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ORIGINAL PAPER

Evidence for association of *SLC7A9* gene haplotypes with cystinuria manifestation in *SLC7A9* mutation carriers

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Abstract Cystinuria is a complex genetic disorder. In the present study, we report on the strict linkage disequilibrium of *SLC7A9* mutations with the wild type *SLC7A9* haplotype of 15 single nucleotide polymorphisms (SNPs) and their effect on cystinuria manifestation and classification. Specifically, screening for mutations and polymorphisms was performed in the family members of ten cystinuric patients with *SLC7A9* gene mutations. The molecular genetic and clinical data of cystinuric patients and their relatives were combined to construct the *SLC7A9* SNP haplotypes and evaluate the manifestation of the disorder in carriers for a *SLC7A9* gene mutation. It was found that all carriers of a *SLC7A9* mutation manifested cystinuria if their normal allele had non-wild type nucleotides in two or more of the identified polymorphic sites. Subsequently, the polymorphic background of the *SLC7A9* gene probably affects the expression of the disorder in *SLC7A9* mutation carriers and points to a revised genetic classification of cystinuric patients.

Keywords Cystinuria · *SLC3A1* gene · *SLC7A9* gene · Inheritance · Genetic classification

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Introduction

Cystinuria is one of the most common inborn errors of metabolism [1]. Mutation and linkage analyses have demonstrated that the disorder is heterogeneous. In almost half of the patients, the disease is caused by mutations in the *SLC3A1* (OMIM 104614) gene which is considered to be responsible for cystinuria type A and follows a complete recessive mode of inheritance. In the other half of the patients, the disease is caused by mutations in the *SLC7A9* (OMIM 604144) gene that is considered to be responsible for cystinuria type B presumably inherited with an incomplete dominant manner. Patients classified under the questionable cystinuric type AB are believed to have both genes involved in the pathogenesis of the disorder [2].

To date, the widely used classification of cystinuric patients as ''type I'' (fully recessive) or ''non-type I'' (incompletely recessive) is based on the urinary cystine levels. ''Type I'' heterozygotes have normal urinary cystine excretion, while ''non-type I'' heterozygotes present moderate to high urinary cystine hyperexcretion [3]. However, any screening method of urinary cystine concentration can be affected by dietary preferences, sodium intake and fluid intake [4, 5]. In contrast, molecular genetic criteria are not prone to acquirable individual differences and tend to be more

concrete and strict for the classification of cystinuric patients. Therefore, a more detailed genetic classification of cystinuric patients than those previously reported as type A, B, and AB is probably needed, taking into account not only mutations but also the polymorphisms of cystinuric genes.

In recent literature, relationship has been reported to exist between certain single nucleotide polymorphism (SNP) haplotypes and imbalanced gene expression [6, 7]. In the present study, we explored the issue of cystinuria phenotypic variability in regard to *SLC7A9* SNP haplotypes, due to the highly polymorphic background of the *SLC7A9* gene [8, 9], and sought to classify cystinuric patients with more solid molecular genetic criteria.

Patients and methods

Screening for *SLC7A9* gene mutations and polymorphisms was performed in the family members (25 first-degree relatives, 14 second-degree relatives, and 6 spouses) of ten previously studied cystinuric patients with *SLC7A9* gene mutations [8]. All subjects gave informed written consent in accordance with the Helsinki Declaration 1964 (revised 2001).

The *SLC7A9* SNP haplotypes were determined in patients and their relatives by associating the identified *SLC7A9* polymorphic backgrounds among the members of each family.

Cystine concentration in urine samples of the relatives of cystinuric patients was determined as previously described and also their clinical history of urololithiasis was taken [4, 8].

In addition, the TFSEARCH software was used in order to identify potential *cis*-acting transcription factors (enhancer*) in the 5′- and 3′-regulatory end of the *SLC7A9* gene and in its introns [10, 11]. Statistical analysis was performed by the Arlequin software appropriate for genetic population studies.

