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CTNS Mutations in African American Patients with Cystinosis

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Cystinosis, an autosomal recessive lysosomal storage disorder, is rarely diagnosed in African Americans. The disease results from mutations in the gene *CTNS*; at least 55 such mutations have been reported. By far the most common is a 57,257-bp deletion of Northern European origin encompassing most of the *CTNS* gene. We performed mutation analysis on DNA from four African American patients with cystinosis. In one individual with classical, nephropathic cystinosis, we identified a new molecular defect, i.e., a homozygous GT→CC substitution at the +5 position of IVS 5 of *CTNS* (IVS 5+5 GT→CC). The out-of-frame splicing of exon 5 creates a null allele consistent with the patient's severe phenotype. Two patients were heterozygous and one homozygous for the common 57-kb deletion allele, reflecting the admixture of African and Northern European gene pools in North America. The two African Americans heterozygous for the 57-kb deletion were also hemizygous for a 928G→A change, associated with ocular or nonnephropathic cystinosis. These two individuals are the only known African Americans with ocular cystinosis. We conclude that the diagnosis of cystinosis should be entertained in African Americans with symptoms of the disease, and that mutation analysis for the 57-kb deletion should be considered in this group of patients.

Key Words: **ocular cystinosis; splicing; mutation analysis; African American.**

Cystinosis is a rare autosomal recessive storage disorder due to deficient transport of the disulfide amino acid cystine across lysosomal membranes (1–3). Excessive accumulation of cystine within cellular lysosomes progresses to crystal formation and ultimately leads to cell death and tissue destruction (4). Cystinosis initially presents in infancy with renal tubular Fanconi syndrome and growth retardation (5,6). Subsequent clinical manifestations include hypothyroidism, photophobia due to corneal cystine crystal accumulation, renal glomerular failure by approximately 10 years of age, and post-renal transplant complications such as a distal vacuolar myopathy (7), swallowing difficulty (8), pancreatic insufficiency (9,10), central nervous system deterioration (11,12), primary hypogonadism in males (13), and impairment of pulmonary function (14). While most cystinosis patients follow the classic, infantile nephropathic course described above, occasional patients exhibit intermediate or adolescent cystinosis and do not develop renal failure until the second or third decades (4,5). Other rare individuals have ocular or nonnephropathic cystinosis and never suffer renal disease; their only symptom is photophobia (4,5). In general, patients with nephropathic cystinosis have 5–23 nmol half-cystine/mg protein in their polymorphonuclear leukocytes and cultured fibroblasts (normal, ≤0.2), while those with intermediate cystinosis have 2–5 nmol half-cystine/mg protein and those with ocular cystinosis have 1–2 nmol half-cystine/mg protein (4). In patients with nephropathic cystinosis, early intervention with cysteamine, a cystine-depleting agent (15), retards glomerular deterioration and enhances growth (16,17). In

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all cystinosis patients, cysteamine eyedrops can dissolve corneal cystine crystals and will effectively alleviate photophobia (18,19).

The cystinosis gene, *CTNS,* encodes a 367-amino acid peptide called cystinosin which presumably functions as the lysosomal cystine carrier (20). *CTNS* is located on chromosome 17p13 (21), has 12 exons, and produces a 2.6-kb transcript. The most commonly observed mutation among Northern Europeans is a 57,257-bp deletion encompassing the first 9 exons of *CTNS* and approximately 36 kb of upstream sequence (20,22,23). In a recent report, this deletion was identified in 121 of 216 (56%) cystinosis alleles surveyed (24). At least 55 different mutations in *CTNS* have been identified (20,22,23,25–27), but only one African American mutation, 1209C→G (Y290X), has been reported (22). Here we describe the *CTNS* genotypes of four additional African American patients, two with classical nephropathic cystinosis and two with the ocular, nonnephropathic variant. The mutations include the common Northern European 57-kb deletion, a novel splicing mutation, and a 928G→A substitution previously found in several different nonnephropathic cystinosis patients (28).

PATIENTS AND METHODS

Patients. Patient 1 is an African American female with renal tubular Fanconi syndrome and growth failure in infancy (29). She was diagnosed with nephropathic cystinosis at 2 years of age based upon the presence of cystine crystals on renal biopsy. Consanguinity was denied. Leukocyte cystine content was 5.4 nmol half-cystine/mg protein and fibroblast cystine was 8.2 nmol half-cystine/mg protein. The patient was subsequently lost to medical follow-up.

