Am. J. Hum. Genet. 57:781–788, 1995

Molecular Genetics of Cystinuria: Identification of Four New Mutations and Seven Polymorphisms, and Evidence for Genetic Heterogeneity

Paolo Gasparini,[1] Maria Julia Calonge,[2,3] Luigi Bisceglia,[1] Jesus Purroy,[2] Irma Dianzani,[5] Angelo Notarangelo,[1] Ferran Rousaud,[4] Michele Gallucci,[6] Xavier Testar,[3] Alberto Ponzone,[5] Xavier Estivill,[2] Antonio Zorzano,[3] Manuel Palacin,[3] Virginia Nunes,[2] and Leopoldo Zelante[1]

[1]Servizio di Genetica Medica, IRCCS-Ospedale "CSS" San Giovanni Rotondo; [2] Departament de Genetica Molecular, Institut de Recerca Oncologica, L'Hospitalet de Llobregat, [3]Departament de Bioquimica i Fisiologia, Facultat de Biologia, Universitat de Barcelona, and [4]Servicio de Nefrologia IUNA, Fundacion Puigvert, Barcelona; [5]Istituto di Clinica Pediatrica, Università degli Studi di Torino, Torino; and [6]Divisione di Urologia, Ospedale "Cristo Re," Rome

Summary

A cystinuria disease gene (rBAT) has been recently identified, and some mutations causing the disease have been described. The frequency of these mutations has been investigated in a large sample of 51 Italian and Spanish cystinuric patients. In addition, to identify new mutated alleles, genomic DNA has been analyzed by an accurate and sensitive method able to detect nucleotide changes. Because of the lack of information available on the genomic structure of rBAT gene, the study was carried out using the sequence data so far obtained by us. More than 70% of the entire coding sequence and 8 intron-exon boundaries have been analyzed. Four new mutations and seven intragenic polymorphisms have been detected. All mutations so far identified in rBAT belong only to cystinuria type I alleles, accounting for ~44% of all type I cystinuric chromosomes. Mutation M467T is the most common mutated allele in the Italian and Spanish populations. After analysis of 70% of the rBAT coding region, we have detected normal sequences in cystinuria type II and type III chromosomes. The presence of rBAT mutated alleles only in type I chromosomes of homozygous (type I/I) and heterozygous (type I/III) patients provides evidence for genetic heterogeneity where rBAT would be responsible only for type I cystinuria and suggests a complementation mechanism to explain the intermediate type I/type III phenotype.

Introduction

Cystinuria (MIM 220100) is a heritable disorder of amino acid transport, transmitted as an autosomal recessive trait. The disease is one of the most common genetic disorders, with an overall prevalence of 1/7,000, ranging from 1/2,500 in Israeli Jews of Libyan origin to 1/15,000 in the United States, according to newborn-screening programs (Segal and Their 1989). Cystinuria is caused by the defective transport of cystine and dibasic amino acids through the brush border epithelial cells of the renal tubule and intestinal tract (Rosenberg et al. 1965). Three types of classic cystinuria have been described (Rosenberg et al. 1966*a*). Type I heterozygotes show normal amino aciduria, whereas types II and III heterozygotes show high or moderate hyperexcretion of cystine and dibasic amino acids, respectively. In contrast to type I and II homozygotes, type III homozygotes show an increase in cystine plasma levels after oral cystine administration. These differences were thought to be due to allelism of the same gene (Rosenberg et al. 1966*b*).

rBAT, named in Genome Data Base "SLC3A1," is a human kidney cDNA that elicits the transport of cystine, dibasic, and some zwitterionic amino acids via a $b^{0,+}$-like transport system in *Xenopus* oocytes (Bertran et al. 1993; Lee et al. 1993). This gene is expressed in the brush border plasma membrane of both the proximal straight tubules of the nephron and the small intestine (Furriols et al. 1993; Pickel et al. 1993)

Recently we have identified six cystinuria-specific missense mutations in the rBAT gene in Italian and Spanish cystinuric patients. The most frequent mutation, M467T, reduces the amino acid transport activity associated with rBAT in *Xenopus* oocytes, thus demonstrating the direct involvement of rBAT in determining the disease (Calonge et al. 1994). Four additional mutations were recently reported by Pras et al. (1994*b*). Nevertheless, the majority of cystinuric chromosomes had still to be characterized in our population and correlated with one of the three disease phenotypes.

