing that *SLC3A1* has greater genetic heterogeneity than *SLC7A9* in the ICC database.

The present search for mutations was very careful (SSCP, DHPLC, direct sequencing, and specific search for large rearrangements of *SLC3A1* and *SLC7A9*) and covered the whole coding region and all intron/exon boundaries. The unexplained cystinuria alleles (13.2%) may be the result of mutations in intronic or promoter regions not yet identified in the two known cystinuria genes or they may reflect unidentified genes. Also, *SLC3A1* or *SLC7A9* polymorphisms in combination with cystinuria specific mutations might result in full blown cystinuria, for the following reasons.

First, the promoter region of these genes was not analysed in this study, but a small contribution of mutations in these regions is expected. Indeed, two cystinuria specific sequence variants in the promoter region of *SLC3A1* have been reported,¹⁹ but functional studies should be done to confirm these mutations.

Second, intronic mutations affecting sequences other than the splice-site consensus ones could explain these alleles, but RNA studies would be necessary to assess this.

Third, a third cystinuria gene cannot be discounted but it would be relegated to a very small proportion of patients (no mutations were found in any of the two cystinuria genes in only 3% of the probands), and seems improbable. Co-immunoprecipitation studies and the *SLC7A9* KO model showed that the protein products of *SLC3A1* (rBAT) and *SLC7A9* (b^{0,+}AT) heterodimerise in mouse kidney brush border membranes, but a significant part of rBAT heterodimerises with an unknown light subunit (X).^{12 32} This may also be true in human kidney brush borders.¹² Therefore, the gene coding for X could be a candidate for cystinuria. However, the similar hyperexcretion of amino acids detected in urine of patients AA and BB does not support this hypothesis. If X were coded by a cystinuria gene one would expect a greater degree of aminoaciduria in patients AA than in patients BB—b^{0,+}AT mutations would affect system b^{0,+} only, whereas rBAT mutations would affect system b^{0,+} and the transport activity of the rBAT/X heteromeric complex.

Finally, *SLC3A1* or *SLC7A9* polymorphisms in combination with cystinuria specific mutations might result in full blown cystinuria, as has been suggested.³³ Cystinuria association and functional analysis of these polymorphisms are required to demonstrate their contribution to the disease.

Some heterozygotes for the *SLC3A1* mutation dupE5-E9—which results in the duplication of the rBAT fragment E298-D539—presented with phenotype non-I (table 6). All other *SLC3A1* mutations, including very severe ones (for example, c.163delC which produces a frameshift with an early stop codon, eliminating the transmembrane and the extracellular domains of rBAT (p.Q55fsX51); or c.431_2055del, which eliminates 80% of the rBAT residues towards the C-terminus (p.G144_C685del)) showed phenotype I.^{6 18 22 25 34} This indicates that mutation dupE5-E9 has a dominant negative effect on the rBAT/b^{0,+}AT heteromeric complex.

Because 14% of *SLC7A9* heterozygotes showed phenotype I we proposed a classification of cystinuria on the basis of genotype rather than phenotype.²⁸ Rozen and coworkers identified two type I heterozygotes carrying the *SLC7A9* mutations I44T and P261L, respectively.²⁴ Here we report 10 more of these *SLC7A9* mutations. Four showed phenotype I in several carriers (A182T, T123M, G63R, and V170M). Mutations A182T and T123M affect non-conserved amino acid residues in the family of the light subunits of HATs. Moreover, A182T is a mild mutation with aberrant trafficking

