

# A Combined Approach to the Molecular Analysis of Cystinuria: from Urinalysis to Sequencing via Genotyping

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## Abstract

**Background:** Cystinuria is an autosomal recessive disease that is manifested by kidney stones and is caused by mutations in two genes: *SLC3A1* on chromosome 2p and *SLC7A9* on chromosome 19q. Urinary cystine levels in obligate carriers are often, but not always, helpful in identifying the causative gene.

**Objectives:** To characterize the clinical features and analyze the genetic basis of cystinuria in an inbred Moslem Arab Israeli family.

**Methods:** Family members were evaluated for urinary cystine and amino acid levels. DNA was initially analyzed with polymorphic markers close to the two genes and *SLC7A9* was fully sequenced.

**Results:** Full segregation was found with the marker close to *SLC7A9*. Sequencing of this gene revealed a missense mutation, P482L, in the homozygous state in all three affected sibs.

**Conclusions:** A combination of urinary cystine levels in obligate carriers, segregation analysis with polymorphic markers, and sequencing can save time and resources in the search for cystinuria mutations.

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Cystinuria is an autosomal recessive disease manifested by the formation of kidney stones [1]. Cystine stones were recognized as early as 1810 and cystinuria was one of the first four diseases described by Sir Archibald E. Garrod in 1908 as “inborn errors of metabolism” [2]. The disease is caused by a defect in the renal reabsorption of cystine and the dibasic amino acids – lysine, arginine and ornithine [3]. Cystine, which has a low solubility, can precipitate and form renal stones, causing pain, obstruction and sometimes renal failure [4]. Despite the availability of therapy with D-penicillamine, alpha-mercaptopyronylglycine and urinary alkalinization agents, cystinuria is still a major cause of morbidity [5]. Cystinuria is responsible for 6–8% of kidney stones in children and is the leading single gene cause of heritable urolithiasis in adults [6]. In 1994 the first cystinuria gene located on chromosome 2p was cloned [7]. The gene, named *SLC3A1*, encodes a 685 amino acid protein called rBAT. A second gene, *SLC7A9*, was cloned in 1998 and encodes a 487 amino acid protein termed b<sup>0</sup>+AT [8]. Together, the two proteins form the active transporter located at the brush border of the proximal tubule. Over 50 mutations have been described in *SLC3A1* [9] and over 30 in *SLC7A9* [10]. Cystinuria is prevalent worldwide with an estimated frequency varying between 1:15,000 in the

United States and 1:50,000 in Japan [11,12]. In Israel, cystinuria is found among various ethnic groups. Mutations in *SLC3A1* were found in Ashkenazi\*, Persian and Yemenite Jews, Druze, and Moslem Arabs [13]. However, the disease is by far more common among Libyan Jews, with an estimated prevalence of 1:2500 and a carrier rate of 1:25 [14]. A founder mutation in *SLC7A9*, V170M is responsible for these high rates [8,15]. Another founder mutation was found in Ashkenazi Jews although the frequency of cystinuria in this ethnic group is much lower [13].

Previously, classical cystinuria was classified into three types based on urinary excretion of cystine and the dibasic amino acids in obligate heterozygotes. Type I heterozygotes show a normal urinary amino acid pattern whereas type II and III heterozygotes exhibit high and moderate levels of excretion, respectively [16]. It was originally supposed that mutations in the *SLC3A1* gene are responsible for type I disease while mutations in *SLC7A9* cause non-type I disease (type II and III). However, recent studies have not supported this traditional classification. Although *SLC3A1* is associated almost invariably with type I disease, *SLC7A9* mutations have been found in all three subtypes [10,17,18]. Therefore, a new classification based on molecular analysis and not on the urinary amino acid profile has been proposed. Type A disease is due to mutations in the *SLC3A1* gene, type B disease results from mutations in *SLC7A9*, and type AB disease from mutations in both genes [18].

In this report we describe an *SLC7A9* mutation in an Arab Moslem family suffering from cystinuria, associated with normal or very mildly elevated urine cystine levels in the obligate carriers.

