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Analysis of the *CTNS* Gene in Nephropathic Cystinosis Mexican Patients: Report of Four Novel Mutations and Identification of a False Positive 57-kb Deletion Genotype with LDM-2/Exon 4 Multiplex PCR Assay

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Objective: Identify *CTNS* gene mutations in nephropathic cystinosis Mexican patients. *Subjects and Methods:* Eleven patients were included, nine presenting infantile nephropathic cystinosis and two siblings with the juvenile phenotype. The common 57-kb deletion was detected by multiplex PCR using large deletion marker-2 (LDM-2)/exon 4 set primers. Those alleles negative for 57-kb deletion were screened by single strand conformation polymorphism (SSCP) and subsequent direct sequencing. *Results:* In our sample, five mutations previously reported are identified: 57-kb deletion, EX4_EX5del, c.985_986insA, c.357_360delGACT, and c.537_557del. We detect a false assignation of 57-kb deletion homozygous genotype by using the LDM-2/exon 4 primers. In addition, four novel and severe mutations are identified: c.379delC, c.1090_1093delACCAinsCG, c.986C>G (p.T216R), and c.400+5G>A. *Conclusions:* Our sample of Mexican patients display allelic heterogeneity as compared to European or North American cystinosis cases. The identification of novel mutations might suggest the presence of exclusive American *CTNS* alleles in Mexican population. In order to prevent the false positive assignation of 57-kb deletion genotype, as caused by the presence of another type of intragenic *CTNS* gross deletion, we propose to analyze a different control *CTNS* exon to those originally reported in both LDM multiplex PCR assays, especially when parental DNA samples are not available.

Introduction

Cystinosis is a rare autosomal recessive lysosomal storage disorder characterized by impaired transport of free cystine out of lysosomes (Kalatzis and Antignac, 2002). Three clinical variants of cystinosis have been reported in the literature (Gahl *et al*., 2002). The most common one is the infantile nephropathic form (MIM 219800), which results in an end-stage renal failure around the age of 10 years; two less severe forms are juvenile nephropathic or late onset form (MIM 219900) and the ocular or adult nonnephropathic form (MIM 219750). Cystinosis has a birth prevalence of about 1 in 100–200,000 in European or U.S. populations (Gahl *et al*., 2001), although in Mexico its frequency is unknown. The gene responsible (*CTNS*, 17p13) is transcribed into a ~2.6-kb mRNA (Town *et al*., 1998; Touchman *et al*., 2000) and codes for cystinosin, a transmembranal lysosomal cystine transporter (Attard *et al*., 1999). To date, around 86 cystinosis-causing *CTNS* mutations have been reported (http://www.hgmd.cf.ac.uk/). The most prevalent mutation is a large 57-kb deletion (Town *et al*., 1998; Touchman *et al*., 2000) that is present in a homozygous state in 33–44% of the Caucasian patients (Shotelersuk *et al*., 1998). Several studies have reported *CTNS* allelic frequencies in various populations (Shotelersuk *et al*., 1998; Town *et al*., 1998; Kleta *et al*., 2001), and Mexican patients have been occasionally included in some of these series (Shotelersuk *et al*., 1998); however, the mutational spectrum in cystinotic patients from Mexico remains unknown. Here we describe the molecular analysis of nine Mexican families with cystinosis.

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Subjects and Methods

Patients

Nine patients with infantile nephropathic cystinosis (INC) from eight nonrelated families and two siblings with clinical features of juvenile nephropathic cystinosis (JNC), referred to us by the Asociación Mexicana de Cistinosis, were analyzed (Table 1). All patients and their parents gave their informed consent for the present investigation. The members of the families herein studied had a Mexican origin. Consanguinity is reported in only one family, where two siblings are affected with INC phenotype. Genomic DNA from leukocytes from all patients and their parents was obtained by "salting-out" method (Puregene™ Blood kit; Gentra Systems, Minneapolis, MN).

