Rice S, Sayer JA. Understanding the molecular genetics of the tubular transport disorder cystinuria. OA Nephrology 2013 Apr 01;1(1):2.

Competing interests: none declared.

Conflict of interests: none declared.

All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript.

All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.
Understanding the molecular genetics of the tubular transport disorder cystinuria

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Abstract

Cystinuria is an inherited renal disorder resulting in urinary wasting of dibasic amino acids and often the formation of cystine stones. Mutations in two genes underlie this condition, namely \textit{SLC3A1} and \textit{SLC7A9}. Point mutations, multi-exon deletions and duplications and genomic rearrangements have been described leading to a loss of function of their encoded proteins. \textit{SLC3A1} encodes rBAT whilst \textit{SLC7A9} encodes the light chain \textit{b0,+AT}. Together these proteins allow proximal tubular absorption of cystine, ornithine, lysine and arginine. Clinical and biochemical definitions of disease types, which can be confusing, can now be replaced with the molecular genetic characterisation of these genes. This will hopefully allow new insights into genotypical and phenotypic variants of this disease.
Introduction

Cystinuria was first reported in the literature as far back as 1810 [1], yet it was another century later when Sir Archibald Garrod made the crucial link between the observations on this chronic stone-forming condition with Mendel's theories of genetic inheritance [2]. Although at the time the pathology was attributed to a metabolic defect [3], the evidence of a familial inheritance pattern was widely accepted.

By 1958, several integral discoveries in determining the cause of this disease had been made. Firstly, in 1947, microbiologist Hier reported that cystinuric patients also had elevated urinary levels of arginine, ornithine, and lysine, whilst only poorly-soluble cystine was a component of the calculi [3, 4]. The second key breakthrough in uncovering the basis of stone formation was made by Charles Dent in 1951, when he reported the presence of hexagonal crystals that had formed through cystine deposition in the renal tubules, not, as previously suspected, in the bladder. In the same report he concluded that the basis of the disease pathology was attributable to a lack of reabsorption of cystine [5]. This finally led to the understanding that the cause of elevated amino acids in the urine was a defective transport system, as opposed to a metabolic pathway. In the 1990s the cloning of system $b^{0,+}$ in *Xenopus laevis* oocytes proved to be the final piece in the puzzle[6, 7][8].

System $b^{0,+}$ is the only transport system known to reabsorb cystine from the filtrate in the proximal tubule [9]. The functional system consists of two transmembrane subunits. The first of these subunits, rBAT, an 85 kDa Type-II Membrane Glycoprotein, is comprised of only one transmembrane domain,
rendering it incapable of stimulating substrate transport when expressed alone [10]. Sharing structural homology to the heavy chain of system y’L (SLC3A2), rBAT has been proposed to act as a “modulator” of transport, trafficking the second subunit, b0,+AT, to the membrane [11]. b0,+AT is the light chain of the system (40-50kDa) and it forms the transport pore via twelve transmembrane helices [12]. The two proteins, covalently linked by a disulphide bond between two cystine residues, mediate high affinity exchange of basic and neutral amino acids [11]. Defects in either of the two subunits can cause an abnormality in transport, decreasing the reabsorption of largely-insoluble cystine from the renal proximal tubule. This leads to precipitation of the cysteine dimer and the subsequent formation of renal calculi.

During the 1990s linkage analysis identified the two genes involved in cystinuria through maximum LOD scoring of microsatellite markers. The “rBAT” gene was located to the short arm of chromosome 2, coinciding with the position of a known solute carrier gene SLC3A1 [13]. Comprised of ten exons, SLC3A1 spans more than 45kb, and is specifically located at region 2p21 (Figure 1A) [14]. In 2000, a second causative gene, SLC7A9 on Chromosome 19q13.11, was identified in cystinuric patients, now known to encode light chain b0,+AT (Figure 1B).

