Urol Res (2003) 31: 417–425
DOI 10.1007/s00240-003-0366-6

ORIGINAL PAPER

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Urinary excretion of total cystine and the dibasic amino acids arginine, lysine and ornithine in relation to genetic findings in patients with cystinuria treated with sulfhydryl compounds

Received: 3 March 2003 / Accepted: 6 August 2003 / Published online: 25 October 2003

Abstract Advances in molecular genetics have brought a deeper understanding of cystinuria. This autosomal recessive disease, which is caused by a defective tubular reabsorption of cystine and the three dibasic amino acids arginine, lysine and ornithine, results in a lifelong risk of renal stone formation because of the low solubility of cystine in urine. Mutations detected within the two genes known to be associated with cystinuria, *SLC3A1* (related to type I) and *SLC7A9* (related to non-type I), cannot, however, in all cases explain the disease. Inasmuch as a high urinary concentration of cystine is the basis of stone formation in these patients, our aim was to measure urinary total cystine, arginine, lysine and ornithine, in patients currently lacking a full genetic explanation for their disease. Thirty-three patients with cystinuria who were on long-term treatment with tiopronin or D-penicillamine were divided into two groups. Group 1 comprised eight patients who carried mutation in one of the *SLC3A1* alleles and two patients who completely lacked mutations both in the *SLC3A1* and the *SLC7A9* genes, that is genetic findings discordant with the increased urinary excretion of cystine and the dibasic amino acids in these patients. Group 2 comprised 23 patients homozygous for mutations within *SLC3A1*, that is genetic findings in accordance with the excretion pattern of classic type I cystinuria. When the two groups were compared, Group 1 had a significantly higher total urinary excretion of cystine ($p < 0.01$) as well as of arginine, lysine and ornithine ($p < 0.05$) than Group 2. Also, when the two patients without mutations were excluded from the calculations, there still was a significant difference in the urinary excretion of total cystine ($p < 0.05$). This suggests that the two patients without any detected mutations in the two known cystine transport genes also contributed to the difference. These unexpected findings indicate that an additional gene or genes participate in the urinary cystine reabsorption in the cystinuric patients who currently are without a full genetic explanation for their disease.

Keywords Cystinuria · Urinary cystine · Amino acid transport · *SLC3A1* · *SLC7A9* · Inherited disease

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Introduction

Cystinuria is a genetic defect of the reabsorption of cystine and the dibasic amino acids arginine, lysine and ornithine in the renal proximal tubules. Because of the low solubility of cystine in urine, a lifelong risk of stone formation ensues.

The prevalence of cystinuria varies from 1/15,000 in the United States to 1/2,500 in Libyan Jews [32, 53], and in a review from St. Bartholomew's Hospital in London it was reported that about 25% of symptomatic patients presented with their first stone in the 1st decade of life and that 30–40% had the first stone in their teens [49]. The treatment of patients with cystinuria aims at reducing the urinary concentration of cystine and increasing the solubility of free cystine. This goal can be achieved by a high fluid intake and by alkalinization of

urine, as shown by Dent and Senior [14]. Such a regimen is, however, far from sufficient in all patients [13]. The introduction of the sulfhydryl compounds D-penicillamine and tiopronin (2-mercaptopropionylglycine) made it possible to reduce the urinary concentration of free cystine by forming soluble disulfide complexes between cystine and tiopronin or between cystine and D-penicillamine [9, 10, 11, 12, 27, 33, 34, 38].

Three subtypes of cystinuria have so far been identified: types I, II and III [46]. Type I heterozygous patients show a normal aminoaciduria, whereas patients with type I homozygous disease have highly elevated levels of cystine and dibasic amino acids in urine.

Type II heterozygous patients show highly and Type III moderately increased excretions of cystine and the dibasic amino acids. Type III differs from type II in that cystinuric patients show elevated plasma cystine concentrations following an oral cystine load.

Type I cystinuria is inherited in an autosomal recessive manner, whereas types II and III show an incomplete recessive inheritance.

In 1992 several laboratories independently reported a 2.3-kb renal cDNA that induced a high affinity, sodium independent uptake of cystine and dibasic amino acids, rBAT (related to $b^{0,+}$ amino acid transport) [2, 51, 54]. The corresponding gene *SLC3A1* was mapped to chromosome *2p21* [31, 55] and a cystinuria locus was localized to the same chromosomal region [42]. The genetic locus of type II and type III was later mapped to chromosome *19q3* [3, 52]. In 1999, the non-type I cystinuria gene *SLC7A9* was identified [16].