Detection of the common 57-kb cystinosis-causing deletion

PCR detection of the 57-kb deletion was initially performed in all DNA samples included in our study by using the primers located across the deletion breakpoint designated as "large deletion marker-2" or LDM-2, which yield a 442-bp PCR product in those alleles with the 57-kb deletion. In addition to the LDM-2 primers, exon 4 primers could be used to coamplify a fragment of 250 bp from nondeleted alleles, so this diagnostic test allows an easy identification of patients with none, one, or two alleles bearing the common 57-kb cystinosis-causing deletion (Anikster *et al*., 1999a). Due to inconsistencies in the assignation of the 57-kb deletion genotype while considering the parents of case 3 (Table 1), it was necessary to carry out another multiplex PCR analysis with an alternative set of deletion-specific primers designated "large deletion marker-1" or LDM-1 (423 bp), and to ascertain the presence of a nondeleted allele, oligonucleotides for amplification of microsatellite $(GT)_n$ D17S829 marker located at intron 3 (~266 bp) were also included (Anikster *et al*., 1999a).

Mutation screening by SSCP analysis and direct sequencing

In those alleles without the 57-kb deletion, mutation screening of coding exons 3–12 and *CTNS* promoter by using primers reported by Rupar *et al.* (2001) and Phornphutkul *et al.* (2001), respectively, was performed by SSCP analysis in 1× MDE™ gels (BioWhittaker Molecular Applications, Rockland, ME) stained with silver nitrate (Silver Stain Kit™; Bio-Rad Laboratories, Hercules, CA). Exons with abnormal electrophoretic patterns were directly sequenced in both strands in an ABI PRISM™ Model 377 sequencer (Applied Biosystems, Foster City, CA).

RT-PCR analysis

Total RNA was isolated from leukocytes from patient 6, his father, and a healthy control by using the RNAeasy Mini Kit (Qiagen, Hilden, Germany). *CTNS* cDNA amplification was carried out with 5′-CCTCTTCCAGTAACATTGAGG-3′ (exon 2) as forward primer (Kleta *et al*., 2001) and R79 5′-CGCGTGCAGGCTGAAGAAGA-3′ as reverse primer (exon 9) (Attard *et al*., 1999), using the One-Step RT-PCR with Platinum® Taq kit (Invitrogen, www.invitrogen.com) yielding a 722-bp product. RT-PCR–amplified fragments were gel extracted and purified by using QiaexII™ Gel Extraction Kit (Qiagen) and directly sequenced.

Table 1. Relevant Data of Mexican Cystinosis Patients Studied

Case	*Phenotype*	*Mutation allele 1*	*Mutation allele 2*	*Predicted effect on mRNA or protein sequence*
1	INC	57-kb deletion	57-kb deletion	Lack of *CTNS* mRNA
2	INC	57-kb deletion	?[a]	Allele 1: lack of *CTNS* mRNA Allele 2: unknown
3	INC	57-kb deletion	EX4_EX5del	Allele 1: lack of *CTNS* mRNA Allele 2: premature stop codon (E21fsX69)
4, 5 (two siblings)	INC	c.985_986insA (exon 9)	c.985_986insA (exon 9)	Premature stop codon (T216fsX227)
6	INC	c.357_360delGACT (exon 3)	c.400+5G>A[b] (intron 3)	Allele 1: T7fsX13 Allele 2: exon 3 skipping
7, 8 (two siblings)	JNC	c.537_557del (exon 5)	c.1090_1093delACCAinsCG[b] (exon 10)	Allele 1: in-frame deletion of seven amino acids in N-terminal domain (163_P73del) Allele 2: premature stop codon (T251fsX294)
9	INC	c.379delC[b] (exon 3)	c.986C>G[b] (exon 9)	Allele 1: premature stop codon (L14fsX14) Allele 2: missense mutation (p.T216R)
10	INC	?[a]	?[a]	Unknown
11	INC	?[a]	?[a]	Unknown

[a]Unknown mutation.
[b]New mutations identified in the present study.
INC: Infantile nephropathic cystinosis.
JNC: Juvenile nephropathic cystinosis.