Patient 2, a 3 and 3/12-year-old boy, was diagnosed with cystinosis at less than 1 year of age due to failure to thrive, renal tubular Fanconi syndrome, and elevated leukocyte cystine levels (1.0 and 1.3 nmol cystine/mg protein). A detailed history of his biological family is not available. He has typical corneal crystals. Oral cysteamine therapy was initiated shortly after 1 year of age, but he appears refractory to cystine depletion, with leukocyte cystine concentrations ranging from 4.2 to 16 nmol/mg protein despite doses of cysteamine recommended for his size. The last value was obtained while the patient was in the hospital receiving 60 mg cysteamine/kg/day. This child's current serum creatinine is 1.0 mg/dL, reflecting deteriorating renal function, and he remains at less than the third percentile for height, weight, and head circumference. He receives all his medications, including the usual electrolyte replacements and cysteamine, via gastrostomy tube.

Patients 3 and 4 are 12- and 16-year-old African American brothers diagnosed with ocular cystinosis at 11 and 16 years of age due to photophobia and a long history of conjunctival injection. Two younger siblings are unaffected. A history of consanguinity was denied. The patients showed no physical abnormalities except corneal crystals in both eyes. They exhibited no radiological signs of rickets and their bone ages were not retarded. Growth and development were normal, and there were no pigment abnormalities. Plasma creatinine values were 0.79 and 0.95 mg/dL for patients 3 and 4, respectively. Creatinine clearances were 98 and 107 mL/min/1.73 m^2. The leukocyte cystine levels were 1.35 and 0.94 nmol half-cystine/mg protein for the younger brother and 1.56 and 1.04 nmol half-cystine/mg protein for the elder brother on two separate measurements.

CTNS gene analysis. Genomic DNA was obtained from whole blood using a standard extraction method (30). Patients were screened for the presence of the common 57-kb deletion using PCR amplification across the deletion (24). The forward primer, LDM_{3F}, was 5′-CAATGGGCTCACAGGTACC-3′, and the reverse primer, LDM_{3R}, was 5′-GACCGGCTATGCTCATAGG-3′. The coding region of *CTNS,* comprising exons 3–12, was amplified using primers and conditions as previously described (22). Reverse-transcription (RT) PCR was performed after total RNA extraction from cultured fibroblasts using Trizol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized by reverse transcription using 5 μg of total RNA, according to the manufacturer's protocol (GIBCO-BRL, Grand Island, NY). PCR amplification of the cDNA was performed using 2 μL of first-strand cDNA, 1X PCR buffer (GIBCO-BRL), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer, and 2.5 U *Taq* DNA polymerase in a total volume of 50 μL. An initial denaturation step of 94°C for 30 s was followed by 30 PCR cycles, each with a denaturation step of 94°C for 30 s, annealing of 55°C for 40 s, and an extension of 72°C for 2.5 min. Amplification cycles were followed by an elongation step of 72°C for 10 min. PCR products were electrophoresed in 1% agarose and stained with ethidium bromide. The primers

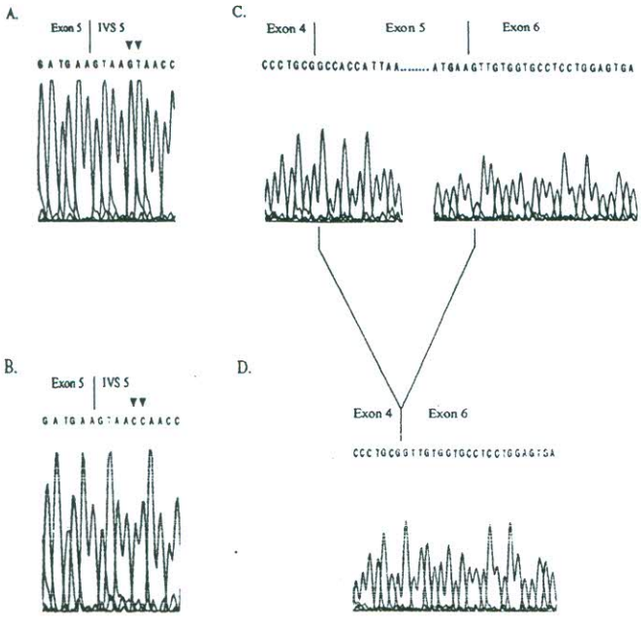

FIG. 1. Sequence analysis of genomic DNA and cDNA of the *CTNS* gene in patient 1. Nucleotide sequence from a normal control (A) and patient 1 (B), showing the 3′ end of exon 5 and the 5′ end of IVS 5. Arrowheads indicate the G and T nucleotides at the +5 and +6 positions of the normal control and the homozygous CC substitution in patient 1. The cDNA sequence of portions of *CTNS* exons 4, 5, and 6 of a normal control (C) and patient 1 (D) illustrate defective splicing of exon 5 in patient 1.

