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A novel missense mutation of *SLC7A9* frequent in Japanese cystinuria cases affecting the C-terminus of the transporter

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Cystinuria is caused by the inherited defect of apical membrane transport systems for cystine and dibasic amino acids in renal proximal tubules. Mutations in either *SLC7A9* or *SLC3A1* gene result in cystinuria. The mutations of *SLC7A9* gene have been identified mainly from Italian, Libyan Jewish, North American, and Spanish patients. In the present study, we have analyzed cystinuria cases from oriental population (mostly Japanese). Mutation analyses of *SLC7A9* and *SLC3A1* genes were performed on 41 cystinuria patients. The uptake of ^{14}C-labeled cystine in COS-7 cells was measured to determine the functional properties of mutants. The protein expression and localization were examined by Western blot and confocal laser-scanning microscopy. Among 41 patients analyzed, 35 were found to possess mutations in *SLC7A9*. The most frequent one was a novel missense mutation P482L that affects a residue near the C-terminus end of the protein and causes severe loss of function. In MDCK II and HEK293 cells, we found that P482L protein was expressed and sorted to the plasma membrane as well as wild type. The alteration of Pro^{482} with amino acids with bulky side chains reduced the transport function of $b^{0,+}$AT/BAT1. Interestingly, the mutations of *SLC7A9* for Japanese cystinuria patients are different from those reported for European and American population. The results of the present study contribute toward understanding the distribution and frequency of cystinuria-related mutations of *SLC7A9*.

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Cystinuria (MIM 220100) is an inherited disorder owing to the defective transport of cystine and dibasic amino acids across the epithelial cells of renal proximal tubule and small intestine.[1] The incidence of cystine crystalluria reported in Western countries and in Japan varied from 15 000:1 to 50 000:1.[2,3] The patients suffer from recurrent nephrolithiasis leading to severe renal dysfunctions for which repeated therapies are imperative.[4] Classical cystinuria has been classified into three types (I, II, and III) based on the excretion of cystine and dibasic amino acids in obligate heterozygotes.[5] Type I heterozygotes show a normal amino-acid urinary pattern, whereas type II and III heterozygotes exhibit high or moderate levels of hyperexcretion of cystine and dibasic amino acids.[5] The discovery of a single-membrane-spanning type II membrane glycoprotein rBAT encoded by *SLC3A1*[6–10] and 12-membrane-spanning protein $b^{0,+}$AT/BAT1 encoded by *SLC7A9*[11–13] has brought a breakthrough in the understanding of the molecular basis of cystinuria and cystine transport in the renal proximal tubules.

The analyses of cystinuria patients have revealed distinct cystinuria-related mutations in *SLC3A1* and *SLC7A9* genes.[14,15] It was originally supposed that mutations of *SLC3A1* and *SLC7A9* genes are responsible for type I and non-type I (type II and III) cystinuria, respectively. However, recent developments in the genetics and physiology of cystinuria have not supported such a traditional classification.[16–18] Although *SLC3A1* is associated with the type I urinary phenotype, *SLC7A9* mutations were found in all three subtypes.[16,17] Therefore, a new cystinuria classification based on molecular analysis and not on urinary amino-acid excretion patterns has been proposed: type A, due to two mutations of *SLC3A1*; type B, due to two mutations of *SLC7A9*; and type AB, with one mutation on each of the above-mentioned genes.[17] For *SLC7A9* gene, International Cystinuria Consortium and Rozen and colleagues identified cystinuria-related mutations mainly from Italian, Libyan Jewish, North American, and Spanish patients

and established the genotype–phenotype relation for *SLC7A9*.[12,16–18] In the present study, we have analyzed cystinuria cases from oriental population (mostly Japanese) and found that the mutations of *SLC7A9* for Japanese cystinuria patients are quite different from those reported for European, North American, and Libyan Jewish. We report here that the most frequent one is a novel missense mutation affecting the C-terminus of the transporter protein (Tables 1 and 2).

RESULTS

Mutations of *SLC7A9* and *SLC3A1* found in cystinuria patients

We studied 41 cystinuria patients from 39 cystinuria families potentially representing 78 independent cystinuria-related alleles. They were subjected to the mutation analysis of *SLC7A9* gene by direct sequencing. The mutations of *SLC7A9* gene found in the cystinuria patients are listed in Table 3. They include one frameshift (1105delA) and one nonsense mutation (W69stop) that produce early stop codons, and seven changes affecting single amino-acid residues (V142A, G195R, L223M, N227D, R333W, R333Q, and P482L). Among them, V142A, L223M, N227D, R333Q, 1105delA, and P482L were novel mutations found for the first time in the present investigation, whereas three mutations (W69stop, G195R, and R333W) were reported previously for the European, North American, and Libyan Jewish population.[12,16,18] The amino-acid alterations except V142A and L223M were not found in 50 normal subjects. V142A and L223M were, in contrast, found in normal subjects without cystinuria phenotype. V142A and L223M were found in 23 and 19 alleles out of 50 normal subjects (100 independent alleles), respectively, suggesting that these amino-acid alterations represent polymorphic variations of *SLC7A9*.