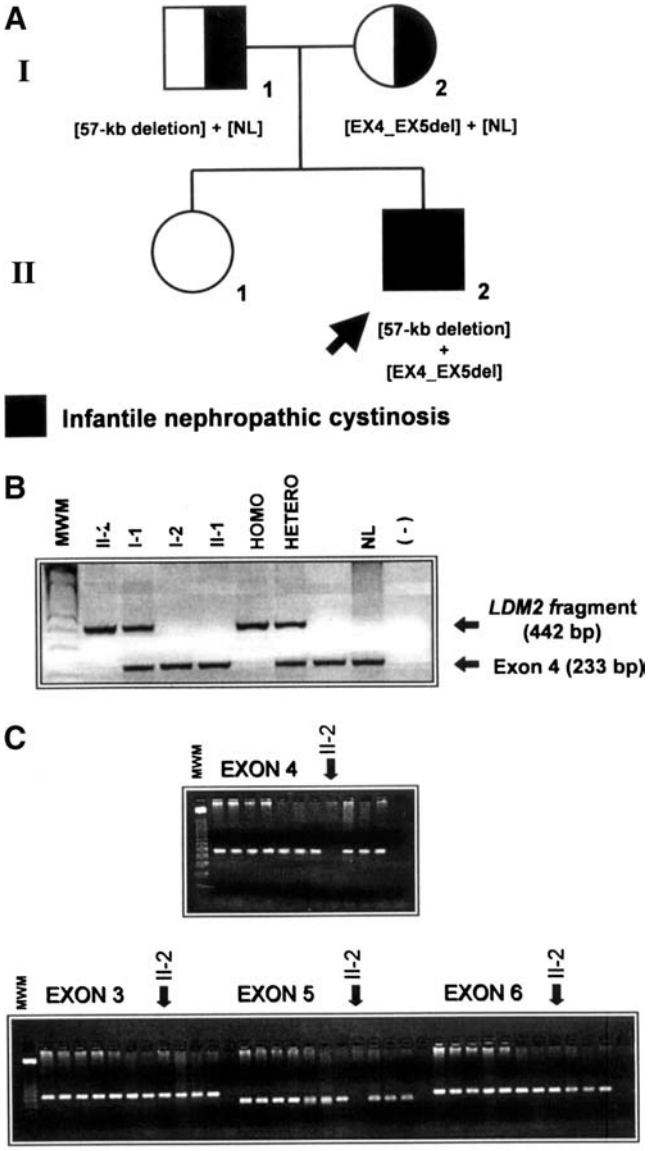

FIG. 1. A false positive homozygous 57-kb deletion genotype assignation in a heterozygous [57-kb deletion]+ [EX4_EX5del] patient (case no. 3, see Table 1). (**A**) Genealogy of case 3. Respective genotypes are indicated. (**B**) Familial results of multiplex PCR analysis with LDM-2/exon 4 primers. Note the apparent 57-kb deletion homozygous genotype in index case (II-2) and the absence of obligate LDM-2 fragment in his mother (I-2). Lane HOMO: control homozygous deletion 57-kb DNA sample; lane HETERO: control heterozygous deletion 57-kb DNA sample. (**C**) Results of amplification of *CTNS* exons 3–6 in II-2, which corroborates a deletion of exons 4 to 5 in the maternal *CTNS* allele of index case.

Results

The phenotype and genotype findings of our patients are summarized in Table 1. Gene mutation nomenclature is according to rules currently accepted (den Dunnen and Antonarakis, 2000), indeed all changes reported herein are described by considering that the A of the ATG start codon is the +1 in the nucleotide numbering.