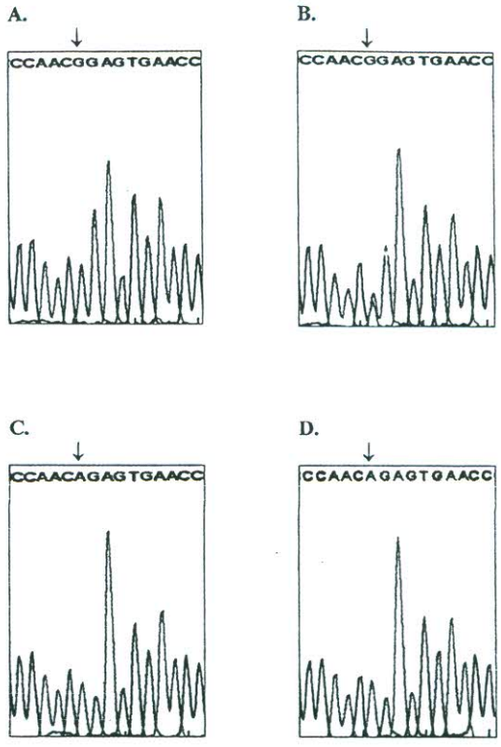

FIG. 3. Sequence analysis of *CTNS* exon 9 showing normal control genomic DNA (A), genomic DNA from the mother of patients 3 and 4 (B), and genomic DNA from patients 3 and 4 themselves (C, D). Arrowheads indicate the 928 position, showing a G in the control (A), both G and A in the heterozygous mother (B), and the mutant A in each hemizygous brother (C, D).

employed for *CTNS* cDNA amplification were 5′-CCTCTTCCAGTAACATTGAGG-3′ (in exon 2) and 5′-AGAAAAGAGATGGCGGTGTC-3′ (in the 3′-UTR), yielding a 1431-bp product. Direct, automated DNA sequencing in both directions was employed using the ABI Prism dRhodamine and Big Dye Terminator cycle sequencing kits (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Northern blot analysis was performed using methods previously described (22).

RESULTS

Patient 1 had a novel *CTNS* splicing mutation. Analysis of genomic DNA revealed a homozygous GT→CC substitution, i.e., IVS 5+5 GT→CC, of *CTNS*. The cDNA product exhibited skipping of exon 5 (Fig. 1). This out-of-frame splicing mutation resulted in the incorporation of six incorrect amino acids beginning with residue 48, followed by a stop codon at residue 54. Northern blot analysis indi-

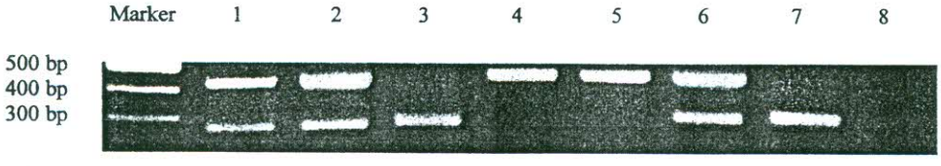

FIG. 2. Agarose gel electrophoresis of multiplex PCR products of patients 2, 3, and 4. The upper band (415-bp) reflects the presence of the 57-kb deletion; the lower band (266-bp) indicates the absence of the 57-kb deletion. Lane 1, patient 3 (heterozygous); lane 2, patient 4 (heterozygous); lane 3, mother of patients 3 and 4 (no deletion); lane 4, patient 2 (homozygous); lane 5, patient known to be homozygous for the 57-kb deletion; lane 6, patient known to be heterozygous for the 57-kb deletion; lane 7, control patient without cystinosis; lane 8, negative control (H_2O, no DNA).

TABLE 1
Known *CTNS* mutations in Cystinosis Patients with African Ancestry

Patient	Type of cystinosis	Allele 1	Allele 2	Amino acid change	Reference
1	Nephropathic	IVS 5+5, GT → CC	IVS 5+5, GT → CC	Skipped exon 5	This report
2	Nephropathic	57-kb deletion	57-kb deletion	Deleted exons 1–10	This report
3	Ocular	57-kb deletion	928 G → A	G197R	This report
4	Ocular	57-kb deletion	928 G → A	G197R	This report
5	Nephropathic	1209 C → G	1209 C → G	Y290X	#22

cated that the fibroblast *CTNS* mRNA of patient 1 had both decreased size and reduced amount compared to normal controls (data not shown).