to the plasma membrane³⁵ and substantial residual transport activity (60%).²¹ Similarly, A126T and P261L, with a single type I carrier identified, also involve non-conserved amino acid residues. Interestingly, patients BB with mixed or type I cystinuria (that is, with type B heterozygotes with phenotype I in their families) also presented with a mild urine phenotype (table 8 and fig 1). This agrees with the mild urine phenotype reported for patients with mixed cystinuria.³⁶ Seven of the 11 patients BB (mixed) and the patient BB (type I) carry A182T or T123M. The mild amino acid transport defect of these mutations could be the underlying cause of the mild urine phenotype of these patients. In contrast, other mutations with phenotype I in heterozygotes (I44T, G63R, G105R, V170M, G195R, and Y232C) involved fully conserved amino acid residues or resulted in large protein changes (W69X, with b^{0,+}AT truncated after the first transmembrane domain; c.614dupA, a mutation shown by mRNA studies to result in skipping of exons 5 and 6 (r.479_709del), producing a frameshift at residue 160 (p.L160fsX1); data not shown). Moreover, G105R and V170M have little amino acid transport function.²¹ Interestingly, a very small proportion of heterozygotes who carry severe and common mutations like c.614dupA (one of 19 carriers) and G105R (one of 32 carriers) had phenotype I, but one quarter of the V170M heterozygotes manifested phenotype I (all from a Libyan Jewish origin). This indicates that, in addition to the residual functional activity of b^{0,+}AT mutants, other factors (environmental or genetic) contribute to the urine phenotype of carriers of *SLC7A9* mutations.

Only three patients in the ICC database are double compound heterozygotes with mutations in both genes (two patients with mutations M467T and dupE5-E9 (AA), and c.789+2T→C (B), and one patient with mutations Y232C and G105R (BB), and M467T (A)). One patient AA(B) has previously been reported but with no description of the associated urine phenotype.³⁷ The degree of aminoaciduria of these type of patient and their relatives in the ICC database indicates that digenic inheritance in cystinuria has only a partial effect on the phenotype, restricted to a variable impact on the aminoaciduria, for the following reasons.

First, in family 77, the severe aminoaciduria in one of the patients AA(B) indicates that the type B mutation c.789+2T→C, associated with high aminoaciduria in heterozygotes, contributes to the urine phenotype; no information is available from the other patient AA(B) as she is on dialysis.

Second, in family 35, the mild aminoaciduria of patient BB(A) indicates that M467T, associated with mild aminoaciduria in homozygotes and compound heterozygotes, does not contribute to the urine phenotype in the patient, but two double compound heterozygotes in this family (M467T/+, G105R/+; that is, AB) showed greater aminoaciduria than the single heterozygotes within the family, suggesting a contribution of both mutations to the urine phenotype.

Third, none of the individuals AB had cystine urolithiasis. Given that almost 90% of the alleles of the ICC cohort of patients have been identified, and that the frequency of type A and B alleles is similar in this cohort, if digenic inheritance was the rule in cystinuria, we would expect 25% AA patients, 25% BB patients, and 50% AB patients. Furthermore, the few AB individuals identified did not have cystine urolithiasis. This indicates that digenic inheritance, affecting the phenotype, is an exception in cystinuria. However, we cannot discard the possibility that some combinations of mutations A and B produce enough cystine hyperexcretion to cause urolithiasis. In this regard, the urolithiasis activity of individuals B/+, with a low penetrance for cystine urolithiasis (three of 192 genotyped patients; 1.7%), might be aggravated by the addition of the effect of one type A allele.

A working hypothesis on the biogenesis of the rBAT/b^{0,+}AT heterodimer and the urine phenotypes in cystinuria may explain the apparent lack of full digenic inheritance in cystinuria. In the first place, the rBAT protein is produced in excess in the kidney, and therefore an rBAT mutation in heterozygosis (for example, the mouse cystinuria model with the *SLC3A1* mutation D140G³⁸ and all human cystinuria rBAT mutations with the exception of dupE5-E9) does not lead to hyperexcretion of amino acids (phenotype I). Secondly; b^{0,+}AT controls the expression of the functional rBAT/b^{0,+}AT heterodimeric complex—interaction with b^{0,+}AT stabilises rBAT and the excess of rBAT is degraded, as shown in transfected cells.^{35–39} As a result, a half dose of b^{0,+}AT (heterozygotes of severe human *SLC7A9* mutations or of the *SLC7A9* knockout mice³²) results in a significant decrease in the expression of rBAT/b^{0,+}AT heterodimer complex (system b^{0,+}), which causes hyperexcretion of cystine and dibasic amino acids. In this scenario, the lack of a full phenotype because of digenic inheritance indicates that in double heterozygotes (AB), the mutated rBAT, with trafficking defects,^{18–40–41} does not interact with, or does not compromise, the heterodimerisation and trafficking to the plasma membrane of the half dose of wild type b^{0,+}AT with the half dose of wild type rBAT. Thus AB individuals behave as B heterozygotes with a variable degree of aminoaciduria, which could be greater than that of the single heterozygotes within the family, depending on the particular combination of mutations. We are currently studying the hypothesis that the phenotype non-I associated to the *SLC3A1* mutation dupE5-E9 reflects heterodimerisation with b^{0,+}AT, which results in a non-active heteromeric complex.

