
Miguel Ángel Alcántara-Ortigoza, Leticia Belmont-Martínez, Marcela Vela-Amieva, and Ariadna González-Del Angel

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 confirmation 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 assignment 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 assignment 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)6 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′-CCTCTTCCGTAACATTTAG-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

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

aUnknown mutation. bNew mutations identified in the present study.
INC: Infantile nephropathic cystinosis. JNC: Juvenile nephropathic cystinosis.
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)/C210 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 mis-spliced 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

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**Results**

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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

Acknowledgments

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References


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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