Identification of the 57-kb deletion: a false positive homozygous 57-kb deletion genotype assignation

The 57-kb deletion was identified in 22.2% of *CTNS* alleles analyzed ($n = 4$ alleles). Case 3 (Table 1) with a [57-kb deletion] + [EX4_EX5del] genotype was initially misclassified as homozygous for 57-kb deletion when using LDM-2/exon 4 primers. Molecular testing of his parents revealed solely the 57-kb deletion carrier status of the father, but no LDM-2 product was observed in the patient's mother DNA sample (Fig. 1). Nonetheless, subsequent proband DNA sample analysis by PCR amplification of exons 3–12 and multiplex PCR with LDM-1/D17S829 primers (Anikster *et al.*, 1999a) failed to amplify exons 4 and 5 (Fig. 1), and revealed integrity of at least one D17S829 *CTNS* allele (data not shown). These results allowed us to define a compound heterozygote genotype in this patient, that is, a 57-kb deletion paternally inherited and an EX4_EX5del in the maternal allele. The same patient was previously reported by Anikster *et al.* (1999b) (personal communication Dr. Gahl) who identified the EX4_EX5del mutation (accession no. CG994863, HGMD®) by analyzing his cDNA.

Small mutations in CTNS *gene: report of four novel and severe mutations*

The SSCP screening allowed us to identify a total of seven small mutations; three of them have been previously reported: c.985_986insA, c.357_360delGACT, and c.537_557del (Anikster *et al.*, 1999b). The four new mutations identified here are c.379delC, c.1090_1093delACCAinsCG, c.986C > G (p.T216R), and c.400+5G > A. Distribution by genotypes is shown in Table 1.

The c.357_360delGACT mutation generates a premature stop codon at position 13 (Town *et al.*, 1998; Shotelersuk *et al.*, 1998). We identified the presence of this mutation in the maternal allele of patient 6 with INC phenotype, who was previously reported by Shotelersuk *et al.* (1998) (personal communication with Dr. Gahl). Moreover, in this patient, we also detect a novel intronic transition c.400+5G > A in his paternal allele. The analysis of c.400+5G > A allele through the Splicing Mutation Analysis Software (https://splice.cmh.edu) (Nalla and Rogan, 2005) indicates that it leads to a leaky 5′ (donor) splice-site at the intron 3 (initial Ri value: 8.9, final Ri value: 5.4, ΔRi:−3.5, percentage change in predicted binding affinity: 8.7%) and do not predict the use of potential cryptic splice-sites in the vicinity to c.400+5G > A mutation. To test the hypothesis of exon 3 skipping, RT-PCR with primers of exons 2 and 9 was carried out in this patient and his father. Two products of 722 bp (transcript "a") and 642 bp (transcript "b") were observed in both as compared with a control individual that only showed a 722-bp product. Sequencing of transcript "b" revealed an exon 3 skipping with consequent loss of the start codon and Kosak sequence, confirming the severe pathogenic effect of c.400+5G>A mutation (Fig. 2). Successful amplification of misspliced transcript "b," both in case no. 6 and his father, suggests an absence of its degradation by the nonsense-mediated mRNA decay. Further, in the absence of quantitative RT-PCR analysis, it is not possible to rule out that