*A regulatory sequence in eukaryotic DNA that may be located at a great distance from the gene it controls. Binding of specific proteins to an enhancer modulates the rate of transcription of the associated gene.

Results

The wild type *SLC7A9* SNP haplotype was found in nine out of the ten patients who were homozygous or compound heterozygous for two *SLC7A9* mutations (wild type of a SNP is the nucleotide of the reference SLC7A9 cDNA sequence: OMIM 604144). With the exception of the distant *SLC7A9* polymorphism 1365C > T, which was found in heterozygosity only in one patient, all other patients carrying *SLC7A9* mutations had the wild type haplotype of the other 15 identified *SLC7A9* SNPs [8].

Combining the molecular genetic and clinical data shown in Table 1, the 33 carriers of a *SLC7A9* mutation were classified into the following three categories:

(a) All the heterozygotes carrying the wild type SNP haplotype in their normal *SLC7A9* allele did not manifest cystinuria ($n = 17$).
(b) Almost half (60%) of the heterozygotes carrying non-wild type SNP in one polymorphic site of their normal *SLC7A9* allele manifested cystinuria ($n = 5$).
(c) All the heterozygotes with two or more non-wild type polymorphic sites on their normal *SLC7A9* allele manifested cystinuria ($n = 11$).

The above observations are further underlined by two subjects with digenic inheritance, who were negative for cystinuria even though their *SLC7A9* mutations show two of the highest physical–chemical dissimilarities according to Grantham's scale [8, 12]. The mother of a cystinuric patient carrying in heterozygosity the mutation N516D of the *SLC3A1* gene and the mutation Y232C of the *SLC7A9* gene was free of cystinuria. In this woman, the mutation Y232C was in *cis*- with 10 non-wild type *SLC7A9* SNPs (rare case), while her normal allele had the wild type *SLC7A9* SNP haplotype which is probably expressed according to the proposed mode of inheritance balancing the mutant allele. The mother of another patient, who carried in heterozygosity the *SLC3A1* mutation F266S and the *SLC7A9* mutation G105R, was also free of cystinuria. In accordance with the above, her normal *SLC7A9* allele having the wild type SNP haplotype is probably expressed.

Taking into consideration the possible functional role of polymorphisms in gene transcription, we searched in the available electronic databases for *cis*-acting transcription factors in the *SLC7A9* gene proximate 5′- and 3′-regulatory and intronic sequences near the identified SNPs. Nineteen enhancers were revealed (maximum score: 91.6, minimum score: 85.1, threshold: 85.0) (Fig. 1). Genetic changes not only in the sequences of the enhancers but also in their vicinity may affect the structure of DNA, altering the interaction between the *cis*- and *trans*-acting transcription factors and leading eventually to the hypothesized allelic imbalanced in *SLC7A9* gene expression.

Table 1 Stratification of *SLC7A9* mutation carriers

No. of SLC7A9 non-WT SNPs		Cystinuria manifestation	Stratification of studied subjects
In cis with the mutant allele[a]	In trans with the mutant allele		
0	0[b]	No	n = 6: G105R/wt, n = 4: R333W/wt, n = 4: 479-1G > C/wt, n = 1: S379R/wt, n = 1: G105R/wt (compound heterozygote with the SLC3A1 gene mutation F266S) The 100% of individuals excreted 20 μg/ml of urinary cystine and had no clinical urolithiasis symptom—negative for cystinuria
≥ 1 (Rarely)	0[b]	No	n = 1: Y232C/wt (compound heterozygote with the SLC3A1 gene mutation N516D) Very rare cases because of the linkage disequilibrium of SLC7A9 mutations with the wild type SLC7A9 SNP haplotype—no cystinuria expression is expected
0[b]	1[b]	Yes/no	n = 4: G105R/wt, n = 1: R333W/wt The 60% of individuals excreted 30 μg/ml of urinary cystine and showed clinical urolithiasis symptoms—Borderline for cystinuria
0[b]	≥ 2	Yes	n = 4: G105R/wt, n = 3: 479-1G > C/wt, n = 2: D233E/wt, n = 1: R333W/wt, n = 1: S379R/wt The 100% of patients excreted ≥ 30 μg/ml of urinary cystine, showed clinical urolithiasis symptoms and cystine stone formation—positive for cystinuria

