

Analysis of *CTNS* gene transcripts in nephropathic cystinosis

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Abstract Nephropathic cystinosis (NC) is an autosomal recessive disorder caused by mutations of the *CTNS* gene that encodes for a cystine transmembrane transporter. Several mutations have been described in the coding and promoter regions of the *CTNS* gene in affected individuals. We selected three patients with NC from two unrelated families, in whom sequence analysis of the *CTNS* gene detected only one or no mutations. Total RNA was isolated from peripheral blood mononuclear cells or fibroblasts and *CTNS* transcripts were analyzed. We observed a skipping of exon 5 (85 bp) in two siblings and an intron 9 retention of 75 bp associated with partial replication of exon 9 in the third patient. Genomic DNA analysis of intron regions surrounding exon 5 showed a point mutation in the hypothetical lariat branch site of intron 4 at position -24 (c.141-24 T>C) in the first two patients and a duplication of 266 bp including a part of exon and intron 9 in the third patient. Analysis of *CTNS* gene transcripts allowed identification of mutations in

patients in whom *CTNS* mutations could not be detected by traditional DNA sequencing. These results support the hypothesis that cystinosis is a monogenic disorder.

Keywords Nephropathic cystinosis · *CTNS* gene · Splicing defect · DNA duplication

Introduction

Nephropathic cystinosis (NC) (OMIM 219800) is a rare autosomal recessive disorder (incidence in 1:100,000–200,000 live births), which presents with renal proximal tubular dysfunction (Fanconi syndrome) during infancy and progresses to end-stage renal failure during the first decade of life if not treated with cysteamine [1]. Over time, patients with NC also present with dysfunction of several organs, including eyes, thyroid, liver, pancreas, male gonad, muscles, and brain [2]. NC is caused by mutations in the *CTNS* gene [3]. It encodes for cystinosin, an amino-acid-cystine carrier that is predicted to have seven transmembrane domains and is primarily expressed in the lysosomal membrane [4]. Lack of cystinosin activity causes cystine accumulation and results in formation of intralysosomal cystine crystals. Approximately 10–20% of *CTNS* transcripts encode for a second cystinosin isoform, termed cystinosin-LKG, which results from an alternative splicing of exon 12 and is expressed in the plasma membrane and other cytosolic vesicles, in addition to lysosomes [5].

The *CTNS* gene maps to chromosome 17p13 and is composed of 12 exons, with the start codon located on exon 3 [3]. The most common *CTNS* mutation in northern Europe is a 57-kb deletion that originated in Germany around AD 500 [1]. To date, more than 90 mutations have been described in the coding region or flanking intronic

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regions of the gene, as well as in the *CTNS* promoter, in patients with cystinosis. Of these, approximately 70 mutations cause NC. These include small deletions or insertions, missense or nonsense mutations, and splicing mutations [3, 6–13].

In clinical practice, the diagnosis of NC is sustained by the demonstration of increased intraleucocyte cystine levels. Genetic tests have primarily a confirmatory value and allow for genetic counseling. Evidence suggests that NC is a monogenic disease, although the mutation detection rate is not 100%. Shotelersuk et al., for example, sequenced the *CTNS* gene in 108 American patients with NC and failed to detect mutations in 19% of affected individuals [6]. The *CTNS* promoter was, however, not analyzed in their study. Similar studies that also included the promoter region showed only heterozygous or no mutations in 18% of 46 Italian patients and in 6% of 108 French patients [9, 11].

In this work, we studied *CTNS* gene transcripts in three patients with NC from two families to search for mutations that were not detected at the genomic level.

Patients and methods

Patients

Among 49 Dutch patients with NC from 44 different families, we selected three patients in whom sequence analysis failed to show homozygous or double heterozygous mutations in the *CTNS* gene (coding regions, intron/exon boundaries, or promoter region). Clinical data of these patients are summarized in Table 1. The diagnosis of NC was established in all patients by demonstration of increased intraleucocyte cystine levels and corneal cystine crystal depositions. Informed consent was obtained to perform the additional genetic studies included in this work.

Peripheral blood mononuclear cell isolation and fibroblasts cell lines

Anticoagulated whole blood samples were diluted with phosphate-buffered saline (PBS) (1:1), layered over Ficoll Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA),

and centrifuged at 1,800 *g* for 20 min at room temperature. The peripheral blood mononuclear cell (PBMC) layer was removed and washed twice with PBS following two centrifugation steps at 250 *g* for 10 min. RNA studies were performed in patient 3 on a previously established fibroblast cell line from that patient [14].