Patient 2 was homozygous for the 57-kb deletion in *CTNS*, as illustrated by multiplex PCR amplification of his gene (Fig. 2). Patients 3 and 4 harbored the common 57-kb deletion on one allele and a different mutation on the other allele, since the mother showed no 57-kb deletion (Fig. 2). Paternal DNA was not available. In order to identify the second mutation, exons 3–12 of the remaining, nondeleted allele were amplified and sequenced in both directions. Genomic analysis revealed a heterozygous base change of 928G→A in exon 9 of *CTNS* in the mother and the same 928G→A substitution in the nondeleted allele of patients 3 and 4 (Fig. 3). This 928G→A substitution, which results in a G197R amino acid change, has been associated with a polymorphism, 843A→G, in two other patients with ocular cystinosis (28). Our patients 3 and 4, however, exhibited the normal, 928A-843A haplotype (data not shown).

DISCUSSION

With an incidence of one in 100–200,000, nephropathic cystinosis is rare in the general population. However, this diagnosis is even more unusual among African Americans. The few reports of cystinosis in this group (29,31–33,35) occurred prior to identification of the cystinosis gene and, therefore, lack molecular correlation. We describe the molecular defects in four African American patients with cystinosis, including two brothers with the extremely rare nonnephropathic or ocular variant.

Patient 2 was homozygous and patients 3 and 4 were heterozygous for the common 57-kb deletion of *CTNS*. This mutation appears to have arisen in Germany approximately 500–700 AD (22,23), with transmission to other regions via migration. It has been reported in two nephropathic cystinosis patients from North Africa, although neither the number of deleted alleles nor the ethnicity of the patients was mentioned (23). We speculate that the presence of this deletion among African American patients reflects the extensive admixture of Northern European and African gene pools that has taken place in North America over the past 300 years.

Patient 1, with classical nephropathic cystinosis, displayed homozygosity for a splicing mutation in *CTNS*, specifically, IVS 5+5 GT→CC (Fig. 1). *CTNS* splicing mutations have been described in all three variants of cystinosis, i.e., classic nephropathic cystinosis (20,22), the intermediate variant (34), and the ocular or nonnephropathic form (28). The mutation in patient 1 represents only the eighth splicing mutation reported among nephropathic cystinosis patients (26). Splicing mutations causing nephropathic cystinosis generally occur early in the *CTNS* coding region and generate premature stop codons that obliterate the putative C-terminal tyrosine-based lysosomal targeting signal. The anticipated null alleles are consistent with the severe phenotype in patient 1.

Patient 2 is a perplexing case, since he has been followed since early childhood on presumably adequate doses of cysteamine, but without significant cystine depletion, and with progression of renal disease. He is homozygous for the 57-kb deletion in *CTNS* (Fig. 2), eliminating the possibility that his lysosomal cystine accumulation is due to a single, non-*CTNS* defect impairing both cystinosin and the lysosomal lysine transporter. Cysteamine depletion of lysosomal cystine occurs via the lysine carrier (36), so a dysfunction of this membrane protein could have accounted for the patient's failure to show cystine depletion. Studies to determine other causes for the patient's failure to respond to cysteamine are ongoing.

Patients 3 and 4 represent the first African Amer-

ican patients known to have ocular or nonnephropathic cystinosis, which has been reported in only approximately 20 individuals worldwide (4). Ocular cystinosis patients have crystals in their corneas, conjunctiva, and bone marrow, but their only symptom is photophobia. It has recently been shown that patients with ocular cystinosis (28), as well as those with the intermediate variant (34), have one "severe" *CTNS* mutation (e.g., the 57-kb deletion or the nonsense W138X mutation) and one "mild" mutation. To date, the only mild mutations known are an IVS10-3C→G splicing mutation (28), an IVS11 + 2T→C splicing mutation (34), a 1308C→G (N323K) substitution (34), and the 928 G→A substitution (28). Remarkably, the 928G→A mutation, which affects an apparently noncritical amino acid outside of the seven transmembrane regions of cystinosin, occurs commonly in patients with ocular cystinosis. In our experience, 7 of 8 patients with ocular cystinosis possess this mutation, including a 38-year old Caucasian woman from California (28), two Caucasian cousins from Texas (28), two Caucasian brothers from Canada (unpublished data), and our two African American brothers. The diverse genetic backgrounds of these individuals suggests that this mutation has occurred repeatedly, indicating a mutation "hot spot," perhaps due to the CpG island located at nucleotide 928. This interpretation is supported by the haplotype of our African American patients, i.e., 928A-843A, which differs from that of ocular cystinosis patients previously reported, i.e., 928A-843G (28). Based upon the mild phenotype of patients hemizygous for 928G→A, we predict that homozygotes for this mutation would be asymptomatic.

Mutation analysis has been performed on one other African American patient, a 27-year-old with nephropathic disease. That individual was homozygous for a 1209C→G (Y290X) substitution (22).

The diagnosis of cystinosis should be suspected in African American children with typical clinical findings. Furthermore, the genotype/phenotype correlations found in the general cystinosis population hold for African American patients as well.

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