A large number of mutations within the *SLC3A1* gene has been reported [1, 3, 5, 7, 15, 20, 21, 24, 25, 36, 43, 47] and more than 30 mutations have been identified within the *SLC7A9* gene [19, 23].

In a group of 53 patients with cystinuria, we previously identified 13 patients with clinically active stone formation who had heterozygous *SLC3A1* mutations (nine patients) or completely lacked mutations in both *SLC3A1* and in *SLC7A9* (four patients), a finding that suggests abnormalities in other genes or aberrant regulation of *SLC3A1* and *SLC7A9* genes [23, 24].

The aim of the present study, which was carried out in 33 patients on long-term treatment with tiopronin or D-penicillamine, was to investigate whether patients who either completely lacked mutations in *SLC3A1* and in *SLC7A9* ($n=2$) or who had a mutation only within one of the *SLC3A1* alleles ($n=8$) had a different excretion of cystine, arginine, lysine and ornithine than patients with homozygosity within the *SLC3A1* gene ($n=23$).

Patients and Methods

Patients

Table 1 summarizes clinical and genetic data of the 33 patients studied. There were 12 women and 21 men from 29 unrelated families. Patients nos. 14 and 15 were sisters, no. 20 was the son of patient 21 and nos. 26–28 were brothers. All patients had the diagnosis of cystinuria based on the demonstration of a high urinary excretion of cystine and dibasic amino acids.

Analysis was carried out with ion exchange chromatography [26, 34]. The patients were not classified according to the criteria of type I or non-type I subtypes, because urinary excretion of obligate carriers were not available for all patients.

All patients were seen by one of the authors (TD), but for practical reasons 13 of the patients had been referred to their respective home clinic. Thus four clinical centres were involved in the investigation.

Basic treatment consisted of hydration (aiming at a fluid intake of more than 2 litres during the day and at least one litre during the night) and alkalinization of the urine (aiming at a urinary pH of at least 7). All patients were on consistent treatment with tiopronin or D-penicillamine.

Twenty-seven of the patients were treated with 2-mercaptopropionylglycine (tiopronin, Thiola, Santen) and six were treated with D-penicillamine (Cuprimine , Merck, Sharp and Dohme). In most patients these compounds were given twice daily. The prescription of Thiola was licensed for each individual patient by the Swedish National Board of Health and Welfare.

The usual interval between outpatient visits was 6 months for 27 of the patients from the two main centres. For six patients, the interval was 12 months. In the majority of the patients, urine was routinely collected as day and night portions for analysis of cystine, as previously described [18]. The total 24-h excretion was calculated for the purpose of this study. In two of the centres (six patients) 24-h collections were used at the time of the study.

The mean age of the patients was 52 years (range, 17–85 years). The mean age at diagnosis was 21 years (range, 1–44 years) and the mean duration of the disease since diagnosis 29 years (range, 2–60 years). In two elderly patients with long-standing clinical disease (nos. 19 and 33), information on the age at diagnosis and accordingly the duration of the disease was less precise. All patients except one with a recent diagnosis (no. 14) were stone formers and 28 had been subjected to endoscopic or open stone surgery and 22 to ESWL (extracorporeal shock wave lithotripsy). Invasive procedures were mainly used before the availability of ESWL, but in 17 cases both treatment modalities had been applied.

In two patients (nos. 5 and 27), unilateral nephrectomy had been carried out because of their stone disease, and one patient (no. 30) suffered from unilateral renal aplasia.

Only three patients had a serum creatinine above normal (115 μmol/l in men and 100 μmol/l in women). In patients nos. 5 and 26, serum creatinine was 140 and 138 μmol/l, respectively. The 24-h creatinine clearance was 59 and 48 ml/min/1.73m^2 body surface area, respectively. Patient no. 33 had a serum creatinine of 135 μmol/l but no assessment of the clearance was available at the time of the study.

The patients participated in the study following informed consent and the study was approved by the Linköping University Hospital Ethical Committee.

Grouping of patients

All SLC3A1 and SLC7A9 exons including exon/intron borders were PCR amplified and all samples were analysed by SSCP and DNA sequencing in a previous study [23, 24]. Based on the genetic findings, two main groups were identified.

Group 1 comprised eight patients in whom mutations were detected within *one* of the alleles of the *SLC3A1* gene and two patients without mutations detected in either the *SLC3A1* or the *SLC7A9* genes. The ten cystinuric patients in this group thus showed *genetic* findings discordant with the increased urinary excretion of cystine and dibasic amino acids.

Group 2 comprised 23 patients, all of whom had homozygous mutations within the *SLC3A1* gene and no detected mutations within the *SLC7A9* gene.