Therefore, to increase this figure, we decided to search for new mutations on genomic DNA using the RNA-SSCP technique (Sarkar et al. 1992; Bisceglia et al. 1994)

Received April 14, 1995; accepted for publication July 18, 1995.

Address for correspondence and reprints: Dr. Paolo Gasparini, Servizio di Genetica Medica, IRCCS, Ospedale Casa Sollievo Sofferenza, I-71013 San Giovanni Rotondo (Fg), Italy.

© 1995 by The American Society of Human Genetics. All rights reserved.

0002-9297/95/5704-0006$02.00

on a panel of 51 Italian and Spanish cystinuric patients. Primers for the PCR reaction have been designed on the genomic structure of rBAT that is now under completion (authors' unpublished data). Moreover, whenever possible, genotype-phenotype correlations have been performed relating the rBAT mutated alleles to one of the types of classic cystinuria described above.

Methods

Patients and Amplification of Genomic DNA

Fifty-one Italian and Spanish cystinuric patients were collected. All patients had typical symptoms of cystinuria. Urinary excretion values of cystine and dibasic amino acids (lysine, arginine, and ornithine) were determined by quantitative ion-exchange chromatography or reverse-phase high-pressure liquid chromatography. Amino acid content measurements were corrected per gram of creatinine. Patients and their relatives were typed according to the classification of Rosenberg and coworkers on the basis of the urinary concentrations of cystine and dibasic amino acids corrected per gram of creatinine (Rosenberg et al. 1966*a;* Sally 1978; Goodyer et al. 1993). Cystinuria patients were assigned as type I/I when their obligate carriers (i.e., parents and offspring) showed normal urinary excretion values. The average and range of urinary cystine and dibasic amino acid levels in the type I heterozygotes of this study (range of the sum between 76 and 632 μmol/g creatinine) agree with values reported elsewhere (Rosenberg et al. 1966*b;* Sally 1978; Goodyer et al. 1993). Obligate carriers showing urinary excretion values higher than the ranges of type I heterozygotes for at least one amino acid and for the sum of urinary cystine plus dibasic amino acids were classified as non–type I heterozygotes. Among them, it was possible to distinguish, according to the range of values reported by Goodyer et al. (1993), between type II (range of the sum between 2620 and 15310 μmol/g creatinine) and type III (range of the sum between 773 and 5055 μmol/g creatinine). Patients for which one parent was type I heterozygote and the other a type III were classified as type I/III, and patients for which the parents were both type III heterozygotes were considered as type III/III. Six patients were generically classified as non–type I (type II/III or type II/II), while a large proportion remained undefined because urinary excretion values of obligate carriers in their families were not available.

Genomic DNA was isolated from peripheral blood lymphocytes according to standard protocols. DNA amplification by PCR was carried out according to standard protocols by incorporating the T7 phage-promoter sequence into one of the PCR primers. In this manner, the amplified product can be processed by RNA-SSCP technique. Ten different pairs of primers were designed after the partial definition of the genomic structure of rBAT. The sequences of the primers, their location, and the size of the amplified products are reported in table 1.

Search for New Mutations

The search for new mutations was carried out by RNA-SSCP technology according to protocols described elsewhere (Sarkar et al. 1992; Bisceglia et al. 1994). After PCR reaction, transcription was carried out with 10 U of T7 RNA polymerase in a final volume of 10 μl containing 10 mM DTT, 40 mM Tris pH 7.5, 6 mM MgCl, 2 mM spermidine, 10 mM NaCl, 5 nmol of each ribonucleoside, 10 U of Rnasin, and O.2 ml of S35 UTP. Two microliters of transcribed RNA were mixed with 48 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. The mixture was heated at 95°C for 6 min and then chilled on ice for 10 min. An aliquot of 4.4 μl was then loaded onto a 6.5% nondenaturing polyacrylamide gel. Electrophoresis was performed at 30 W constant power for 13 h. After electrophoresis, the gel was dried and subjected to autoradiography for 12 h. Samples showing an electrophoretically altered mobility were then sequenced on an automatic sequencer (Applied Biosystem 373A) according to manufacturer's protocols. New mutations and polymorphisms found were also checked by restriction enzyme digestion.

Screening for Known Mutations

The frequency of the mutations previously described in the rBAT gene was assessed by PCR followed by enzyme restriction digestion as described by Calonge et al. (1994). When necessary, a modified primer was designed to construct a restriction site according to the restriction site–generating PCR (RG–PCR) protocol (Gasparini et al. 1992). In both cases, the primers and reaction conditions used were as described by Calonge et al. (1994).