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from PBMCs or fibroblasts using TRIzol[®] reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was reverse-transcribed into complementary DNA (cDNA) using a first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). *CTNS* transcripts were amplified by PCR adding *Taq* DNA polymerase, PCR buffer with magnesium chloride (MgCl₂), deoxyribonucleotide triphosphate (dNTP) (Invitrogen Life Technologies), and primers (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturers' instructions. Primers and PCR conditions for *CTNS* amplification are described in Table 2. PCR products were analyzed using a 1.5% agarose gel with GelRed[™] staining (Biotium Inc., Hayward, CA, USA).

PCR fragments were extracted and purified with gel extraction kit (QIAGEN, Milan, Italy). Transcript sequencing was performed using primers detailed in Table 2 and dye terminator cycles sequencing (DTCS) Quick Start Mix (Beckman Coulter, Brea, CA, USA) at following conditions: 96°C for 30 s, 53°C for 30 s, and 60°C for 240 s for 37 cycles. After purification, samples were sequenced on a CEQ2000XL DNA Analysis System (Beckman Coulter).

Genomic DNA analysis

Genomic DNA was extracted from anticoagulated whole peripheral blood by QIAamp DNA blood kit (QIAGEN) in accordance with the manufacturer's instructions. The *CTNS* gene was amplified using primers and PCR conditions detailed in Table 2. Direct sequencing was performed with the primers described in Table 2 and DTCS Quick Start Mix (Beckman Coulter) at conditions detailed above. Samples were sequenced on a CEQ2000XL DNA Analysis System (Beckman Coulter).

Table 1 Patient characteristics

	Patient 1	Patient 2	Patient 3
Gender	F	M	F
Age of diagnosis (months)	41	48	18
Age at ESRF (years)	12	10.3	14.2
Intraleucocyte cystine (nmol ½ cystine/mg protein)	5.2	7.8	9
Ocular symptoms	Corneal cystine crystals	Corneal cystine crystals	Corneal cystine crystals, band keratopathy

M male, *F* female, *ESRF* end-stage renal failure

Table 2 Primer sequences and polymerase chain reaction (PCR) amplification conditions

Amplified products	Product length (bp)	Primer sequences	PCR conditions
Exon regions			
2–12	1,456	Fw 5′-gagacgctgagagaaccttgc-3′	a.t.: 64°C
		Rv 5′-gagtggctcctctgtccgc-3′	e.t.: 90 s
3–7	382	Fw 5′-ccctgaagctcgtagagaaatg-3′	a.t.: 60°C
		Rv 5′-gatggaccaggccacaaagtag-3′	e.t.: 30 s
7–12	842	Fw 5′-ggatctactttgtggcctgt-3′	a.t.: 60°C
		Rv 5′-gagtggctcctctgtccgc-3′	e.t.: 60 s
Intron regions			
4–5	310	Fw 5′-ctgattaagacaggaaggcc-3′	a.t.: 56°C
		Rv 5′-acctagcattccctaccc-3′	e.t.: 30 s
8–9	585	Fw 5′-gccttgccctgcctacatctc-3′	a.t.: 63°C
		Rv 5′-actcactcatccttggagccc-3′	e.t.: 60 s

Fw forward, Rv reverse, a.t. annealing temperature, e.t. extension time

Results

This study was performed on three patients with a clinical diagnosis of NC who had been previously screened for the *CTNS* gene mutations that revealed only one or no mutated alleles. Patients 1 and 2 are siblings. Previous sequence analysis had shown a heterozygous 57-kb deletion. No mutation was found on the other allele. To identify the second *CTNS* mutation, gene transcripts were analyzed from total RNA extracted from PBMCs. The PCR reaction yielded a 1,456-bp fragment spanning exons 2–12 that contained a 85-bp deletion corresponding to the entire exon 5 (Fig. 1). To highlight this deletion, *CTNS* transcripts were amplified using primers spanning exons 3–7 and were resolved by agarose gel electrophoresis, demonstrating a shorter band compared with control cDNA (Fig. 1). Skipping of exon 5 in these two patients causes a frameshift at position 141 bp, with a premature stop codon at position

160 bp. This splicing defect was further analyzed by sequencing the flanking intronic regions up to 300 bp upstream and downstream of exon 5. The analysis showed a point mutation at position -24 bp (c.141-24 T>C) of intron 4 that was not detected in 100 control chromosomes. The predicted protein generated by exon 5 skipping was truncated at position 53 after introducing six modified amino acids at the carboxy terminal end (wild-type cystinosin: 367 amino acids).