## Methods

### Family and DNA

The family was evaluated at the nephrology clinic at the Sheba Medical Center. The parents, of Arab Moslem origin, are first cousins. The proband (no. 4 in Figure 1 and Table 1), 18 years old, was diagnosed with cystinuria based on symptomatic nephrolithiasis and elevated levels of urinary cystine and the dibasic amino acids. Ultrasound studies performed on his brothers revealed kidney stones in two of them. Urinary cystine levels

\* Of Eastern European origin

**Table 1.** Urinary cystine levels in the family

No.	Family member	Age (yrs)	Urinary cystine levels*
1	Father	44	8
2	Mother	39	22
3	Child	20	112
4	Child	18	169
5	Child	16	5
6	Child	12	107
7	Child	5	4

\* Normal range 3–17  $\mu$ mole/mmol creatinine

in the two brothers were also consistent with cystinuria [Table 1]. Except for the proband, none of the other family members had symptoms suggestive of kidney stones. Ten milliliters of EDTA-treated blood were obtained from each family member and DNA was extracted using a commercial kit.

**Genotyping, sequencing and restriction analysis**

Electronic screening of clone AC019129 containing *SLC3A1* revealed a polymorphic CA repeat that was amplified with the primers 5'-AAATGGCATGGTGTCCAAT-3' and 5'-TGGCCCAGAACTCTTTTGTAT-3'. Screening of clone AC008805 containing *SLC7A9* disclosed a CA repeat amplified with the primers 5'-TATGCTTCCAAGTCAGCAAAAC-3' and 5'-TCTAACTCCATCCAGTGATGC-3'.

Polymerase chain reactions were carried out in a 25  $\mu$ l volume containing 50 ng of genomic DNA, 10 ng of each primer, 0.2 mM dNTP, in 1.5 mM MgCl<sub>2</sub> buffer, with 1.2 U of Taq polymerase. After an initial denaturation of 5 minutes at 94°C, 30 cycles were performed (94°C for 30 seconds, 56°C for 30 sec, and 72°C for 30 sec), followed by a final extension time of 10 min at 72°C.

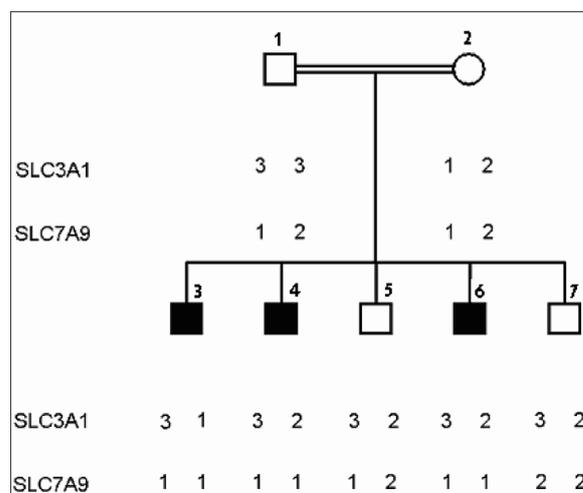
Sequencing of *SLC7A9* was performed as previously described [8]. Genomic DNA was screened for the mutation by testing for the presence of an Hpa II restriction site in a PCR-amplified fragment, using the primers 5'-GTGAACTTTCGAATTCAGTCTAA-3' and 5'-CTAGAACCCCACTCAGAGAACTA-3'.

PCR reactions were carried out in a 25  $\mu$ l volume containing 50 ng of genomic DNA, 10 ng of each primer, 0.2 mM dNTP, in 1.5 mM MgCl<sub>2</sub> buffer, with 1.2 U of Taq polymerase. After an initial denaturation of 5 min at 94°C, 30 cycles were performed (94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec), followed by a final extension time of 10 min at 72°C.