Whilst originally thought to be a completely recessive condition, it has become apparent that some pathogenic mutations have a variable penetrance and can cause a cystinuric phenotype as an autosomal dominant trait [15]. Based on this, cystinuria patients are often classified as Type I or Type II / III (referred to as Type non-1). Patients were defined to be Type I if their parents excreted normal amounts of cystine, the cystinuria phenotype running as a recessive trait. Hence the
heterozygote relatives of the affected probands showed normal urinary amino acid profiles[16].

Types II or III (Type non-1) are defined if parental cysteine excretion was either greatly (II) or moderately (III) increased. Therefore heterozygote relatives also have elevated urinary levels of arginine, cystine, lysine and ornithine, indicating a dominant inheritance pattern[16]. Of the Type non-1 cystinurics, type II cystinuria typically displays a more severe phenotype than Type III [17]. Additionally, “mixed” type cystinuria patients are those with obligate heterozygous parents with and without a phenotype. Given this complex and imprecise nomenclature, there is room for confusion in classifying cystiniuric patients on phenotype alone.

However, following the relatively recent identification of the two genes involved in cystinuria, a second, more robust classification system has been introduced. Patients with mutations in \textit{SLC3A1} are classed as type “A”, and those with mutations in \textit{SLC7A9}, type “B”. Thus, patients can be classified as Type AA, BB, or AB etc. depending on the genotype of their specific mutations[18]. For example two \textit{SLC3A1} mutant alleles would be referred to as AA (Table 1). It is also noteworthy that, not all individuals with genotypically proven cystinuria develop cystine stones, indicating that other factors, perhaps environmental or related to risk-modifying genes, may contribute to the risk for calculus formation in these patients.

Cystinuria may occur in the Hypotonia-Cystinuria Syndrome (Figure 1A). Here, a microdeletion involving part of the \textit{SLC3A1} and \textit{PREPL} genes on 2p21 gives rise to
more complex phenotypes. In addition to cystine stones, affected patients have infantile hypotonia and poor feeding during infancy [19, 20].

Discussion

To date, two large-scale multinational studies of cystinuria, employing both probands and obligate heterozygote relatives, have been undertaken to determine the correlation between phenotype and genotype[15, 17] (Table 2). The initial indication seemed to be that Type-I cystinuria patients had mutations in SLC3A1, and Type non-I patients had mutations in SLC7A9. This implied that Type-I (autosomal recessive) cystinuria, was equivalent to Type A, and Type non-I equivalent to Type B. However, the findings of both of these studies indicated an overlap between clinical and biochemical phenotype and the A and B classification system based upon genotype. Several Type B probands had relatives heterozygous for SLC7A9 mutations with a clinically and biochemically silent phenotype. Additionally, a heterozygous duplication of exons 5-9 of SLC3A1 was identified in four subjects, yet they showed a varying degree of phenotype, indicating Type non-I disease [15].

In 2001, the largest attempt at functional characterisation of cystinuria-causing mutations was carried out [21]. A multi-national cohort was screened for mutations in both genes using single-strand conformation polymorphism (SSCP) or RNA-SSCP before direct sequencing of amplicons displaying abnormal electrophoretic motility. The cohort from this functional study, consisting of 175 probands and their known heterozygous relatives, was divided into Type Non-1 and untyped probands[21]. Of the 114 Type Non-1 chromosomes studied, 90 (79%) were found to have a mutation in SLC7A9. Theoretically, if one considers SLC7A9
mutations to be autosomal dominant, all patients in this cohort were solved. However, if the un-typed probands are considered, this is no longer the case. Here, 13 of the 54 studied (24%) had a mutation in SLC7A9. Similarly, 32% had a mutation in SLC3A1, whilst none of the probands had a digenic genotype, a finding rarely reported in the literature [21] (Table 2). Considering these patients as a whole, and eliminating the “Type” classification, it would seem that some of these mutations are behaving as a dominant trait with an unknown modifying factor, or third gene, influencing the penetrance of phenotype. To further emphasise this, the variability in biochemical phenotype of probands with the same genotype was reported[21].