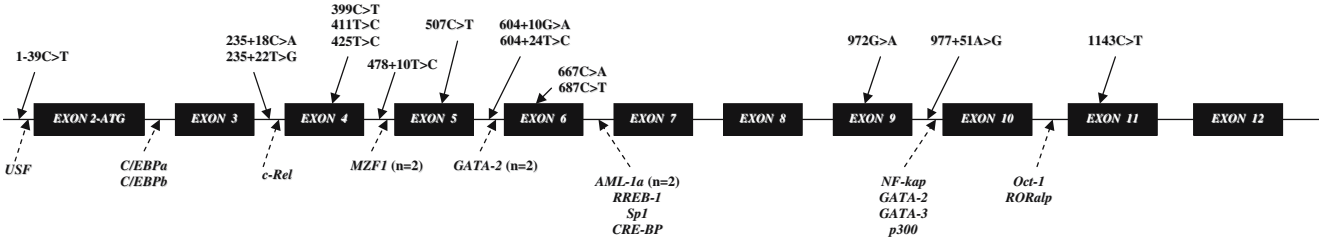
wt wild type

[a]In this column in the total number of genetics changes we should take into account the existence of a SLC7A9 mutation which must be added to the number of polymorphisms

[b]The SLC7A9 allele that is probably preferred to be transcripted

Discussion

All subjects carried the *SLC7A9* mutations in linkage with the wild type *SLC7A9* SNP haplotype. The linkage disequilibrium of the *SLC7A9* mutations with the wild type *SLC7A9* SNP haplotype probably cannot be attributed to a recent acquisition of the *SLC7A9* mutations in our carrier population [2]. In a previous study, we found that the mutation G105R of the *SLC7A9* is the most frequent in our patients [8]. Subsequently, the G105R may represent the oldest mutation and therefore it was more susceptible to genetic



Transcription factor	Putative recognition site*	Transcription factor	Putative recognition site*
USF	From c.-184G to c.-177G	*CRE-BP*	From c.704+52T to c.704+59A
C/EBPa	From c.87+34A to c.87+47G	*AML-1a*	From c.704+38T to c.704+43T
C/EBPb	From c.87+34A to c.87+47G	*NF-kap*	From c.977+4G to c.977+13C
c-Re1	From c.236-24G to c.236-15C	*p300*	From c.977+15C to c.977+28G
MZF1	From c.478+53T to c.478+60A	*GATA-2*	From c.977+49C to c.977+58C
MZF1	From c.479-15T to c.479-8C	*GATA-3*	From c.977+49C to c.977+57C
GATA-2	From c.604+82G to c.604+91C	*Oct-1*	From c.1075-38G to c.1075-26T
GATA-2	From c.605-63G to c.605-54C	*RORa1p*	From c.1075-32A to c.1075-20T
AML-1a	From c.704+2T to c.704+7T	*CRE-BP*	From c.704+52T to c.704+59A
RREB-1	From c.704+29G to c.704+42G	*AML-1a*	From c.704+38T to c.704+43T
Sp1	From c.704+29G to c.704+38T		

* The position of the putative recognition site was numbered taking the adenine of the ATG codon of SLC7A9 cDNA sequence as number 1.