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Supplementary material: Supplementary figures and tables can be accessed at the JMG website (www.jmg.com/supplemental)

Authors' affiliations

M Font-Llitjós, M Jiménez-Vidal, V Nunes, Centre of Medical and Molecular Genetics (IRO), Hospital Duran i Reynals, Barcelona, Spain
M Jiménez-Vidal, M Palacín, Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona
L Bisceglia, M D Perna, L Zelante, Medical Genetics Service, IRCCS-Hospital "CSS", San Giovanni Rotondo, Italy
L de Sanctis, Department of Paediatrics, Regina Margherita Children's Hospital, University of Torino, Italy
F Rousaud, Urology Service Fundació Puigvert, Barcelona

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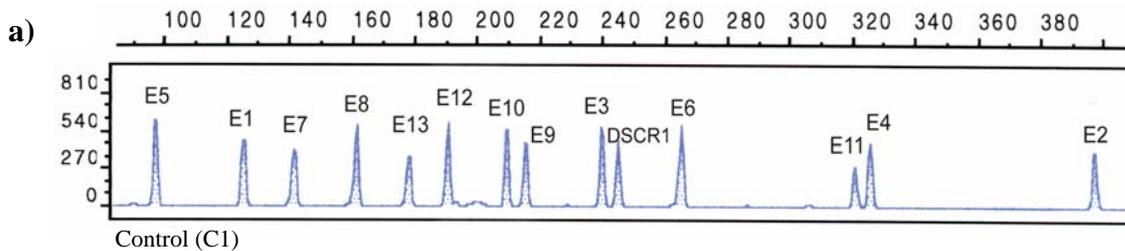
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Figure 1s.

Semi-quantitative Multiplex PCR analysis of SLC7A9 in controls. a) Chromatogram of a control (C1). The assignation of areas under peaks was made by GENESCAN™ software. Peak (SLC7A9 exons E1 to E13 and DSCR1-E5) sizes (top ruler in bp) and quantities (left ruler) were determined using the same software and the area of each peak was divided by the external double dose control (exon 5 of DSCR1) (internal to the reaction). We considered that the area of each peak obtained by semiquantitative PCR would be a reliable indicator of the amount of DNA in each exon, so we would see a clear difference in ratios wherever there was a large rearrangement. b) Three unrelated normal controls (C1, C2 and C3) are shown as an example of the reproducibility of the method. The values presented here were obtained as follows: The area below each peak is a measure of the amount of DNA present. These areas were divided by that of the internal double dose control (exon 5 of DSCR1). A mean of the values was obtained for each exon. Last, each value is divided by the mean of its corresponding exon. If all the samples are similar for one particular exon, as is the case with the normal controls, all the values are around 1. When a value is 0 we suspect a complete deletion, when it is 0.5 we suspect a deletion in heterozygosis and when it is 2 or larger we suspect that the sample has a duplication for this exon.



b)