It has been hypothesised that the apparent variability in penetrance in cystinuria could be due to the presence of mutations outside of the open reading frame (ORF), or in an unidentified second light chain that may associate with rBAT[15]. It has recently been reported that throughout the literature the mutation detection rate in genotyping studies of cystinuria patients is an average of 85% [22].

From the published literature, it is apparent that the most common method for mutation detection in cystinuria has been SSCP, an inexpensive mutation detection method (Table 2). Originally a technique only feasible for detecting mutations in DNA fragments of up to 200bp in length, this simple technique has now been to allow sequence lengths of up to 500bp to be analysed [23]. Despite this, the sensitivity of the technique remains under question with reports of mutation detection rates of around 80% [24]. Other, less frequently-employed, techniques include real time or quantitative polymerase chain reaction (q-PCR), and enzymatic digest of heteroduplexes. Enzymatic digest, unlike SSCP, allows localisation of single base pair mismatches within sequences of up to 1.5kb in length [25]. This technique,
however, also fails to reach a sensitivity rate of 100%, and is unable to detect large-scale gene deletions or duplications.

Multiplex ligation probe analysis (MLPA) has unfortunately rarely been used for the detection of mutations in \textit{SLC3A1} and \textit{SLC7A9}. This technique, unlike the other methods, allows quantification of whole exon duplications or deletions, and copy number variation [26]. Using traditional detection techniques, hemizygous mutations combined with a deletion may appear as homozygous mutations, which can potentially lead to confusion. Although probe synthesis is costly, and would not allow the detection of mutations in the probe annealing sequence, when used in combination with SSCP or enzyme digest, this could potentially allow identification of close to 100% of mutations within coding regions of the \textit{SLC3A1} and \textit{SLC7A9} genes.

Due to the apparent “missing heredity” in cystinuria, the search for other gene involvement has been theorized. Genetic linkage analysis in cystinuria patients maps genetic involvement solely to Chromosomes 2p21 and 19q13.11, the locations of \textit{SLC3A1} and \textit{SLC7A9}, respectively (Figure 1).

This hypothesis of a modifier or third gene ignited the search for other solute carrier genes present at these loci. To date, two putative genes have been reported. Firstly, \textit{SLC7A10} encoding Asc-1, a high affinity D- and L-serine transporter; and then \textit{SLC1A5}, encoding ASCT2, related to classical amino acid transport system ASC[27].

\textit{SLC7A10} was suggested as a potential candidate for several reasons. First, it maps to 19q13.11, and was likely to have evolved through a \textit{SLC7A9} gene duplication. Additionally, \textit{SLC7A10} mRNA has been localised to the kidney; and cystine is a substrate for Asc-1[28, 29]. The first investigation into this putative
cystinuria gene was carried out by Leclerc et al. in 2001 on ten unrelated cystinuria patients. In total, five variants were found in the coding regions and intron-exon boundaries of SLC7A10. Two of these were also found in control populations; the third an intronic mutation was predicted not to affect splicing; the fourth was a synonymous-coding mutation S56S; and the fifth was a missense mutation E112D[29]. A second study by Palacin et al. in 2004 was also conducted to investigate the involvement of this gene. Twenty-two unrelated European patients were included in the cohort on the basis that they were cystinuric with less than two identified mutations in SLC3A1 or SLC7A9. No pathogenic mutations were identified in SLC7A10. The group functionally characterised the three previously-identified mutants through radiolabelled uptake in transfected HeLa cells, and found no decrease in Asc-1 function when compared to the wild type protein [28]. It was also identified that the renal Asc-1 mRNA was absent from the proximal tubule, the only site of cystine reabsorption [28]. The conclusion of these two studies was that there is no involvement of SLC7A10 in cystinuria.

Elsewhere, two “non-type 1” families presenting linkage to 19q13, but lacking any discovered abnormalities in SLC7A9 were investigated to detect mutations in SLC1A5 [27]. Again, no pathogenic mutations were identified in this candidate gene.