In a separate comparison, Group 1 was divided in two subgroups: Group 1a comprised two patients without any evident mutations and Group 1b comprised eight patients with mutation in

Table 1 Clinical and genetic data of 33 patients with cystinuria treated with sulfhydryl compounds

	Patient	Sex	Age	Age at diagnosis	History of stone surgery	History of ESWL	SH substance	Dose (mg)	Mutation(s) within *SLC3A1*	
Group 1	1	F	57	28	No	Yes	Tiopronin	3,000	None detected	Group 1a
	2	M	58	28	Yes	Yes	Tiopronin	1,500	None detected	
	3	F	66	34	Yes	No	Tiopronin	2,000	M467T/wt	Group 1b
	4	M	36	20	Yes	Yes	Tiopronin	3,000	M467T/wt	
	5	M	80	27	Yes	No	Tiopronin	1,500	M467T/wt	
	6	F	45	18	Yes	No	Tiopronin	2,750	N253 K/wt	
	7	M	24	11	Yes	Yes	Tiopronin	4,500	R227 W/wt	
	8	M	22	20	Yes	Yes	Tiopronin	2,000	S547L/wt	
	9	M	27	22	No	Yes	Tiopronin	750	S547L/wt	
	10	M	47	18	No	No	D-penicillamine	2,500	1132+2 T-C/wt	
Mean (SD)			46 (18)	23 (6)			Tiopronin dose: 2,333 mg (1,047 mg)			
Group 2	11	M	77	32	Yes	Yes	Tiopronin	2,250	1999–2000 del TT/ 1999–2000 del TT	
	12	M	57	3	Yes	No	Tiopronin	500	M467T/A584T	
	13	M	47	34	No	Yes	Tiopronin	1,750	M467T/F599S	
	14[a]	F	20	18	No[b]	No[b]	Tiopronin	500	M467T/M467T	
	15[d]	F	22	19	Yes	Yes	Tiopronin	2,000	M467T/M467T	
	16	F	39	18	Yes	Yes	Tiopronin	2,500	M467T/M467T	
	17	F	44	18	Yes	No	Tiopronin	3,000	M467T/M467T	
	18	F	67	19	Yes	No	Tiopronin	1,000	M467T/M467T	
	19[c]	F	85	Uncertain	No	Yes	Tiopronin	500	M467T/M467T	
	20[d]	M	17	1	Yes	No	Tiopronin	1,750	M467T/M467T	
	21[e]	M	51	25	Yes	No	Tiopronin	3,250	M467T/M467T	
	22	M	30	19	No	Yes	Tiopronin	1,500	M467T/Y259X	
	23	F	40	13	Yes	No	Tiopronin	3,000	Y151C/1500+1 T-G	
	24	M	79	19	Yes	Yes	Tiopronin	1,750	Y151C/M467T	
	25	M	39	6	Yes	No	Tiopronin	2,500	Y151C/Y151C	
	26[f]	M	60	18	No	No	Tiopronin	2,250	Y151C/Y151C	
	27[f]	M	70	13	Yes	Yes	Tiopronin	2,500	Y151C/Y151C	
	28[f]	M	72	23	Yes	Yes	Tiopronin	1,500	Y151C/Y151C	
	29	F	55	23	Yes	Yes	D-penicillamine	1,250	1136+2 T-C/M467T	
	30	M	73	25	Yes	Yes	D-penicillamine	1,000	M467T/M467T	
	31	M	79	44	Yes	Yes	D-penicillamine	750	M467T/Q510R	
	32	M	49	29	Yes	No	D-penicillamine	1,000	Y151C/M467T	
	33[c]	F	80	Uncertain	Yes	Yes	D-penicillamine	500	Y151C/Y151C	
Mean (SD)			54 (20)	20 (10)[b]			Tiopronin dose: 1889 mg (838 mg) / D-penicillamine dose: 900 mg (255 mg)			

SD standard deviation.
[a] Sisters.
[b] No history of stone formation at the time of the study.
[c] Patients no. 19 and 33 excluded from calculation.
[d] Son of patient no. 21.
[e] Father of patient no. 20.
[f] Brothers.

only one of the *SLC3A1* alleles. This step was taken in order to obtain as genetically homogeneous groups as possible. Group 1a, however, consisted of only two patients and was not used for any statistical considerations.

Analytical methods

Analyses of cystine and dibasic amino acids

Free urinary cystine was measured with ion-exchange chromatography at the Department of Clinical Chemistry, University Hospital, Malmö, Sweden. The urine samples were frozen without preservative within 24 h after delivery and transported to the laboratory in a frozen state. Prior to chromatography, an internal standard of aminoethylcysteine was added to each sample and urinary proteins were precipitated with sulfosalicylic acid. Separation and detection of analysed constituents were carried out with an amino acid analyser (Biochrom 20, Pharmacia Amersham Biotech, Uppsala, Sweden).