Exon	Genescan Data			Divided by DSCR1-E5			Average	Ratio/Average		
	C1	C2	C3	C1	C2	C3	C1 + C2 + C3	C1	C2	C3
1	3448	4477	4315	1.32	1.21	1.32	1.28	1.03	0.94	1.03
2	2834	3891	4089	1.09	1.05	1.25	1.13	0.96	0.93	1.11
3	5247	4606	6182	2.01	1.25	1.89	1.72	1.17	0.73	1.1
4	3566	4227	4615	1.36	1.14	1.41	1.3	1.04	0.87	1.08
5	3555	5420	4979	1.36	1.46	1.52	1.45	0.94	1.01	1.05
6	5059	6381	6218	1.94	1.73	1.9	1.86	1.04	0.93	1.02
7	3046	3859	3642	1.17	1.04	1.11	1.11	1.06	0.94	1.00
8	3973	4910	4565	1.52	1.33	1.39	1.41	1.08	0.94	0.98
9	2445	3677	3656	0.94	0.99	1.12	1.02	0.92	0.97	1.1
10	2480	3842	3642	0.95	1.04	1.11	1.03	0.92	1.01	1.07
11	2343	3085	2895	0.9	0.83	0.88	0.87	1.03	0.95	1.01
12	3704	4969	4406	1.42	1.34	1.35	1.37	1.04	0.98	0.99
13	2722	3686	3493	1.04	1.00	1.07	1.04	1.00	0.96	1.03
DSCR1-E5	2613	3700	3273	1	1	1	1	1	1	1

Figure 2s. Genomic region of *SLC7A9* rearranged in patients with the 5 kb deletion and the 5 kb duplication. We have detected two novel large mutations in two Spanish families: a 5kb deletion (c.1224+4166_c.1399+119del) and a 5 kb duplication (c.1224+4166_c.1399+119dup) both affecting exon 12, originated by the crossing over of two 195 bp sequences (Repeat 1 and 2), separated by 4778 bp, which differ by 1 nucleotide. Both mutations have been confirmed by standard PCR method using primers which specifically amplify the mutated allele in both cases. Primers sequences of F11, F13, R10d and R13 are listed in Materials and Methods.