Despite the lack of evidence for a third gene involvement in cystinuria, the fact remains that patients without known mutations in SLC7A9 still show linkage to 19q13. It seems likely that undiscovered mutations may lie in promoter regions or within intronic regions of SLC7A9, but there remains the possibility that additional genetic variants may be responsible for a cystinuria phenotype.
The existence of an undiscovered gene is further supported by the strong possibility of a second light chain harnessed by rBAT. This is implied by the specific distributions of the mRNA encoding individual subunits of system \( b^{0,+} \) in the renal proximal tubule. The renal proximal tubule can be divided into distinct sequential segments along its length, based upon cellular composition. A distinct tubular gradient in rBAT expression (S1>S2>S3) can be visualised through blotting analysis in both human and mouse tissue, as can that of \( b^{0,+} \): S3>S2>S1 [30]. Despite the co-expression of rBAT and \( b^{0+} \)AT in the epithelium of the tubule, their exact expression patterns are directly opposing. Within the kidney, 90% of cystine reabsorption occurs in the early proximal tubule, correlating directly with rBAT expression patterns. The additional knowledge that rBAT shows high structural homology to \( SLC3A2 \) (Chromosome 11q12.3), which has six known associated light chains, strengthens the possibility that an additional light chain for rBAT may exist.

A pragmatic approach to the molecular genetic investigation of cystinuria can be taken. Following a clinical suspicion of the disease and confirmation of either cystine calculi or biochemical evidence of cystinuria a molecular genetic diagnosis should be sought. Prior to proceeding, a family pedigree and biochemical screening of at risk relatives will allow some insights into the pattern of disease (autosomal dominant with partial penetrance or autosomal recessive). For families with an autosomal dominant pattern of inheritance screening of the \( SLC7A9 \) gene should be undertaken first, before proceeding to \( SLC3A1 \). Sanger sequencing of all coding regions combined with MLPA to look for exon deletions / rearrangements would be a traditional approach which is likely to detect most mutations. Other alternative methodologies may also be used such as qPCR and microarray typing for detection of
multi-exon and whole gene imbalances. Confirmation of segregation of identified mutations is also important. Where there seems to be an autosomal recessive pattern of inheritance, screening of *SLC3A1* should be undertaken, again combined with MLPA to look for deletions and rearrangements of exons. *SLC7A9* should then be screened to examine for digenic inheritance of mutant alleles. For practical purposes it makes sense to screen both genes simultaneously as important genotype information will be missed if single genes are selected. Segregation of mutations using parental DNA samples should be performed where available \[31\]. This traditional approach may soon be overtaken by whole exome sequencing (WES) and whole genome sequencing, where the different challenges of ensuring good sequence coverage of target genes and appropriate data handling and pipelining are prominent.

Our knowledge of cystinuria has increased dramatically since the disease was first reported to the Royal Society in 1810, particularly so in recent years due to a greater understanding of the human genome and the effect mutations can have on the function of transport proteins. However, for more than a decade there have been reports on cystinuria suggesting reasons for incomplete detection of pathogenic mutations: a variability in penetrance due to unknown modifying factors; mutations outside of the ORF; abnormalities in sequence that cannot be detected by the current methods employed; and the involvement of a third gene, still unidentified. Despite years of hypothesis, evidence to explain this missing heredity still evades our grasp, and regardless of the ever-expanding collection of data on cystinuric patients and their specific mutations.
What remains vital is that the genetic investigation of cystinuric patients is correlated with segregation analysis where available and precise clinical and biochemical phenotypic information. This will ensure that with the increasingly sophisticated methods of genomic analysis being applied to inherited diseases we will be able to make sense of the data and put it into a meaningful context to promote better clinical management of cystinuria patients.

**Funding**

This project is supported by the Wellcome Trust and the Northern Counties Kidney Research Fund.