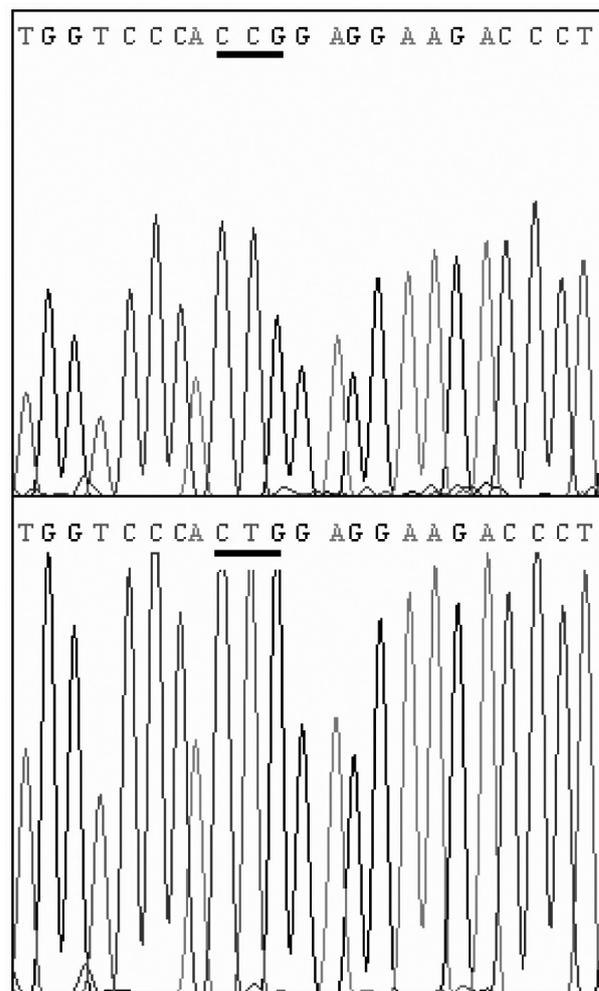
**Results**

Genotyping with the two polymorphic markers located very close to *SLC3A1* and *SLC7A9* excluded linkage in the former and disclosed full segregation in the latter [Figure 1]. Sequencing of *SLC7A9* in one patient revealed a C to T change at position 1085 in exon 10 [Figure 2], resulting in a proline to leucine change very close to the C terminus of the protein. This sequence

PCR = polymerase chain reaction



**Figure 1.** Family pedigree and genotypes. Full segregation is noted with the *SLC7A9* marker (chromosome 19q). All the patients are homozygous for allele 1 while the unaffected sibs are not. No segregation was observed with the *SLC3A1* marker (chromosome 2p).



**Figure 2.** The P482L mutation: CCG in the wild type (top) changes to CTG in the patient (bottom) and as a result proline changes to leucine.

variation abolishes an Hpa II restriction site in the carrier alleles. Using this restriction assay we found full segregation of the mutation in the family. The mutation was not found in 100 control chromosomes of Arab origin.

## Discussion

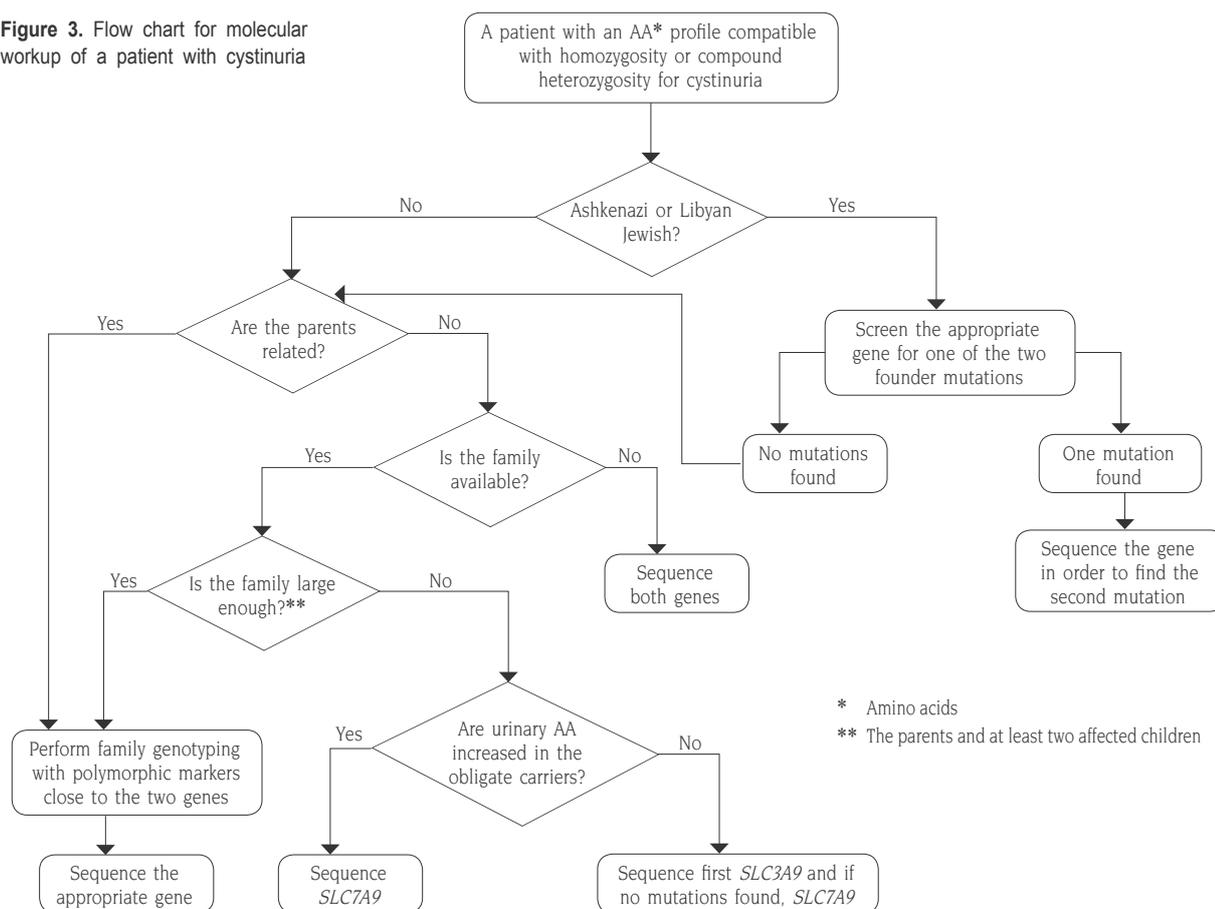
In this report we analyzed a family with cystinuria and found a missense mutation at the C terminus of the b<sup>o</sup>+AT protein. The mutation, which was recently described by Shigeta et al. [19], is highly prevalent among cystinuria patients in the Far East. Screening of 41 cystinuria patients from Japan and Korea revealed 25 homozygotes and 6 heterozygotes for the P482L mutation. Co-expression of b<sup>o</sup>+AT protein containing the P482L mutation with rBAT in COS-7 cells resulted in a more than 90% decrease in the cystine transport activity compared to the wild type and provided evidence that this mutation causes severe loss of function. Despite being prevalent in the Far East this mutation has not been described in Europe or in the western hemisphere. At this stage it is difficult to determine if the mutation results from an ancient common founder of the Far East families and of the Arab Israeli family, or represents a particular mutable point in the coding sequence. Haplotype analysis using intragenic single nucleotide polymorphisms may resolve this issue.