Results

The majority of the rBAT coding regions (~73% of the total), almost half of intron/exon boundaries (8 of 18), and some intron sequences (on average, 50 bp on each side of the exons) have been investigated in a sample of 51 Italian and Spanish cystinuric patients, including the 13 patients analyzed in our previous work (Calonge et al. 1994). The method of choice was the RNA-SSCP technique.

Table 2 summarizes the overall number of new mutations and polymorphisms detected in rBAT gene and described in the present paper. So far, four new mutations and seven polymorphisms have been identified.

Table 1

Description of rBAT Primers Utilized in the Present Work

Fragment	Size	Primer	(nucleotide position)
1	370	GAAGGCACTCCGAAGACATAA	(−32; −12)
		AGAGAGGGCAATGATGGCTA[a]	(333; 315)
2	208	AACTGGACTACATCACAGCT	(443; 462)
		TCATCCCCATCTTGCCCACT[a]	(610+35; 610+16)
3	212	TTAGCCATTACTGTGCCTGG	(611−82; 611−63)
		TTCATGGGTACAGTCATGCC[a]	(793; 774)
4	203	ACGGTTCTGGCTCACAAAG[a]	(900; 918)
		TAACTGGTATCTCAGGCTGAG	(1011+86; 1011+66)
5	218	ACATTCATATAGAGCGAGCTG[a]	(1012−73; 1012−53)
		AGCCTGGGAAGAAATGAGAGC	(1136+37; 1136+17)
6	290	CCTACATCTTGTACATGCAAG	(1137−94; 1137−74)
		GGCTAGTTTTCCAATCCAGGA[a]	(1308; 1278)
7	179	GCGTTTGGGGAATCAGTATG	(1365; 1384)
		ATAGCTGTGATGAATAGTC[a]	(1500+38; 1500+20)
8	136	GTAAATCAGGACCAAAGCACAT[a]	(1501−72; 1501−50)
		CTATTGTCCCACTGCATTGGT	(1541; 1521)
9	336	AAGACTCAGCCCAGATCGGC[a]	(1624; 1643)
		CGTGTTGTGTTCAAAGATGAG	(1958; 1936)
10	344	GAGAGCTGGATGGCATCGA[a]	(1751; 1769)
		GCCAGTGTCTTCATCTCTTC	(2088; 2069)

[a] Primer to which the T7 phage-promoter sequence has been added, to perform RNA-SSCP analysis.

Description of New Mutations

A deletion involving the nucleotide A at position 1749 of rBAT gene sequence (1749delA) was found in 1 chromosome of an Italian patient and in none of the 100 normal chromosomes analyzed. This frameshift mutation leads to a stop signal 14 codons downstream and can be detected by RG-PCR protocol creating a *Dde*I restriction site (table 2).

A C→T substitution at nt position 1093 of the gene was found in one affected chromosome of Italian origin. This point mutation changes an arginine codon (CGG) to a tryptophan one (TGG) at position 365 (R365W). It was found in a patient who was also carrying the mutations R181Q and T652R. Because parents are deceased, it was not possible to define which of the three mutations cosegregates with the disease. Mutation R365W destroys a *Msp*I and creates an *Alw*NI site and was never found on 108 normal chromosomes. We were not able to classify the cystinuric chromosomes carrying either of these new mutations into one of the three different biochemical types of cystinuria.

A T→C substitution at nt 1943 of rBAT gene sequence was also detected. The normal codon TTT, which codes for the amino acid phenylalanine at position 648, changes to a TCT serine codon (F648S). It was found in a type I affected chromosome and never in normal ones (0/108). The mutation can be analyzed using the RG-PCR method creating a *Dde*I restriction site. Mendelian inheritance from the parents was confirmed by PCR followed by enzymatic digestion.