The location of the *SLC7A9* mutations is shown at the corresponding amino-acid residues in the 12-transmembrane (TM)-domain model of $b^{0,+}$AT/BAT1 protein in Figure 1. Five cystinuria-specific missense mutations were localized within the putative TM domains 5 and 6 (G195R and N227D), in the putative intracellular loop between TM8 and TM9 (R333W and R333Q), or in the C-terminus (P482L). The one single nucleotide deletion is localized to the portion corresponding to the putative intracellular loops between TM8 and TM9 (1105delA). Three mutations (G195R, R333W, and R333Q) alter amino-acid residues that are conserved for all the human members of the heterodimeric amino-acid transporter family (Figure 1).

Among 41 cystinuria patients examined in the present study, we found mutations of *SLC3A1* in five cases. Two cases without any alterations in *SLC7A9* gene possessed mutations for *SLC3A1*: one as a homozygote for the deletion of T at nucleotide 1820; the other as a compound heterozygote for V183A (T548C)/C673R (T2017C); nucleotide numbers refer to GenBank accession no. NM_000341 for rBAT cDNA.[19] Among four cases with only polymorphic changes (V142A and/or L223M) in *SLC7A9*, two cases possessed mutations for *SLC3A1*: one as a homozygote for the insertion of TA at nucleotide 1898; the other as a compound heterozygote for V183A (T548C)/L346P (T1037C). The other two cases with only polymorphic changes in *SLC7A9* did not possess *SLC3A1* mutations. *SLC3A1* mutations were not found in the cases with cystinuria-specific mutations of *SLC7A9* except one P482L homozygote that also possesses I445T (T1334C) mutation in *SLC3A1*.

Functional analysis of *SLC7A9* mutations

All the *SLC7A9* mutations found in the present study were examined for amino-acid transport activity. As shown in Figure 2, the cystinuria-specific mutations such as W69stop, G195R, N227D, R333Q, R333W, 1105delA, and P482L exhibited remarkable decrease in cystine transport activity compared with wild-type $b^{0,+}$AT/BAT1. In contrast, V142A and L223M, which were also found in normal subjects, did not affect or only slightly decreased the cystine transport activity compared with wild-type $b^{0,+}$AT/BAT1 (Figure 2). We also constructed V142A/L223M double mutant, which contains both V142A and L223M alterations because they are possibly located in the same allele. As shown in Figure 2, even the double mutation for V142A and L223M did not severely affect the functional activity.

Table 1 | Primers used for amplification of *SLC7A9* exons and their direct sequencing

	Sense primer	Antisense primer	Size of amplified fragment
Exon 2	5′-GAGCTTGCACTTGCGTCTTG-3′	5′-AATCAAAGAGTACATCTTCTGCCG-3′	299[a]
Exon 3	5′-TGGCCTTCTGGGCTGGGTC-3′	5′-AAGAGGGATACTGGCAGGGT-3′	307
Exon 4[b]	5′-AGCCTCCGGTGGGAGGAAG-3′	5′-GAGTCCCCAGACACCCTCTG-3′	388
Exon 5[b]	5′-AAAGGAGACTCTCTCCAGGG-3′	5′-ATGCTTCCTTGGAGATGGGCT-3′	292
Exon 6	5′-CCATCTTTCCCGTGGAGATACA-3′	5′-CAAACCCCAGAAAGGAGAACTC-3′	279
Exon 7	5′-CCACTAGCAGGGCCATTCAC-3′	5′-CGGGAAGGGCATCATGGAATAC-3′	316
Exon 8[b]	5′-CTGAACGTGGGTCTCCGTG-3′	5′-ACCTCCAGTGCTGACACCTG-3′	235
Exon 9	5′-CTCTTGGAGGCCGAGAAAGAC-3′	5′-GGGTGTTATTGCTTTCGCCGC-3′	214
Exon 10	5′-TGGTCTGCACTCTGGTCAGC-3′	5′-GGCATCTGGGTCATTTGGAAG-3	236
Exon 11	5′-CTTCTTCGGTCTTCTGTGAC-3′	5′-CTAGAAGGCATGCCCCTAGC-3′	314
Exon 12	5′-AGGGGGTACATGGAGTTCATAC-3′	5′-GTGACAGAGGTCTTGGAGTC-3′	366
Exon 13	5′-CAGGGTCTAGGTGACGCATC-3′	5′-TCAGCTGACTTGGCTACAAGAG-3′	218

[a]The size of the fragments amplified by PCR using sense and antisense primers described is indicated (bp).
[b]The primers for exons 4, 5, and 8 are identical to those for reference International Cystinuria Consortium.[12]