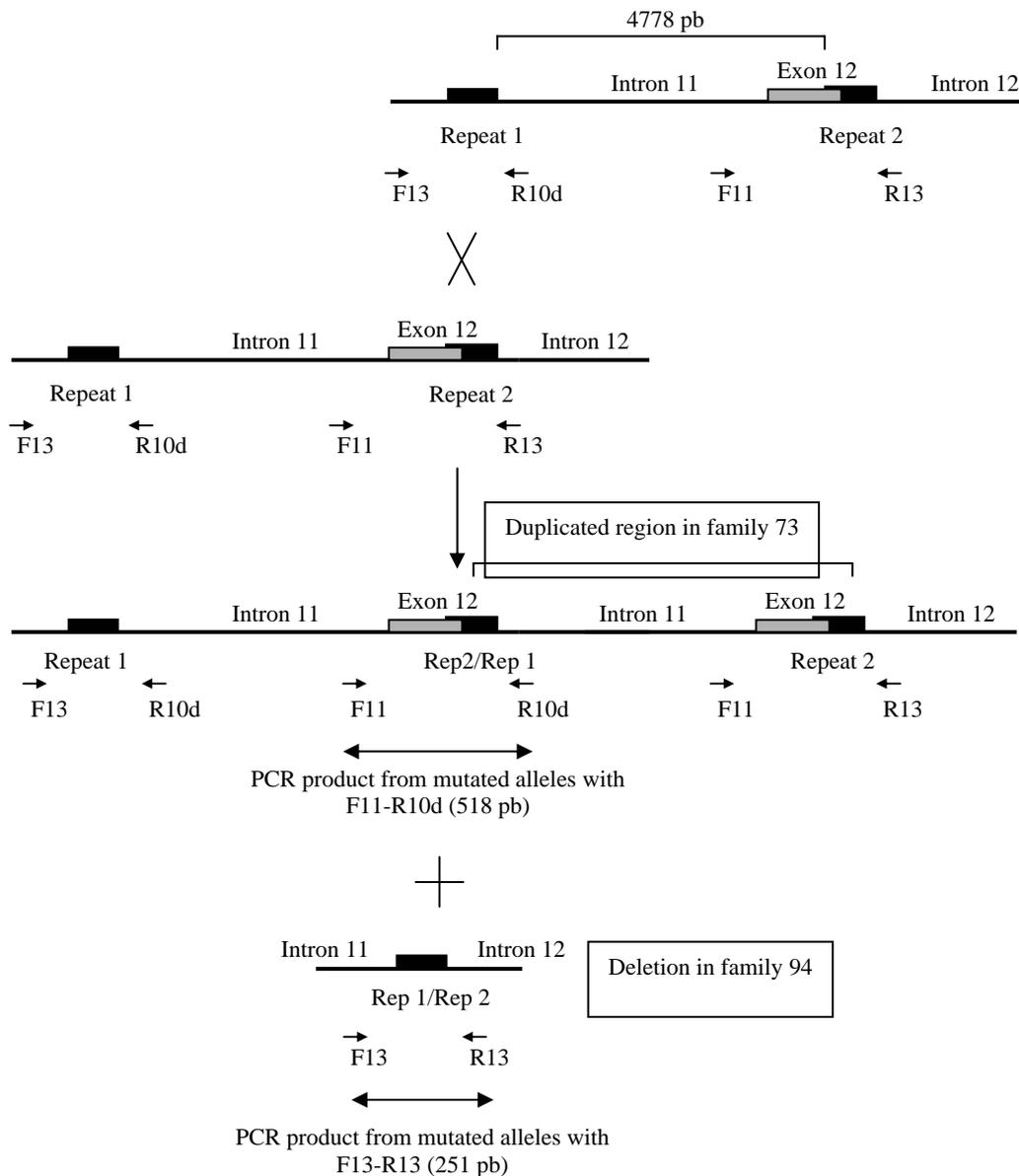


Figure 3s. (Supplementary Materials)

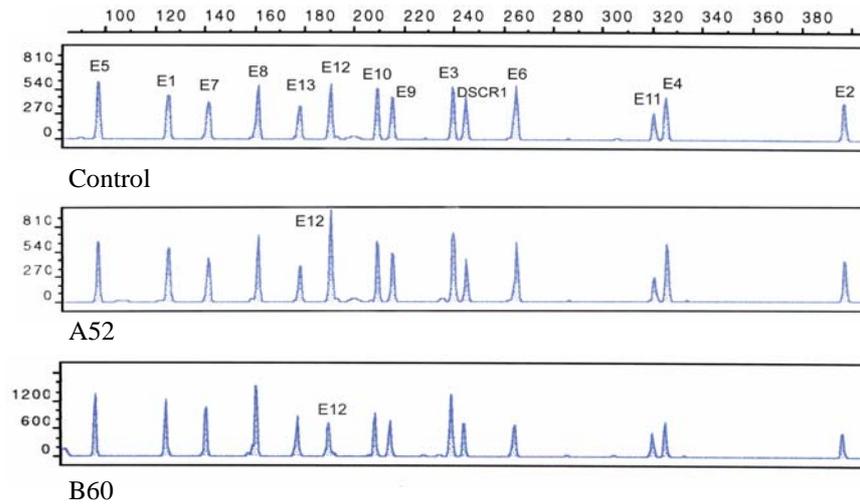


Figure Results semi-

	Family 73						Family 94
<i>SLC7A9</i>	A50	A51	A52	A53	A54	A55	B60
Exon 1	0.99	1.11	0.79	0.94	0.71	0.87	1.03
	1.09	0.88	1.00	0.83	0.83	0.78	1.08
Exon 2	1.04	1.06	1.04	1.24	1.04	1.16	1.03
	0.70	0.76	0.77	0.77	0.75	0.72	1.23
Exon 3	1.27	1.37	1.24	1.56	1.39	1.57	1.19
	0.91	1.13	1.11	1.24	0.95	1.11	1.40
Exon 4	1.26	1.25	1.10	1.36	1.14	1.28	1.07
	0.93	0.82	0.87	0.88	0.78	0.83	1.32
Exon 5	0.79	1.25	0.75	0.92	0.78	0.94	0.84
	1.08	0.83	0.93	0.74	0.72	0.80	1.16
Exon 6	1.06	1.33	1.03	1.24	1.04	1.24	1.07
	0.90	0.88	0.88	0.85	0.74	0.81	1.38
Exon 7	0.91	1.06	0.91	1.10	0.87	1.03	1.10
	0.96	0.88	1.08	0.89	0.70	0.77	1.02
Exon 8	0.91	1.04	0.89	1.21	0.95	1.07	1.11
	1.15	1.00	1.03	0.90	0.57	0.61	1.23
Exon 9	1.02	1.16	0.99	1.16	1.02	1.09	1.18
	0.95	0.98	0.98	0.95	0.94	0.99	1.35
Exon 10	1.13	1.24	0.99	1.11	1.07	1.18	1.15
	0.93	1.01	0.95	1.00	0.95	1.03	1.04
Exon 11	1.05	1.21	1.01	1.07	0.90	1.05	1.07
	1.01	0.91	0.97	0.85	0.97	0.91	1.05
Exon 12	1.59	1.05	1.48	1.06	0.95	1.56	0.63
	1.47	0.91	1.37	0.78	0.89	1.40	0.51
Exon 13	0.90	1.08	0.92	0.89	0.92	0.98	1.09
	1.02	1.01	0.91	0.88	0.93	0.92	0.97