The fourth mutation is a T→C substitution at nt position 1744 of the gene. This point mutation changes a tyrosine codon (TAC) to a histidine one (CAC) at position 582 of the rBAT gene sequence (Y582H). It was found in an affected chromosome of a type I patient carrying on the other chromosome the M467T mutation. None of the 100 normal chromosome analyzed carried this alteration. This mutation destroys a *Rsa*I and creates an *Apa*LI restriction site. Mendelian inheritance from the parents was confirmed by PCR followed by enzymatic digestion. An overall distribution of the mutations here identified and of those previously described, along a schematic representation of the rBAT protein, is given in figure 1. This model of the rBAT protein, containing four membrane-spanning domains, is based on limited proteolysis and peptide-directed anti-rBAT antibody studies (Mosckovitz et al. 1994), as initially proposed for the rat rBAT protein itself (Tate et al. 1992). No mutations have yet been found in the first, second, or fourth transmembrane domain. The stop codon or the frameshift mutations R270X, 1306insC, and 1749delA result in the lost of 3, 2, or 1 transmembrane domains, respectively.

Description of Sequence Variants and Polymorphisms

Seven mutations were detected. Four of them were found only on affected chromosomes, while the remaining three were also found on normal ones (table 2). At nt 114 of the rBAT sequence a substitution C→A (114 C→A) was detected in 20 (.21) of 94 affected chro-

Table 2

Mutations and Polymorphisms in the rBAT Gene

Name	Effect on Coding Sequence	Nucleotide Change	Restriction Site		Frequency		RNA-SSCP Fragment	Primer Pairs	Nucleotide Position
			Created	Destroyed	Cystinurics	Noncystinurics			
Missense:									
R365W	Arg→Trp at 365	C→T at 1093	*Alw*NI	*Mps*I	1/142	0/108	5	5′-GTACCATGACTTCACCACCA-3′	1038–1057
								5′-AGCCTGGGAAGAAATGAGAGC-3′	1137+37–1137+17
Y582H	Tyr→His at 582	T→C at 1744	*Apa*LI	*Rsa*I	1/142	0/100	9	5′-CAGCCACTATGTTGTGTAC(T)C-3′	1728–1748
								5′-GAGATTCTTCGTGTTGTG(C)TCA-3′	1965–1944
F648S	Phe→Ser at 648	T→C at 1943	*Dde*I[a]	...	1/142	0/108	10	5′-CAGCCACTATGTTGTGTAC(T)C-3′	1728–1748
								5′-GAGATTCTTCGTGTTGTG(C)TCA-3′	1965–1944
Frameshift, deletion, insertion:									
1749delA	Frameshift	del of A at 1749	*Dde*I[a]	...	1/142	0/100	9	5′-CAGCCACTATGTTGTGTAC(T)C-3′	1728–1748
								5′-GAGATTCTTCGTGTTGTG(C)TCA-3′	1965–1944
Polymorphisms:									
114A/C	No aa change	A or C at 114	*HinD*III[a]	...	20/94	27/100	1	5′-GAAGGCACTCCGAAGACATAA-3′	−32–−12
								5′-CTTCAGGTTGTCTGTT(A)AGCT-3′	135–115
231T/A	No aa change	T or A at 231	*Dde*I	...	12/102	0/160	1	5′-GAAGGCACTCCGAAGACATAA-3′	−32–−12
								5′-CCATCCTTGTTACTGTCCTT-3′	413–394
1136+3 delT	5′ intron 6	T or G at 1136+3	*Mae*III	...	3/102	5/50	5	5′-GTACCATGACTTCACCACCA-3′	1038–1057
								5′-AGCCTGGGAAGAAATGAGAGC-3′	1137+37–1137+17
1398C/T	No aa change	C or T at 1398	*Nde*I[a]	...	1/102	0/100	7	5′-AATCAGTATGTCAACGTGATG(C)A-3′	1375–1397
								5′-GCTACAATATTTCCCATTCC-3′	1472–1453
1473C/T	No aa change	C or T at 1473	*Alu*I	...	1/102	0/102	7	5′-AATCAGTATGTCAACGTGATG(C)A-3′	1375–1397
								5′-ATAGCTGTGATGAATAGTC-3′	1500+20–1500+38
1854A/G	M or I at 618	A or G at 1854	*BsmA*I[a]	...	29/94	34/100	10	5′-CAGCCACTATGTTGTGTAC(T)C-3′	1728–1748
								5′-GAATTGGTACTTAACCTT(G)TCT-3′	1877–1855
2189C/T	3′-UTR	C or T at 2189	*Bstu*I[a]	...	29/94	34/100	...	5′-GTATACCTCGTGTTAGGCAC-3′	2043–2062
								5′-CATTTGAGAACCTTTCTTAA(C)GC-3′	2190–2212

NOTE.—Description of new mutations and polymorphisms identified in cystinuric patients. The nucleotide changes are referred according to the International Nomenclature on Human Mutations. The nucleotides are counted considering as number 1 the adenine of the first ATG codon or rBAT cDNA sequence.