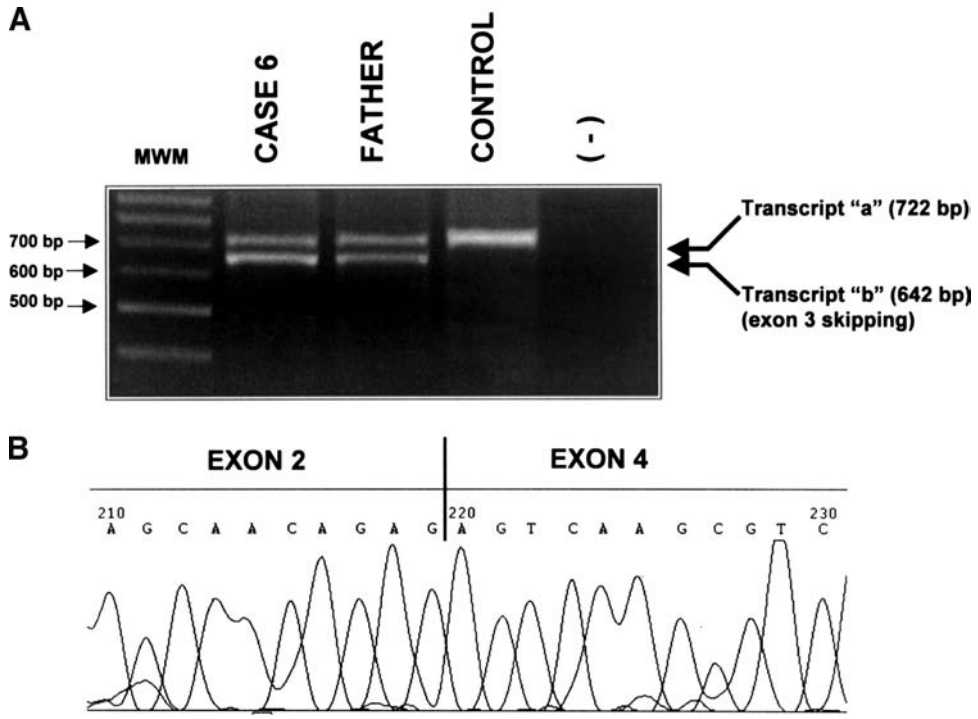

FIG. 2. (**A**) RT-PCR analysis of case 6, his father, and a healthy control. Note the presence of an additional RT-PCR product (transcript "b" above 600 bp) in samples from the patient and his father, and its absence in the normal control. (**B**) Sequence analysis of transcript "b" from the patient illustrates the defective splicing of exon 3 due to the presence of the c.400+5G>A mutation. MWM: 100 bp leader.

the patient still presents small amounts of normally spliced transcript, since the Splicing Mutation Analysis Software indicates a decreased, but not abolished, activity for donor site of intron 3.

The in-frame deletion c.537_557del that eliminates seven amino acids (ITILELP) of N-terminal domain of cystinosin was identified in two brothers (cases 7 and 8) from the only family with JNC included in this study. The second *CTNS* mutation identified in these patients is a novel severe indel-type mutation, c.1090_1093delACCAinsCG, situated at exon 10.

Discussion

Here we report the molecular analysis of nine Mexican families with cystinosis. We found that the 57-kb deletion is less frequent in *CTNS* Mexican alleles (22.2%) than in North European or North American ones (65–76%) (Anikster *et al*., 1999b; Forestier *et al*., 1999; Kiehntopf *et al*., 2002). Still, it is comparable to that reported in an Italian cohort (17%) (Mason *et al*., 2003). The presence of 57-kb deletion in our sample supports that Mexican population has an admixture with European or Spanish gene pools (Shotelersuk *et al*., 1998).

We emphasize the possibility of errors in genetic counseling derived from a false assignation of 57-kb deletion homozygous genotypes by the use of only the LDM-2/exon 4 primers in those patients whose parents are not available for molecular study; this could be solved by PCR amplification of a different control *CTNS* exon to those originally proposed in both LDM multiplex PCR assays. Interestingly, Kiehntopf *et al.* (2002) referred two patients classified as homozygous for 57-kb deletion (LDM-1 fragment positive without amplification of D17S829 locus), but when their mothers were analyzed, the obligate LDM-1 junction fragment was not present; the authors attributed this outcome to the poor quality of the DNA samples. Nonetheless, our findings open the possibility that other deletions exist in these mothers (for example, the P11 deletion) (Town *et al*., 1998; Forestier *et al*., 1999) that cannot be identified when used only LDM-1/D17S829 primers.