### Table 1: Nomenclature of cystinuria phenotypes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Type I (autosomal recessive pattern)</th>
<th>Type non-I (autosomal dominant pattern with incomplete penetrance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Type II</td>
<td>Type III</td>
</tr>
<tr>
<td>Genotype</td>
<td>$A$</td>
<td>$B$</td>
</tr>
<tr>
<td>Possible genotypes</td>
<td>$A, AA, B, BB, AB, AAB, ABB$</td>
<td></td>
</tr>
</tbody>
</table>

- Gene (Protein) for Type I: $SLC3A1$ (rBAT, heavy chain)
- Gene (Protein) for Type II: $SLC7A9$ (b$^{0,+}$AT, light chain)
Table 2: A summary of identified mutations in cystinuria studies.

<table>
<thead>
<tr>
<th>Country</th>
<th>Alleles</th>
<th>Mutations</th>
<th>Solved (%)</th>
<th>A (%)</th>
<th>B (%)</th>
<th>AB (%)</th>
<th>AAB (%)</th>
<th>BBA (%)</th>
<th>Methods</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>328</td>
<td>282</td>
<td>86</td>
<td>44</td>
<td>54</td>
<td>0</td>
<td>~0.5</td>
<td>~0.5</td>
<td>SSCP, DHPLC, qPCR, MLPA</td>
<td>15</td>
</tr>
<tr>
<td>Italy, Spain</td>
<td>376</td>
<td>302</td>
<td>80</td>
<td>45</td>
<td>53</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>SSCP, Sanger sequencing</td>
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</tr>
<tr>
<td>Spain</td>
<td>240</td>
<td>129</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SSCP, RNA-SSCP</td>
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<tr>
<td>USA</td>
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<td>14</td>
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<td></td>
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<td></td>
<td>SSCP</td>
<td>21</td>
</tr>
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<td>14</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RNA-SSCP, restriction assay</td>
<td>22</td>
</tr>
<tr>
<td>Japan</td>
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<td>8</td>
<td>14</td>
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<td>RNA-SSCP</td>
<td>23</td>
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<td>34</td>
<td>52</td>
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</tr>
<tr>
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<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RNA-SSCP, restriction enzyme, MLPA*</td>
<td>25</td>
</tr>
</tbody>
</table>

Solved: percentage of patients having either a homozygous or compound heterozygous mutation in SLC3A1, a single mutation in SLC7A9 or digenic mutations in both SLC3A1 and SLC7A9
Column A: mutation(s) in *SLC3A1*; Column B: mutation(s) in *SLC7A9*; Column AB: mutation(s) in both *SLC3A1* and *SLC7A9*; Columns AAB & BBA: three mutations identified in the respective genes.

SSCP: single-strand conformation polymorphism; DHPLC: denaturing high performance liquid chromatography; qPCR: quantitative polymerase chain reaction; MLPA: multiplex ligation probe analysis.

*MLPA carried out in just one family to investigate an apparent loss of heterozygosity.*

Yellow rows indicate studies which only investigated *SLC7A9*; grey boxes indicate studies which only investigated *SLC3A1*: in these studies the overall mutation detection percentage could not be calculated and boxes are coloured black.
Figure legends

Figure 1: Chromosomal regions and candidate genes associated with cystinuria

A. Schematic of Chromosome 2. The SLC3A1 gene, encoding rBAT is found on Chromosome 2p21, next to the PREPL gene. A contiguous gene deletion syndrome which involves SLC3A1 and PREPL give rise to the Hypotonia-Cystinuria Syndrome (HCS).

In atypical HCS an additional gene CAMKMT (alias C2orf34) is deleted. In the 2p21 deletion syndrome a further neighbouring gene PPM1B is also involved.

B. Schematic of Chromosome 19. The SLC7A9 gene is found on Chromosome 19q13.11 and encodes the protein b0+AT. Within the same region (although not neighbouring) lies SLC7A10, encoding the neutral amino acid transporter (y+ system) member 10 (alias asc-1). Mutations in SLC7A10 have not been associated with cystinuria.

SLC1A5 on Chromosome 9q13.32 was screened for mutations in cystinuria patients demonstrating linkage to Chromosome 19q13, however no mutations were found.