[a] Enzymes that recognize a restriction site created by a mutagenesis primer according to RG-PCR protocols. The frequency, in our populations, of each one of the four mutations here described has been evaluated on a sample of 142 cystinuric chromosomes arising from 71 patients attending our centers.

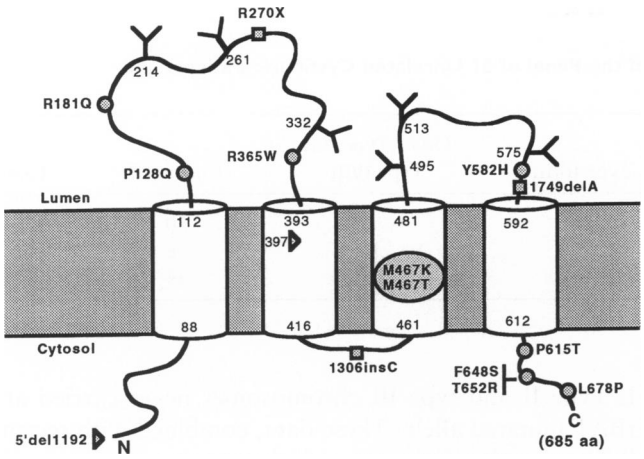

Figure 1 Localization of cystinuria-specific rBAT mutations along a schematic representation of the model of the rBAT protein suggested by limited proteolysis and peptide-directed anti-rBAT antibody studies (Mosckovitz et al. 1994). Missense mutations (P128Q, R181Q, R365W, Y582H, P615T, F648S, T652R, and L678P) are shown by circles, and stop codons or small deletions or insertions (R270X, 1306insC, and 1749delA) are shown by squares. A large deletion (5'del1992) involving the N-termini with loss of the first six exons and of the first transmembrane domain is comprised between two triangles. Two mutations (M467T and M467K) are located in the third transmembrane domain, while no mutations have yet been found in the first, second, or fourth transmembrane domain. Mutations R365W, Y582H, 1749delA, and F648S are described in this paper for the first time. Mutations R181Q, M467T, M467K, P615T, T652R, and L678P were previously described by our group (Calonge et al. 1994), while mutations 5'del1192, P128Q, R270X, and 1306insC were described by Pras et al. (1994*b*).

mosomes and in 25 (.27) of 94 normal ones. It can be analyzed by RG-PCR, creating a *Hin*dIII restriction site. This frequent polymorphism has a PIC value of .32.

A T→A substitution at nt 231 (231 T→A) was found in 12 affected chromosomes in complete linkage disequilibrium with the presence of the mutation M467T. It could be easily detected by *Dde*I restriction digestion.

A deletion of a T three bases downstream the nt 1136 was found in an Italian family (1136+3delT). This mutation, located at the splicing junction site, could increase the strength of the splicing itself, as deduced by a computer-model analysis performed using the SIGNAL program (Staden 1984). The mutation creates a *Mae*III restriction site.

A nucleotide substitution C→T was found at position 1398 of an Italian affected chromosome (1398 C→T). Being a third base-position change it does not modify the amino acid codon. It could be analyzed by RG-PCR protocols (*Nde*I restriction digestion).

A C→T substitution was found at nt position 1473 (1473 C→T), on one affected chromosome. It can be investigated by restriction digestion (*Alu*I site).

An A→G substitution was detected at nt position 1854 (1854 A→G). This sequence polymorphism leads to an amino acid change from an isoleucine to a methionine. It is frequent on both affected (29 of 94) and normal (34 of 100) chromosomes and can be easily analyzed by RG-PCR, creating an *Bsm*AI site.

A T→C substitution in the 3′ UTR of the gene at nt position 2189 (2189 T→C) was also found. This polymorphism is in complete linkage disequilibrium with the 1854 A→G polymorphism. Both of these two last polymorphisms have a PIC value of .35.

Genotype-Phenotype Relationships

Table 3 reports the distribution of the rBAT mutated alleles in the different types of cystinuria. Seventeen of 102 affected chromosomes carry an identified mutation. Among them, nine corresponded to the M467T mutation (9 [.53] of 17), which is so far the most common cystinuric mutation in the Spanish and Italian population.