At Tel Hashomer we encounter about 10 new cystinuria patients each year, the vast majority of whom are Ashkenazi or

Libyan Jewish [E. Pras, personal communication]. Since a founder mutation has been described in each of these two ethnic groups, mutation screening is straightforward and simple in Ashkenazi and Libyan Jewish patients. However, if the founder mutation is not found on both chromosomes or if the patient belongs to other ethnic groups, the use of the flow chart presented in Figure 3 could save considerable time and resources when trying to identify the disease-causing mutation. Indeed, in the family described in this report, one of the two obligate heterozygotes (parents) displayed urinary cystine levels within the normal range while the second had levels slightly above normal. Since no founder mutations have been documented in the Israeli Arab population and since classification of this family based on urinary amino acid profile was inconclusive, we first analyzed the family with two polymorphic markers very close to the two genes. Both markers are located less than 0.5 Mb from the gene and therefore recombination events that would obscure segregation are very unlikely (less than 0.5%). Indeed, only one of the markers showed segregation with the phenotype and therefore only the gene close to this marker was sequenced. As expected in a consanguineous family, the patients displayed homozygosity for the marker close to the disease-causing gene, thus providing an additional clue as to which of the genes should be sequenced.

In contrast to the normal or very low levels of urinary cystine that we found in the heterozygotes, P482L heterozygotes

**Figure 3.** Flow chart for molecular workup of a patient with cystinuria



described by Shigeta and co-workers [19] displayed a very wide range of urinary cystine levels – from normal to very high, sometimes overlapping those of homozygotes for this mutation. A number of P482L heterozygotes were reported to have renal stones. These findings are very similar to those reported for Libyan Jewish cystinuria. Heterozygotes carrying the *SLC7A9* V170M mutation also exhibited a wide variance in urinary amino acid profile and about 15% of them suffer from renal stones [20]. The reason for the wide range in urinary amino acid profile in individuals sharing the same mutation is unknown, but it probably involves the influence of modifier genes and environmental factors. Consanguineous marriages are common in the Arab villages in Israel and therefore clusters of cases of rare autosomal recessive diseases are commonly observed. Defining these genetic disorders and detecting the genes and mutations that cause them are essential in order to offer precise genetic counseling for family planning and eventually decrease the number of patients.

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