Fig. 1 Nineteen putative *cis*-acting transcription factors were found in the proximate 5′- and 3′-regulatory and intronic sequences of the SLC7A9 gene, in the vicinity of the identified polymorphisms that were in linkage disequilibrium

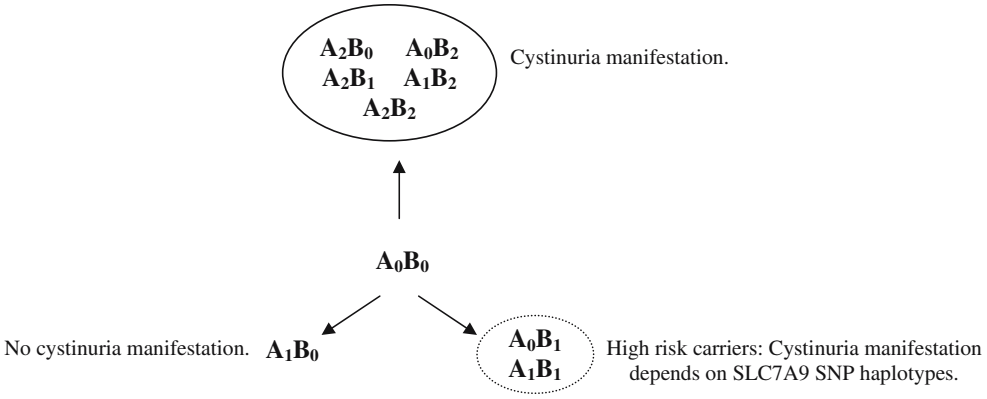


Fig. 2 Algorithm for cystinuric patient classification. The *A* corresponds to the SLC3A1 gene and the *B* corresponds to the SLC7A9 gene. The index next to *A* and *B* shows the number of mutant alleles in each gene (0, 1, or 2). The types outside cycles do not cause cystinuria, the types inside the dotted cycle may cause cystinuria which depends on SLC7A9 SNP haplotypes, and the types inside the plotted cycle will cause cystinuria

events (e.g., recombinations, genetic drift) that could affect its linkage disequilibrium with the wild type *SLC7A9* SNP haplotype.

Furthermore, the combination of the genetic and phenotypic characteristics of the carrier relatives of cystinuric patients and the investigation for *cis*-acting transcription factors in the regulatory sites of the *SLC7A9* gene revealed that the allele with the fewer genetic changes may have an epistatic role on the expression of the other *SLC7A9* allele having more nucleotide substitutions. This mode of inheritance may explain why only a number of heterozygotes for a *SLC7A9* mutation manifest cystinuria [13]. The reported incomplete dominant mode of inheritance of the *SLC7A9* gene may be the result of the polymorphic background in the *SLC7A9* alleles. This may also explain why some cystinuric patients with digenic inheritance remain undiagnosed and are represented with low prevalence even though *SLC3A1* and *SLC7A9* genes have a similar contribution to the disease [2, 14].

Consequently, we propose a modification of the previously reported genetic classification of subjects carrying *SLC7A9* and/or *SLC3A1* mutations taking also into account the presence of the wild type *SLC7A9* SNP haplotype (Fig. 2). This modification adds to the clinical prognosis of cystine stone formation in *SLC7A9* heterozygotes. However, the above preliminary results should be tested by functional analyses in a larger group of *SLC7A9* mutation carriers, whose origin is not restricted to one country. Specifically, the non-silent *SLC7A9* polymorphisms 425T > C and 667C > A, the variant 1-39C > T in 5′-regulatory gene sequence, and the intronic variants 235 + 18C > A, 235 + 22T > G, 478 + 10T > C, 604 + 10G > A, 604 + 24T > C, and 977 + 51A > G could be among the first polymorphisms that should be studied for their role in *SLC7A9* alleles' transcription imbalance.

To conclude, recently it has been reported that heterozygotes for *SLC26A5* mutations exhibit a variable degree of hearing loss, whereas heterozygotes for *SLC22A12* mutations demonstrate a gene dosage effect reflected in differences in serum urate levels [15, 16]. The aforementioned relationship in heterozygous individuals between the *SLC7A9* SNP haplotypic background and the gene's allelic imbalance may further exist in other *SLC* genes [17]. Therefore, the findings of the present study are helpful to predict the risk of an *SLC7A9* heterozygote to manifest cystinuria and also to direct future studies concerning the function of other *SLC* transporters and the pathogenesis of related disorders.

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