3s.
of two

quantitative multiplex PCR analysis in families 73 and 94. Individuals A52 and B60 have an abnormal pattern (up): A52 have approximately double dose for exon 12 and B60 have half of the dose for exon 12 comparing with the control. The experiment was run with two replicas including all the members of family 73 and B60 from family 94, and the results were confirmed (bottom). The double dose for exon 12 is observed in

A50 (father), A52 (proband) and A55 (brother) (in bold). The segregation in family 94 could not be performed because there was no DNA of other members.

Table 1s. Primers used to amplify the *SLC7A9* exons for DHPLC analysis

The size of the amplified fragment and the resolution temperature (Tr) is indicated.

Fragment	exon	Sequence (5' -> 3')	Size (bp)	Tr (°C)
1	2	For: ATGACTGACTTTGACTCTGGG Rev: TCTTCTGCCGTGTCAATAGGG	258	63
2	3	For: CGCCCTCTTCCTTCCTCC Rev: TAGCAGCTGCCTGGCGTG	266	64/63
3	4	For: AGCCTCCGGTGGGAGGAAG Rev: GAGTCCCCAGACACCCTCTG	230	64
4	4	For: AAAGGAGACTCTCTCCAGGG Rev: AGCCCATCTCCAAGGAAGCA	244	64
5	5	For: TCCCGTGGAGATACACACTCA Rev: TGGAGTTAAAGTCACCTGGAG	310	63
6	6	For: AGTCAAGGTGTGTGACGCTTG Rev: AGGAGAAGAGAAATCAGGCTG	252	61
7	7	For: CTGAACGTGGGTCTCCGTG Rev: ACCTCCAGTGCTGACACCTG	161	59
8	8	For: GACCCTCAGGACTCATCTCG Rev: TAGCTGTGTGTCTTCCTCGGG	255	63
9	9	For: GGAGCACAAGTCCTCAGTGG Rev: GCCTTGAAGATAGGCTGGTAG	236	61
10	10	For: ATGACTGACTTTGACTCTGGG Rev: TCTTCTGCCGTGTCAATAGGG	230	60
11	11	For: TTCGGTCTTCTGTGACATGAG Rev: AGCCACTCGTGACTCTGGG	272	58
12	12	For: ATGATTGAAATTGGAGGAGGG Rev: TGGAGTCAGGACAGGTGAGG	390	60
13	13	For: CCTCACCCACAACA ACTCC Rev: AAATTCAGCTGACTTGGCTAC	231	59

Table 2s Primers used to amplify the *SLC7A9* exons together with the external double dose control *DSCR1* gene

Amount refers to picomoles of each pair of primers per 20 μ l reaction. *) Primers labeled with FAM fluorescence (Lifetechnologies). *DSCR1* primers are used as a double dosage control.