The ninhydrin complexes of amino acids were detected spectrophotometrically at 570 nm, and their concentrations calculated by an EZChrom intergration software (Scientific Software USA). The mixed disulfides cysteine-tiopronin and cysteine-penicillamine were also separated in the same chromatogram.

To compensate for intraindividual variations, we used the mean of four 24-h excretion values of total cystine, as obtained from analysis of urine during a period of 2–4 years. In the case of the dibasic amino acids, results were available from only one 24-h urine sample.

Total urinary cystine

The total urinary cystine was calculated as the sum of free cystine and the amount of cystine corresponding to the cystine in the mixed tiopronin-cysteine and D-penicillamine-cysteine compounds. Thereby one mole of the mixed disulfide was assumed to correspond to half a mole of cystine. The formula of total urinary cystine thus becomes [34, 35]:

$$\text{Total cystine} = \text{Free cystine} + \frac{\text{Mixed disulfide}}{2}$$

Genetic analyses

Blood samples were obtained from the patients and genomic DNA was isolated from peripheral blood lymphocytes. SLC3A1 and SLC7A9 exons were PCR amplified and all samples were analysed by SSCP and DNA sequencing as previously described [23, 24].

Statistics

The Mann-Whitney U-test was used for group comparisons. Box plots were used for graphical presentation. A p-value < 0.05 was considered statistically significant.

Results

Table 2 shows individual 24-h urinary excretion data of total cystine, arginine, lysine and ornithine and Table 3 shows the median excretion values as well as 25th and 75th percentiles in Groups 1, 1b and 2. In Figures 1–4 urinary findings in Group 1 and 1b are compared with those in Group 2.

The median total urinary excretion of total cystine was 4,631 μmol (25th percentile, 4,275 μmol and 75th percentile, 5,388 μmol) in Group 1 and 4,069 μmol (25th percentile, 2,754 μmol and 75th percentile, 4,268 μmol) in Group 2 (Fig. 1). This difference was statistically significant ($p < 0.01$).

Table 2 Individual urinary excretion data for total cystine, arginine, lysine and ornithine of 33 cystinuric patients treated with sulfhydryl compounds

Patient	Urinary excretion in micromoles			
	Total cystine[a]	Arginine[b]	Lysine[b]	Ornithine[b]
1	4,275	3,471	9,156	1,684
2	5,388	8,426	18,736	8,426
3	3,348	1,074	6,266	1,071
4	4,874	1,320	7,742	1,470
5	5,585	3,872	13,331	3,955
6	3,833	4,141	9,899	2,448
7	5,437	9,883	17,394	9,883
8	4,492	6,702	18,508	4,149
9	4,354	5,632	12,114	2,698
10	4,770	8,874	16,501	4,406
11	3,394	5,266	10,787	2,394
12	4,142	2,071	8,194	2,445
13	4,611	2,296	9,792	1,806
14	4,616	1,500	7,363	1,320
15	3,587	1,138	8,808	840
16	4,348	947	10,458	1,898
17	4,122	3,318	11,595	3,095
18	2,378	747	5,965	1,540
19	908	54	1,302	190
20	4,069	2,642	11,099	2,421
21	5,109	1,376	9,561	2,107
22	4,258	4,082	17,073	2,827
23	3,813	4,336	8,769	2,207
24	4,216	2,765	7,250	2,703
25	5,012	8,086	9,987	4,031
26	2,541	3,255	6,834	1,696
27	4,191	4,250	9,623	1,876
28	4,272	6,273	10,301	2,829
29	2,382	603	5,336	750
30	3,680	1,934	7,666	2,785
31	2,077	2,413	6,192	1,043
32	3,425	1,994	11,848	2,309
33	1,687	984	2,050	788

[a] Excretion data represents the mean value of four 24-h urine collections.
[b] Excretion data based on a single 24-h urine collection.

Figure 2 shows that the urinary total cystine excretion in Group 1b was significantly higher than in Group 2 ($p < 0.05$).

The urinary excretion of dibasic amino acids is given in Table 2.

As shown in Fig. 3, the urinary excretion of arginine, lysine and ornithine was significantly higher in Group 1 than in Group 2 ($p < 0.05$).

The exclusion of the two patients without mutations in *SLC3A1* and *SLC7A9* did not dramatically alter the statistical conclusion (Fig. 4).