On the other hand, the novel c.400+5G>A mutation identified in the present study corroborates that the substitution of the highly conserved+5 guanine, present in the majority of human introns, might produce deleterious splicing (Krawczak *et al*., 2007). Actually, Kleta *et al.* (2001) reported a mutation also revealing substitution of a guanine at position+5, a homozygous IVS5+5GT>CC double substitution, which is accompanied by the skipping of exon 5 in the cDNA of an Afro-American patient with INC.

Patients with late-onset cystinosis forms usually bear one "mild" mutation on one allele and either a "mild" or a "severe" mutation on the other allele (Anikster *et al*., 1999b). In addition, the in-frame deletion c.537_557del has been described in a homozygous state in patients with INC (Shotelersuk *et al*., 1998) as well as in late-onset cystinosis cases in

conjunction with severe, or unknown mutations (Attard *et al*., 1999); thus there is not a clear genotype–phenotype correlation (Attard *et al*., 1999; Kalatzis *et al*., 2004). However, the residual transport activity conditioned by the hypomorphic allele p.I67_P73del (c.537_557del) (Kalatzis *et al*., 2004) appears to be responsible for the late-onset nephropathic phenotype observed in our patients (cases 7 and 8). Although these patients have a severe mutation c.1090_1093de lACCAinsCG (T251fsX294) on the other allele. This novel indel type mutation, whose deleterious effect expected, is an alteration in the reading frame of cystinosin at the fourth trade mark (TM) domain and hence the mutated protein, if stable, would lack the GYDQL lysosomal sorting signal at C-terminal tail. Accordingly, the c.1090_1093delACCAinsCG mutation might condition an INC phenotype.

The other two novel mutations that we observed in patient 9 predict a severe deleterious effect. Specifically, the c.379delC frameshift mutation, located at exon 3, converts the leucine 14 CTG codon into a TGA stop codon (L14fsX14), and the transversion c.986C > G (p.T216R), located at exon 9, substitutes a highly conserved threonine 216 residue for an arginine in the third TM domain; the predicted effect of the presence of a basic amino acid in this domain is to abolish the transport activity as has been demonstrated by *in vitro* functional studies for the mutation Q222R (Kalatzis *et al*., 2004) associated to severe phenotypes (Kalatzis *et al*., 2002). Besides, arginine substitutions in other TM domains (e.g., W182R, G308R, G337R, and G339R) have been identified in patients with severe cystinosis phenotypes (Shotelersuk *et al*., 1998; Rupar *et al*., 2001; Mason *et al*., 2003).

The identification of four novel and severe mutations could indicate the presence of exclusive American *CTNS* mutated alleles in Mexican population, although more extensive studies and haplotype analysis must be required to support this hypothesis.

In five *CTNS* alleles of three patients, the responsible mutation was not identified perhaps due to the SSCP technique limitations (Mason *et al*., 2003) or because they might be localized in intronic *CTNS* regions or in exons 1 and 2 not analyzed in this study; however, as far as we know, mutations in these exons have not been previously reported in cystinotic patients. Although our sample is small, we have shown that the spectrum of *CTNS* mutations in the examined patients is heterogeneous in contrast to that observed in other European or North American samples (Shotelersuk *et al*., 1998; Town *et al*., 1998; Anikster *et al*., 1999b). This is according to data previously described for some Mendelian diseases in Mexican population (Nicolini *et al*., 1995).

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Electronic-Database Information

Website https://splice.cmh.edu (Splicing Mutation Analysis Server).

Website http://www.hgmd.cf.ac.uk/(The Human Gene Mutation Database® at the Institute of Medical Genetics in Cardiff).

Website http://www.ncbi.nlm.nih.gov/Omim (Online Mendelian Inheritance of Man, for infantile, juvenile nephropathic and ocular non-nephropathic cystinosis [MIM 219800, MIM 219900 and MIM 219750, respectively]).

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