Exon	Name	Sequence (5' -> 3')	Size (pb)	Amount (pmoles)
1	P6IF1(-)*	CATTTCTAGGGTTGGACCGTG	127	2.8
	P6IR1(-)	GGCCAGGAGAGCCATGAG		
2	P6IF1N	ATGACTGACTTTGACTCTGGG	400	2
	P6IR1b*	TCTTCTGCCGTGTCCTAGG		
3	P6IF2*	CTCCTAATCTTTCTGTGCCTCA	242	2.35
	P6IR2b	GGTAGCAGCTGCCTGGCGT		
4	P6F3*	GGAGCTTGGCACAATGATCAC	328	2.1
	P6IR3c	GCTCTCACCAGAGACTCACT		
5	P6F4*	CTTCCAGTGTTTCATCTCGACA	98	2.6
	P6R13	ATGGCCACGATCACCAGCTTG		
6	P6IF5	TCCCGTGGAGATACTCA	267	1.85
	P6IR5*	CCCAGAAAGGAGAACTCATTGT		
7	P6IF6	AGTCAAGGTGTGTGACGCTTG	143	1.5
	P6IR6*	CAGGAGAAGAGAAATCAGGCT		
8	P6D8	CGGCGTGCTACATCCTCATGA	163	1.15
	P6IR7*	CAGGAGAAGAGAAATCAGGCT		
9	P6IF8	GACCCTCAGGACTCATCTCG	217	1.35
	P6IR8*	TAGCTGTGTGTCTTCCTCGG		
10	P6IF9*	GGAGCACAAGTCCTCAGTGG	211	2.1
	P6IR9	GCCTTGAAGATAGGCTGGTAG		
11	P6IF10*	TTCGGTCTTCTGTGACATGAG	323	2.75
	P6IR10b	GTCAGATTGGAAGTGAAGGCA		
12	P6IF11	ATGATTGAAATTGGAGGAGGG	192	1.5
	P6IR11*	GCTTGCTGATGATTGGAGCCA		
13	P6IF12*	CCCTCACCCACAACAACCTCC	180	2.2
	P6IR12	AAATTCAGCTGACTTGGCTAC		
Control	DSCR1-E5F	GTCAAGGGTCATATGCAGGTA	247	1.9
(DCSR1)	DSCR1-E5R*	GAACCCAACCTCACCTGAGCA		

Table 3. Cystinuria-specific mutated alleles identified in the 164 probands studied of the International Cystinuria Consortium (ICC) database.

One M467T allele (*SLC3A1*) and one c.789+2T>C allele (*SLC7A9*) are not included because they are the third mutated allele in patients BB(A) and AA(B) respectively. *) Two mutations in the same chromosome. #) Renamed *SLC7A9* mutations following The Human Genome Variation Society nomenclature (www.genomic.unimelb.edu.au/mdi/mutnomen/) (e.g., for the common mutation c.800-801insA, also named c.799-800insA, the new name is c.614dupA). \$) Two c.604+1G>A alleles have been considered in one patient even though hemizygosity due to deletion in the other *SLC7A9* allele is not discharged. &) *SLC3A1* deletions with unidentified boundaries. (n), number of independent alleles. F, frequency. Previous identified mutations in the ICC database shown in this table have been published elsewhere ^{2,7,8,15,16,17,18}.

Type A mutations (<i>SLC3A1</i>)				Type B mutations (<i>SLC7A9</i>)			
Nucleotide change	Amino acid change/ Predicted consequence	(n)	F	Nucleotide change	Amino acid change/ Predicted consequence	(n)	F
c.410A>G	D137G	1	0,008	c.977+3A>T#	A290fsX45	1	0,006
c.1699_1700 delAG	R567fsX8	1	0,008	c.1265_1266 delTG	L424fsX62	1	0,006
c.664delT	W222fsX3	1	0,008	c.1399+1G>T	447fsX22	1	0,006
c.542G>A	R181Q	1	0,008	c.475_477dupGCC#	A158dup	1	0,006
c.1702G>A	G568L	1	0,008	c.579_581dupCAT#	I193dup	1	0,006
c.1998C>G	C666W	1	0,008	c.151delT	S51fsX38	1	0,006
c.1364C>T	S455L	1	0,008	c.368_369 delCG#	T123fsX84	1	0,006
c.1035G>A	D338fsX4	1	0,008	c.501_504 delACTG#	L168fsX19	1	0,006
c.1043A>C	H348P	1	0,008	c.630_633 delTTTC#	F211fsX51	1	0,006
c.446T>A	L149Q	1	0,008	c.813delC#	N271fsX6	1	0,006
c.368T>G	M123R	1	0,008	c.376G>A#	A126T	1	0,006
c.1690C>T	L564F	1	0,008	c.947C>T	A316V	1	0,006
c.1230C>A	N410K	1	0,008	c.1144G>A#	A382T	1	0,006
c.1865T>C, c.1520C>T	L622X, S507L*	1	0,008	c.171C>T	N58_G79 del22	1	0,006
c.566C>T	T189M	1	0,008	c.586C>T	L196_G202 delfsX5	1	0,006