Table 1 shows the administered doses of sulfhydryl substances. It is thereby evident that the patients in Group 1 had a higher mean tiopronin intake (2,333 mg) than those in Group 2 (1,889 mg).

The percentage of patients treated with tiopronin was 90% in Group 1 and 78% in Group 2 and the percentage of patients with increased serum creatinine in

Table 3 Urinary excretion data for total cystine and dibasic amino acids in Group 1, 1b and 2

	Group 1			Group 1b			Group 2		
	($n=10$)			($n=8$)			($n=23$)		
	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile
Total urinary cystine excretion (μmol)	4,631	4,275	5,388	4,631	4,093	5,156	4,069	2,754	4,268
Urinary arginine excretion (μmol)	4,886	3,741	8,426	4,886	2,596	7,888	2,296	1,198	3,839
Urinary lysine excretion (μmol)	12,722	9,156	17,394	12,722	8,821	16,947	8,808	6,938	10,419
Urinary Ornithine Excretion (μmol)	3,327	1,684	4,406	3,327	1,959	4,277	2,107	1,375	2,638

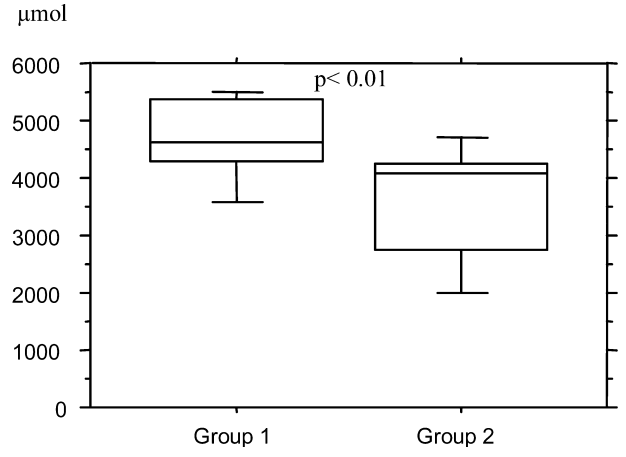

Fig. 1 Box plot of 24-h urinary excretion of total cystine (μmol) in Groups 1 and 2. Group 1, patients with one mutated allele in *SLC3A1* only or no detected mutation in either *SLC3A1* or *SLC7A9* ($n=10$). Group 2, patients with both alleles in SLC3A1 mutated ($n=23$). Box plot, *Horizontal lines* represent 10th, 25th, 50th, 75th and 90th percentile

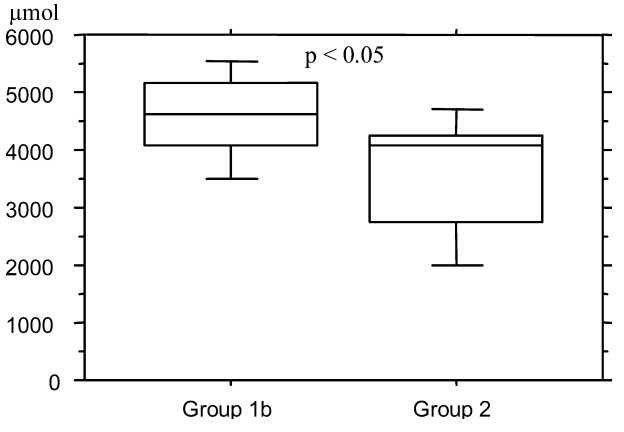

Fig. 2 Box plot of urinary total cystine excretion (μmol) in Groups 1b and 2. Group 1b, patients with one mutated allele in *SLC3A1* only ($n=8$). Group 2, Patients with both alleles in *SLC3A1* mutated ($n=23$)

each group was 10% and 9%, respectively. Only one patient in Group 1was without a history of active stone removal (10%). Two similar patients (9%) were identified in Group 2. In Group 1, all patients were stone formers but in Group 2 one patient (no. 14), in whom the diagnosis had recently been established, had not yet formed urinary stones.

There was no statistical difference between Groups 1 and 2 in terms of urinary volumes, neither in the samples used for calculation of the total urinary cystine excretion, nor in the samples used for calculation of the excretion of the three dibasic amino acids (data not shown).

The most frequently displayed mutation in *SLC3A1* was M467T, occurring in 30% of the patients in Group 1 (Table 1). Thirty nine percent of the patients in Group 2 showed M467T in both alleles of the *SLC3A1* gene and 30% of the patients in this group had M467T in one allele and another mutation in the other allele.

Inasmuch as the two patients without mutations were excluded from the statistical analysis, their data are not found in Figs. 2 and 4, but shown in Table 2.