Genomic DNA analysis failed to reveal any *CTNS* gene mutations in patient 3. *CTNS* transcripts were amplified from skin fibroblasts and demonstrated a homozygous duplication located in the exon 9 region (Fig. 1). Specifically, the messenger RNA (mRNA) sequence of exon 9 was followed by a 75-bp sequence corresponding to a portion of intron 9, located 118 bp downstream to the exon donor splicing site and by a repeat sequence of the last 73 bp of exon 9. At the genomic level, this transcript corresponded

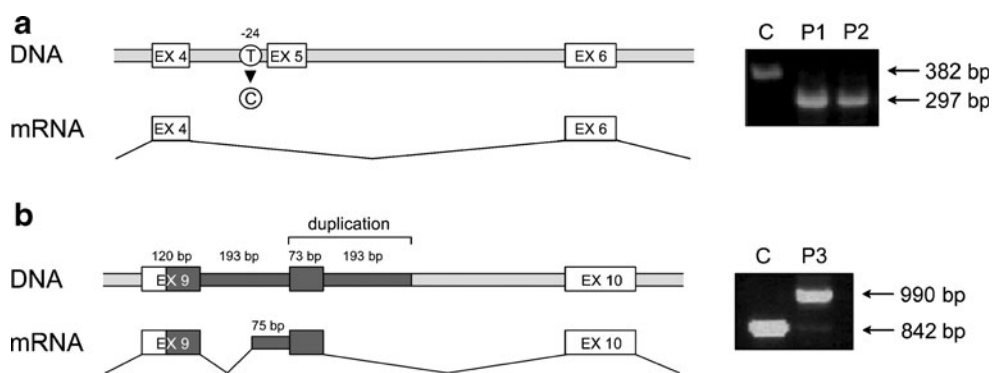


Fig. 1 Detected DNA and messenger RNA (mRNA) changes. **a** Patients 1 and 2 had a T>C substitution at position -24, upstream of exon 5 (c.141-24 T>C) that was not detected by standard sequencing. This mutation causes a skipping of exon 5 in the *CTNS* gene transcripts, as demonstrated by direct sequencing and agarose gel electrophoresis after reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the region spanning exons 3–7. The smaller band (297 bp) corresponds to the amplicon lacking exon 5.

b Patient 3 had a duplication of the genomic DNA sequence encompassing the last 73 bp of exon 9 and the first 193 bp of intron 9, shown in dark gray. This duplication causes an additional splicing of the mRNA transcript, with the incorporation of a portion of intron 9 followed by a repeat sequence corresponding to the duplicated portion of intron 9. By agarose gel electrophoresis using primers spanning exons 7 to 12, a larger 990 bp band is observed

to a duplication of the last 73 bp of exon 9 and the first 193 bp of intron 9, which introduces an additional splicing site that removes the first 118 bp of intron 9. When resolved on an agarose gel, the amplicon of patient 3 produced a larger band that included 148 additional nucleotides (Fig. 1). The predicted protein was truncated at position 232 by a frameshift that generated 5 mutated amino acids, followed by a stop codon.

Discussion

In this work, two novel mutations in the *CTNS* gene were detected by studying *CTNS* mRNA transcripts in patients in whom traditional genomic sequencing failed to detect mutations in one or both alleles. Specifically, a splicing defect and a DNA duplication were identified. Analysis of gene transcripts may be limited by tissue-specific expression patterns. Fortunately, the *CTNS* gene is ubiquitously expressed, and accumulation of increased cystine has been demonstrated in various tissues and cell lines, including fibroblasts, proximal tubular cells, and PBMCs [15–17].

In all our patients, the clinical diagnosis of NC was confirmed by measuring intraleucocyte cystine levels. The splicing defect in patients 1 and 2 was located in an intronic region that is not routinely investigated (T>C substitution 24 bp upstream of the splicing acceptor site of intron 4). This mutation is located at a hypothetical lariat branch site and probably plays an important role in the splicing of exon 5. Lariat branch sites are crucial for appropriate splicing of pre-mRNAs, and point mutations in these regions have been shown to cause abnormal mRNA splicing [18–20]. The 266-bp DNA duplication in patient 3 could not be detected using standard sequencing primers spanning exon 9 and its immediate neighboring intronic regions. In fact, this is the first DNA duplication described in patients with NC. Apart from point mutations and small insertion/deletions, all other mutations involve larger deletions [3, 6].

With this study, mutations in the *CTNS* gene were identified in all investigated Dutch patients, with the exception of one individual from whom RNA material was not available. Because the diagnosis of NC is substantiated by very specific findings, such as increased intraleucocyte cystine levels and corneal cystine crystals depositions, failure to discover mutations in these patients can be reasonably explained only by detection failures or by the existence of a second cystinotic gene. Our findings support the hypothesis that cystinosis is a monogenic disorder and suggest that *CTNS* transcripts should be studied when traditional genomic sequencing does not allow mutation detection. In addition, cystinosin-LKG is encoded by an alternative splicing of exon 12 and includes sequences that are located in the originally described 3'

untranslated region of the gene [5]. Although we have never observed mutations in this region (unpublished data), we also systematically include in our sequencing protocol the amplification of cystinosin-LKG-specific sequences.

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