c.1367G>A	R456H	1	0,008	c.712_716 delCTCAA#	N239fsX6	1	0,006
c.1966_1968 delCTT	L656del	1	0,008	c.1224+4166_ c.1399+119del(4972)	K386fsX78	1	0,006
c.2019C>G	C673W	1	0,008	c.1224+4166_ c.1399+119dup(4972)	V409fsX9	1	0,006
c.2033T>C	L678P	1	0,008	c.187G>A#	G63R	1	0,006
c.1355G>A	R452Q	1	0,008	c.131T>C#	I44T	1	0,006
c.1943T>C	F648S	1	0,008	c.1400-3C>G	447fsX22	1	0,006
c.1618_ 2055del	V540_ C685del	1	0,008	c.847C>T	L283F	1	0,006
c.431_2055 del	G144_ C685del	1	0,008	c.155C>T#	P52L	1	0,006
c.766-2A>G	L256_ K297del	1	0,008	c.184G>A	V62M	1	0,006
c.1093C>T, c.1955C>G	R365W, T652R*	1	0,008	c.206G>T#	W69L	1	0,006
c.371A>G	Y124C	1	0,008	c.206G>A#	W69X	1	0,006
c.1383T>G	Y461X	1	0,008	c.695A>G	Y232C	1	0,006
c.1744T>C	Y582H	1	0,008	c.235+3_ c.235+293del	L30fsX9	1	0,006
c.1012-23C>G	D338_Y378 delfsX5	1	0,008	c.1060G>A#	A354T	1	0,006
c.1221dupT	P408fsX18	1	0,008	c.562G>A	V188M	1	0,006
c.503C>A	S168X	1	0,008	c.604+1G>A \$ #	L160X	2	0,013
Del 3'	to be defined ^{&}	1	0,008	c.604+2T>C	L160X	2	0,013
Del 5'	to be defined ^{&}	2	0,016	c.209C>T#	A70V	2	0,013
c.1749delA	T583fsX14	2	0,016	c.583G>A#	G195R	2	0,013
c.388T>C	S130P	2	0,016	c.544G>A#, c.1060G>A#	A182T, A354T*	2	0,013
c.1447G>T	E483X	2	0,016	c.775G>A#	G259R	2	0,013
c.1366C>T	R456C	2	0,016	c.688T>C, 28_30del AGA#	W230R, R10del°	3	0,019
c.892-1332del	E298_ M444del	2	0,016	c.730_732 delGAA#	E244del	3	0,019
c.266T>C	L89P	2	0,016	c.605-3C>A	I203fsX27	4	0,025
c.1084C>T	R362C	2	0,016	c.368G>A#	T123M	6	0,038
c.891+4A>G	L256_K297del	2	0,016	c.544G>A#	A182T	8	0,051
c.1640C>G	S547W	2	0,016	c.614dupA#	L160fsX1	11	0,070
c.451T>A	Y151N	2	0,016	c.508G>A#	V170M	18	0,115
c.1093C>T	R365W	2	0,016	c.997C>T#	R333W	19	0,121
c.1322C>G	P441R	3	0,024	c.311G>A#	G105R	43	0,274
c.1353C>T	R452W	3	0,024		Total	157	1
c.1400T>A	M467K	3	0,024				
c.765+1G>T	L256_K297del	4	0,032				
c.808C>T	R270X	5	0,040				
c.163delC	Q55fsX51	5	0,040				

DupI4-I9	E298_ D539dup	5	0,040
c.647C>T	T216M	8	0,064
c.1400T>C	M467T	33	0,264
Total		125	1