It should be noted that patient no. 19 showed low urinary excretion values. This cystinuric patient, however, had formed cystine stones and also showed the most common mutation (M467T) in both alleles. Furthermore, the four cystine excretion values collected were all at the same level (1,096, 749, 759 and 1028 μmol/24 h, respectively). Because of these findings, the patient was not excluded. Patient no.19 also showed a very low urinary arginine excretion, if this patient's arginine excretion data was excluded from the comparisons, the *p*-value when comparing Groups 1 and 2 was, however, still less than 0.05 and in the comparison between Groups 1b and 2, the *p*-value increased from 0.052 to 0.067.

Discussion

Recent advances in the field of molecular genetics and the delineation of the two known genes *SLC3A1* and *SLC7A9* responsible for cystine transport have greatly improved our understanding of the pathogenesis of cystinuria. The gene products expressed by these two genes seem to have different roles in the transport of cystine and the dibasic amino acids in the apical tubular membrane.

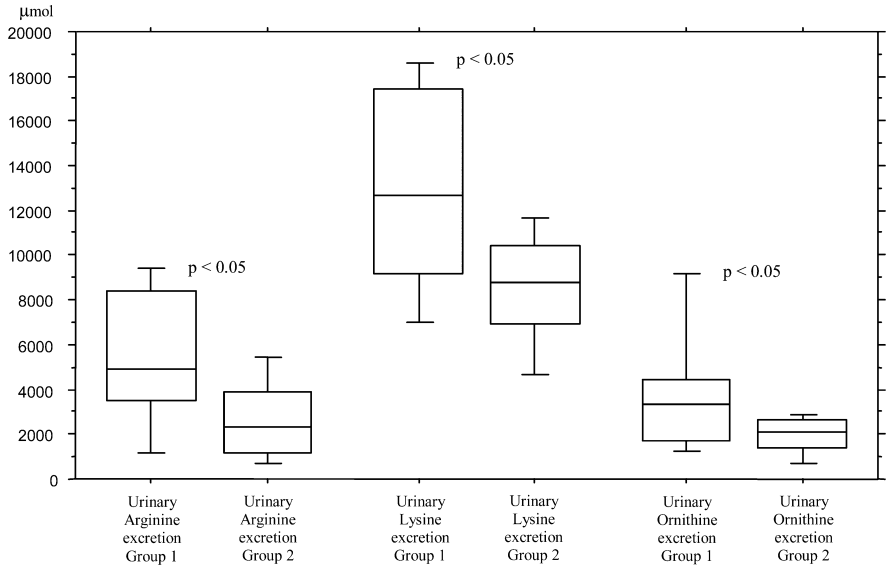

Fig. 3 Box plot of urinary arginine, lysine and ornithine excretion (µmol) in Groups 1 and 2

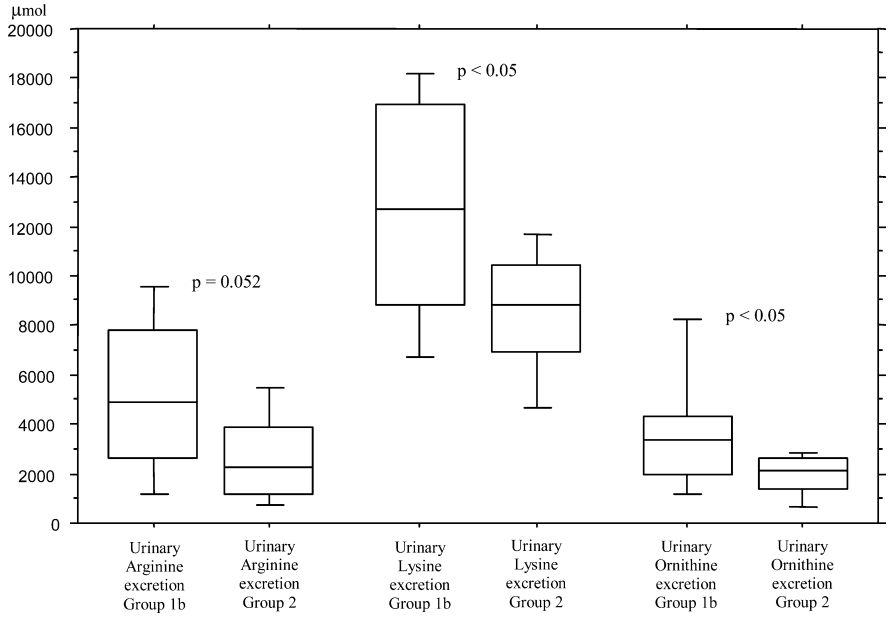

Fig. 4 Box plot of urinary arginine, lysine and ornithine excretion (µmol) in Groups 1b and 2

SLC3A1 has been related to the autosomal recessive type I cystinuria and heterozygotes have a normal amino acid profile in the urine. The gene product of *SLC3A1*, rBAT, possesses only a single transmembrane domain. This is unusual for amino acid transporters, and it has been regarded as a transport activator or modulator and not a transporter in itself [2, 37, 39, 54].