Among patients classified as type I/I homozygous, a proportion of ~50% of affected chromosomes (8 of 16) has been characterized for the presence of mutated alleles in rBAT gene. Three chromosomes arising from patients classified as compound heterozygous type I/III also carried a rBAT mutated allele (3 [.33] of 9). In all cases, the mutated allele belongs to their type I heterozygote parent. Pooling all type I chromosome, the final figure of characterized chromosomes is 44% (11 of 25). A proportion of 28% of type I affected chromosomes carries the M467T mutation (7 of 25). This figure has been further corrected to 24% (12 of 50), adding 25 new type I chromosomes (data not shown). No cystinuria-specific mutation has been detected in the rBAT gene of 37 type II and type III affected chromosomes.

Discussion

After the identification of a gene causing a common genetic disease, the obvious goals are the detection of almost all mutated alleles and the assessment of their frequency in large samples. The knowledge of these data could lead to a rapid and accurate molecular diagnosis of the disease, proper genetic counseling, and the possibility of planning population-screening programs.

At the beginning of the present work, three important data were well established: there was a gene recently identified (rBAT), some different disease-specific mutations in this gene were known, and there was a general agreement that the three cystinuria types were more due to allelism of the same gene than to genetic heterogeneity (McKusick 1992).

Taking this into account, we decided to assess the frequency of the disease-specific mutations previously reported in a large sample of cystinuric patients. This analysis failed to be conclusive, because only few affected chromosomes were positive in the screening. To

Table 3

Description of the Results Obtained after the Mutational Analysis of the Panel of 51 Unrelated Cystinuric Patients and Relationship with the Three Classical Cystinuria Phenotypes

	Type I/I	Type I/III	Type III/III	Other Type II/II Type II/III	Undefined	Total
No. patients	8	9	8	6	20	51
Positive chromosomes	8	3/0	0	0	6	17
Negative chromosomes	8	6/9	16	12	34	85

increase this figure it was decided to search for new mutations by using the RNA-SSCP technology, an accurate and sensitive method able to detect almost all kinds of nucleotide alterations present in a given DNA fragment (Sarkar et al. 1992; Bisceglia et al. 1994). Initially, this strategy was applied to the analysis of patients cDNAs according to our previous experience (Calonge et al. 1994). Soon after, increasing the amount of preliminary information we were producing on rBAT genomic structure, it was possible to screen >70% of coding regions of the gene and 8 intron/exon boundaries directly on genomic DNA. This approach is less time consuming and less expensive than the cDNA approach.

Four new mutations causing disease have been identified. One is a frameshift mutation that leads to a truncated rBAT protein, while the remaining three affect residues that are highly conserved across species (i.e., rat and rabbit). There are some doubts about the role of mutation R365W, found in a patient carrying also the R181Q and T652R mutations. Because none of these alleles has been evaluated by functional studies (i.e., *Xenopus* oocytes expression), it is impossible to establish which one is a protein variant.

The seven intragenic polymorphisms here described are useful tools for linkage analysis and evaluation of informativeness in at-risk families. However, the presence of the nucleotide C at position 2189 is in complete linkage disequilibrium with the presence of the nucleotide A at position 1854, while a T at position 2189 goes with a G at position 1854. At the same time, a C at position 114 is frequently associated with an A at position 1854. This finding reduces the informativeness of these polymorphisms to the information given by any one of them. Nevertheless, in many pedigrees, the segregation pattern of one of these polymorphisms can be sufficient to establish whether rBAT is related to the disease. The combination of these polymorphisms also defines two frequent haplotypes, which are equally distributed on normal and affected chromosomes.

Classifying, on the basis of their urinary parameters, the affected patients in the three different cystinuria phenotypes, we were able to demonstrate that all chromosomes carrying an rBAT mutated allele belong to type I. Type II and type III chromosomes never carried an rBAT mutated allele. These data, combined with recent linkage results (Calonge et al., in press), provide strong evidence for the genetic heterogeneity of cystinuria. Our findings are in contrast with the general agreement of cystinuria as a genetically homogeneous disorder and with a recent report of homogeneity in Jewish families (Pras et al. 1994*a*). But Italians and Spaniards have different genetic backgrounds from those of Jews and are more heterogeneous populations. Moreover, genetic homogeneity in Jewish families can be explained on the basis of phenotypic homogeneity, although no phenotypic characterization of individuals was reported.