$b^{0,+}AT$ (*SLC7A9*), with 12 membrane spanning regions, is believed to be the actual amino acid transporter in the rBAT– $b^{0,+}AT$ complex and *SLC7A9* has been related to non-type I cystinuria, which has an incomplete autosomal recessive inheritance. Heterozygotic patients have an aminoaciduria between normal and the pronounced increments seen in type I.

The mechanisms for amino acid transport might, however, be more complicated than previously thought. In a recent publication, Leclerc and co-workers showed *SLC7A9* mutations in all three cystinuria subtypes [30] and $b^{0,+}AT$ have been shown to be sufficient to catalyse transmembrane amino acid exchange even in the absence of rBAT in HeLa cells [45].

In previous studies of 53 Swedish cystinuric patients, we found that not all patients with cystinuria had a complete genetic explanation of their disease. These patients showed mutations within only one of the *SLC3A1* alleles ($n=8$) or a complete lack of mutations in either the *SLC3A1* or the *SLC7A9* genes ($n=2$) [23, 24]. This finding indicated the involvement of additional genes in accordance with reports by Botzenhart and co-workers, the International Cystinuria Consortium and Schmidt and co-workers [6, 19, 48]. The possibility that other genes are involved in cystinuria is further

emphasized by the fact that immunostaining has revealed different localization of rBAT and $b^{0,+}AT$ along the nephron.

The rBAT protein was found in the apical membrane of the renal proximal tubule, increasing from the S1 to the S3 segment [8, 28, 37, 40, 41].

On the other hand, $b^{0,+}AT$ expression decreased from the S1 to the S3 segment [8, 40, 44], implying that rBAT and $b^{0,+}AT$ may have other complexing partners in the proximal tubule in order to form fully functioning units of cystine and dibasic amino acid transportation. In a recent study by Fernandez and co-workers, $b^{0,+}AT$ heterodimerized exclusively with rBAT. On the other hand, in the same study, complete immunodepletion of $b^{0,+}AT$ did not coprecipitate more than 20–30% of rBAT, and another rBAT-associated subunit may thus be present in the distal part of the proximal tubule [17].

The results of the present study revealed a higher excretion of urinary total cystine in a group of ten cystinuric patients (Group 1) and showed either a complete lack of mutations in the two known cystine transporter genes ($n=2$) or heterozygous mutations within *SLC3A1* ($n=8$) when compared with patients who had homozygous mutations in *SLC3A1* (Group 2).

This difference was also found when Group 1b (the patients with only one of the *SLC3A1* alleles mutated) was compared with Group 2.

We are well aware of the problems associated with the small number of patients in Group 1. It is of note, however, that when patients in Groups 1b and 2 were compared (following exclusion of the two patients in Group 1a), the statistical power was reduced. It is our opinion that this indirect evidence justifies the comparison of Groups 1a, 1b and Group 2. This is further emphasized by the fact that both patients in Group 1a showed excretions of total cystine, arginine and lysine above the median value recorded for each of these amino acids in Group 2.

The differences between the two groups in terms of urinary excretion of the dibasic amino acids arginine, lysine and ornithine were similar to the difference recorded for urinary excretion of total cystine. This finding indicates a common transport of cystine as well as the three dibasic amino acids in the hypothesized, yet unknown, amino acid transport system(s) involved in cystinuria. From the genetic difference between patients in Groups 1a and 1b, however, a different excretion pattern of the dibasic amino acids might be expected. Unfortunately, there were only two patients in Group1a and a statistical comparison was not feasible. For this reason no reliable conclusion can be drawn in this regard.

All but one patient had formed stones and it was thus not possible to discontinue the long-term prophylactic medication. The routine analysis of the disulfides of cysteine-tiopronin and cysteine-penicillamine as part of the clinical management and surveillance of patients offered a possibility to compare the total urinary cystine excretion in the two groups. The concept of total urinary cystine was introduced by Lotz and co-workers in a study of D-penicillamine-treated cystinuric patients in whom treatment with hydration and urine alkalization was insufficient [35]. These authors found that the use of D-penicillamine was effective in lowering the excretion of free cystine. Unexpectedly, they also recorded a reduced excretion of total urinary cystine.