In addition, we demonstrated that mutation M467T is the most common cystinuric allele in our population and that ~50% of all type I cystinuric chromosomes still need to be characterized. Thus, we should further search in the coding regions not yet analyzed (~30%) and in the promoter sequences not yet isolated.

The finding of type I/type III compound heterozygotes patients positive for the presence of rBAT mutations in the type I allele, but negative in the type III one, suggests that cystinuria could be due, in many cases, to complementation, as recently hypothesized by Goodyer et al. (1993), on the basis of biochemical data. In our case, the complementation model provide for an interaction and expression of mutated alleles of two different genes, one for cystinuria type I (rBAT) and the other for cystinuria type III.

Genetic heterogeneity and the complementation model can better explain some biochemical difference detectable among the different cystinuria types. First-degree relatives of type I patients had no abnormal urinary amino acid excretion, while type II and type III heterozygous individuals show increased amounts of cystine and the dibasic amino acids in their urine. Moreover, oral cystine loading fails to raise serum cystine levels in type I and type II patients but results in nearly normal elevation of plasma cystine levels in type III patients, thus demonstrating a different intestinal defect. Genetic heterogeneity also agrees with functional and immunohistochemical data. Studies with the microperfusion of isolated tubules suggest that cystine uptake

occurs by a high-affinity system located in the proximal straight tubule (segment S3) and by a low-affinity system located in the proximal convolute tubule (segment S1 and S2) (Volkl et al. 1982; Shafer et al. 1984). These last portions are responsible for ≤90% of the L-cystine reabsorption, while rBAT, located in the S3-segment of the proximal tubule (Furriols et al. 1993), should be responsible for only the remaining 10%.

Candidate genes for cystinuria type II and type III might be new genes coding for cystine transporters expressed in the S1 and S2 segments of the proximal tubule and/or a functionally associated subunit of an oligomeric rBAT transporter. First, the rBAT transporter is localized to the microvilli of epithelial cells in the proximal straight tubule (i.e., S3 segment of the nephron) where 10%–20% of the renal cystine reabsorption occurs (Furriols et al 1993; Pickel et al. 1993; Silbernagl 1988). In contrast, the proximal convoluted tubule (i.e., S1 and S2 segments of the nephron) is responsible for the bulk of renal cystine reabsorption (80%–90%) (Silbernagl 1988). To our knowledge, these cystine transporters have not been cloned or purified. Second, the rBAT protein and the cell-surface antigen $F2hc are the identified components of a family of proteins involved in dibasic and neutral amino transport. It is interesting that for the 4F2hc antigen and for the rBAT protein association by disulfide bridges to unidentified subunits of 35–40 kDa has been demonstrated or suggested, respectively (reviewed in Palacin 1994). The structure and role of the rBAT and 4F2hc putative subunits remain to be established.

In conclusion, the present paper gives helpful information on how to screen easily a large portion of the cystinuria gene directly on genomic DNA; describes new mutations causing disease and several intragenic polymorphisms helpful for linkage analysis; confirms the M467T mutation as the most common disease mutation in our population; and strongly suggests that only type I cystinuria is due to mutations in rBAT. Finally, for the first time, we provide some evidence for a possible genetic complementation model able to explain the cystinuria type I/type III phenotype.

Acknowledgments

The first two authors contributed equally to the results described here. We thank the families who collaborated in this work, and At. Totaro for his technical assistance. This work was supported in part by Ministero Italiano della Sanità (to P.G.), by Telethon 94 grant E083 (to L.Z.) from Italy, by Direcciòn General de Investigaciòn Cientifica y Técnica Research Grant PB90/0435 and PB93/0738 (to M.P.), by Fundaciò August Pi i Sunyer (grant to V.N. and a fellowship to J.P.), by the Institut Català de la Salut (to V.N. and X.E.), and by Sociedad Espanola de Dialisis y Transplante (to F.R.) from Spain. M.J.C. is recipient of a predoctoral fellowship from the Comissiò Interdepartamental de Recerca i Innovaciò Tecnològica from Catalonia.

References

Bertran J, Werner A, Chillaron J, Nunes V, Biber J, Testar X, Zorzano A, et al (1993) Expression cloning of a human renal cDNA that induces high affinity transport of L-cystine shared with dibasic amino acids in Xenopus oocytes. J Biol Chem 268:14842–14849

Bisceglia L, Grifa A, Zelante L, Gasparini P (1994) Development of RNA-SSCP protocols for the identification and screening of CFTR mutations: identification of two new mutations. Hum Mutat 4:136–140

Calonge MJ, Gasparini P, Chillaron J, Chillon M, Gallucci M, Rousaud F, Zelante L, et al (1994) Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. Nat Genet 6:420–426, 1994

Calonge MJ, Volpini V, Bisceglia L, Rousaud F, de Sanctis L, Beccia E, Zelante L, et al. Genetic heterogeneity in cystinuria: the rBAT gene is linked to type I but not to type III cystinuria. Proc Natl Acad Sci USA (in press)

Furriols M, Chillaron J, Mora C, Castellò A, Bertran J, Camps M, Testar X, et al (1993) rBAT, related to L-cystine transport is localized to the microvilli of proximal straight tubules and its expression is regulated in kidney by development. J Biol Chem 268:27060–27068

Gasparini P, Bonizzato A, Dognini M, Pignatti PF (1992) Restriction site generating-polymerase chain reaction (RG-PCR) for the probeless detection of hidden genetic variation: application to the study of some common cystic fibrosis mutations. Mol Cell Probes 6:1–7

Goodyer P, Clow C, Reade T, Girardin C (1993) Prospective analysis and classification of patients with cystinuria identified in a newborn screening program. J Pediatr 122:568–572

Lee W-S, Wells R, Sabbag R, Mohandas T, Hediger M (1993) Cloning and chromosomal localization of a human kidney cDNA involved in cystine, dibasic, and neutral amino acid transport. J Clin Invest 91:1959–1963

McKusick, V (1992) Cystinuria. In: Mendelian inheritance in man: catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes, 10th ed. John Hopkins University Press, Baltimore and London, p 1322

Mosckovitz R, Udenfriend S, Felix A, Helmer E, Tate S (1994) Membrane topology of rBAT kidney neutral and basic amino acid transporter. FASEB J 8:1069–1074

Palacin M (1994) A new family of proteins (rBAT and 4F2hc) involved in cationic and zwitterionic amino acid transport: a tale of two proteins in search of a transport function. J Exp Biol 196:123–137

Pickel V, Nirenberg M, Chan J, Mosckovitz R, Udenfriend S, Tate S (1993) Ultrastructural localization of a neutral and basic amino acid transport in rat kidney and intestine. Proc Natl Acad Sci USA 90:7779–7783

Pras E, Arber N, Aksentijevich I, Katz G, Shapiro J, Prosen L, Gruberg L, et al (1994*a*) Localization of a gene causing cystinuria to chromosome 2p. Nat Genet 6:415–419

Pras E, Golomb E, Raben N, Arber N, Aksentijevich I, Shapiro J, Harel D, et al (1994*b*) Mutations in the *SLC3A1* gene and the molecular basis of cystinuria. Am J Hum Genet Suppl 55:A236

Rosenberg L, Downing S, Durant J, Segal S (1966*a*) Cystinuria biochemical evidence of three genetically distinct diseases. J Clin Invest 45:365–371

Rosenberg L, Durant J, Albrecht I (1966*b*) Genetic heterogeneity in cystinuria: evidence for allelism. Trans Assoc Am Physicians 79:284–296

Rosenberg L, Durant J, Holland M (1965) Intestinal absorption and renal extraction of cystine and cysteine in cystinuria. N Engl J Med 273:1239–1345

Sally K (1978) Cystinuria genotypes predicted from excretion patterns. Am J Med Genet 2:175–190

Sarkar G, Yoon H-S, Sommer S (1992) Screening for mutations by RNA single strand conformation polymorphism (rSSCP): comparison with DNA-SSCP. Nucleic Acids Res 20:871–878

Segal S, Their S (1989) Cystinuria. In: Scriver CH, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease. McGraw Hill, New York, pp 2479–2496

Shafer J, Watkins M (1984) Transport of L-cystine in isolated perfused proximal straight tubules. Pflugers Arch 401:143–151

Silbernagl S (1988) The renal handling of amino acids and oligopeptides. Physiol Rev 68:911–1007

Staden R (1984) Description of the method used in SIGNAL. Nucleic Acids Res 12:505–519

Tate S, Yan N, Udenfriend S (1992) Expression cloning of a NA+–independent neutral amino acid transporter from rat kidney. Proc Natl Acad Sci USA 89:1–5

Volkl H, Silbernagl S (1982) Mutual inhibition of L-cystine/L-cysteine and other neutral amino acids during tubular reabsorption. Pflugers Arch 395:190–195