Our group noted the same decrease in total urinary cystine excretion when patients were treated with tiopronin in doses above 1,500 mg per day [34]. These findings suggest that the presence of a sulfhydryl compound such as tiopronin or D-penicillamine might interfere with cystine metabolism in a more complex way than by simply forming a disulfide complex [34]. Because of these findings, we looked at the mean dose of the sulfhydryl compounds that was administered in the two groups. As shown in Table 1, the mean dose of tiopronin was lowest in Group 2. The same was true for D-penicillamine, but only one patient in Group 1 was treated with D-penicillamine.

It thus seems that these observations should have decreased rather than increased the differences between the two groups, but Group 1 had a statistically higher excretion of total urinary cystine despite these differences in the dosage of sulfhydryl compounds.

The patients in Group 2 tended to be older than the patients in Group 1, but the percentage of patients with an elevated serum creatinine was comparable in the two groups, and no patient had a serum creatinine above 140 μmol/l. The two groups were also comparable with respect to the need of active stone removal, and in both groups the most frequent mutation was M467T.

From the clinical perspective, studies that relate genetic findings to the actual excretion of cystine and the dibasic amino acids have been scarce in the literature because of the recent identification of the *SLC7A9* gene. In a prospective paediatric study, Goodyer and co-workers showed that classic type I cystinuria was phenotypically distinct from other subtypes of cystinuria [22]. At that time, however, only *SLC3A1* was identified.

Langen and co-workers found in phenotype and genotype analyses only two subtypes of cystinuria, type I and non-type I [29]. The International cystine consortium showed variability in the excretion of cystine and the dibasic amino acids between heterozygous patients with the same *SLC7A9* mutations. These data support the possibility that type II and III cystinuria could be generated by the same mutation. Differences were also demonstrated between different *SLC7A9* mutations in the excretion of these amino acids in non-type I cystinuria.

In a recent study, Strologo and co-workers proposed a classification based on homozygous mutations in *SLC3A1* and *SLC7A9* [50]. Type A was identical to classic type I cystinuria and comprised patients with both alleles in *SLC3A1* mutated and heterozygotes with a normal urinary amino acid profile.

Type B consisted of patients with both *SLC7A9* alleles mutated (that is, homozygous non-type I cystinuria patients); heterozygotes mostly showed elevated levels of cystine and the dibasic amino acids in the urine.

Surprisingly, 14% of the patients with only one *SLC7A9* allele mutated showed a normal urinary amino acid profile. This finding indicates that mutations associated with non-type I cystinuria do not always result in the expected clinical outcome. For unknown reasons, females in type B showed a significantly higher excretion of cystine when compared with males. There were, however, no clinical differences between types A and B and no significant difference in the excretion of cystine and dibasic amino acids, although effects of treatment could not be analysed.

The authors also suggested the existence of type AB caused by one mutation in *SLC3A1* and one mutation in *SLC7A9*, although it was pointed out that the existence of this type needs to be confirmed.

In our study patients were grouped differently, identifying significant differences in the urinary excretion of cystine and the dibasic amino acids between the groups studied.

In conclusion, the group comprising ten sulfhydryl treated patients without a complete genetic explanation of their disease (Group 1) had a statistically higher urinary excretion of total cystine when compared with patients homozygously mutated in *SLC3A1*. This was also the case in the genetically more homogeneous subgroup of patients displaying mutations within one of the two *SLC3A1* alleles (Group 1b). Furthermore, the two patients with no detected mutations within *SLC3A1* and *SLC7A9* (Group 1a) seem to have contributed to the difference found between the two main groups (Groups 1 and 2).

These results suggest that currently unknown gene(s) or aberrant regulation of the identified cystinuria genes are involved in the mechanisms responsible for urinary cystine and dibasic amino acid excretion.

Acknowledgements We thank all patients for their participation in this study. This investigation was supported by grants from FORSS (The Health Research Council in the South-East of Sweden), MFR (the Swedish Medical Research Council), Lisa and Johan Grönberg's Research Fund and Skane County Council Research and Development Foundation. We wish to thank the late Mats Ekberg MD, Department of Internal Medicine, Hässleholm Hospital, Birger Lindergård MD, Department of Nephrology, Lund University Hospital, and Anders Christensson MD, Department of Nephrology and Transplantation, Malmö University Hospital, for valuable help in making the patients medical records available for this study. Ms Iréne Eriksson at the Renal Stone Unit, Division of Urology, Linköping University Hospital is acknowledged for managing patients and blood sampling. We also want to thank Mats Fredriksson, Department of Environmental Medicine, Linköping University Hospital for valuable statistical advice.

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