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Novel Cystatin B Mutation and Diagnostic PCR Assay in an Unverricht-Lundborg Progressive Myoclonus Epilepsy Patient

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Two mutations in the cystatin B gene, a 3′ splice mutation and a stop codon mutation, were previously found in patients with progressive myoclonus epilepsy of Unverricht-Lundborg type [Pennacchio et al. (1996): Science 271:1731–1734]. We present here a new mutation 2404ΔTC: a 2-bp deletion within the third exon of the cystatin B gene in an Unverricht-Lundborg patient. This mutation results in a frameshift and consequently premature termination of protein synthesis. Complete sequencing of the coding region and splice junctions of the cystatin B gene showed that neither of the two previously known mutations was present in this patient. The level of cystatin B mRNA in an immortalized cell line was found to be decreased, as had been reported for other Unverricht-Lundborg patients. The new mutation further supports the argument that defects in the cystatin B gene cause the Unverricht-Lundborg form of progressive myoclonus epilepsy. We describe a simple PCR method which can detect the 2404ΔTC deletion. This assay, together with previously described PCR assays for the other two known mutations, should prove useful in confirming clinically difficult diagnoses of Unverricht-Lundborg disease. Am. J. Med. Genet. 74:467–471, 1997.

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INTRODUCTION

Progressive myoclonus epilepsy (PME) of the Unverricht-Lundborg type (U-L) is an inherited autosomal recessive disorder (gene locus EPM1) characterized by stimulus-sensitive myoclonus, tonic-clonic seizures, and a progressive course beginning about age 6–15 years [Koskiniemi, 1974; Norio and Koskiniemi, 1979]. The disease is rare worldwide but more common in Baltic countries and Finland [Eldridge et al., 1983], affecting approximately 1 in 20,000 individuals in Finland. In the Mediterranean it is known as Mediterranean myoclonus (previously reported as a subgroup of Ramsay Hunt syndrome [Roger et al., 1968; Malafosse et al., 1992]). By genetic linkage analysis the putative EPM1 gene was mapped to a 0.3-cM region on human chromosome 21 [Lehesjoki et al., 1993]. Applying linkage disequilibrium and haplotype analysis to the Finnish population and searching for highly polymorphic markers using a contiguous array of cosmid, BAC, and P1 clones [Stone et al., 1996] narrowed the EPM1 locus to a 175-kb segment [Virtaneva et al., 1996]. Recently, the gene encoding cystatin B, a 98-amino acid cysteine protease inhibitor, was localized to this region, and two cystatin B point mutations in U-L patients were identified: a splice site mutation and a stop codon mutation [Pennacchio et al., 1996]. These nucleotide substitutions, 1925G → C and 2388C → T (GenBank accession no. U46692), are presumed to be responsible for decreased levels of cystatin B messenger RNA in affected individuals.

We describe here the complete sequencing of the cystatin B coding region and splice junctions of an U-L patient who has decreased cystatin B mRNA level but lacks the previously characterized mutations. This patient was found to be heterozygous for a new mutation 2404ΔTC, a two-base pair (bp) deletion in the third exon of the cystatin B gene. The mutation causes a translational frameshift and subsequent protein truncation after 74 amino acids.

We have developed a simple method for detecting the new 2404ΔTC deletion. Enzymatic amplification of genomic DNA with a PCR primer having two mismatched nucleotides and a nonmismatched primer yields a 204-

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TABLE I. Primers for PCR and Sequencing of Cystatin B Gene

Primer	Orientation	Sequence (5′→3′)	Position[a]
pF2	Forward	CTCCGACTGCCCCTTCCCTATC	18–39
51814R1	Reverse	GAGACACAGGGAAAGTTGCCATCT	1250–1227
F11	Forward	CCACCGTACCCAGCTGGAACTGT	1729–1751
R1	Reverse	CGGAGGATGACTTTGTCAGTCTTC	2536–2513
pr194	Forward	GTCCCTTCTTGCGGGGCCACC	244–264
Pr585	Reverse	CCCGAGCGGAGGGAGGCCTCT	635–615
pr38	Forward	GAAGTTCCCTGTGTTTAAGGCCGTG	1958–1982
pr231	Forward	TGTGAGGCATCCCCTGCACATG	2231–2252
pr44	Forward	GCAGCAAGGTGACTTGGGATCAGAGG	2303–2328
pr133	Reverse	GATAAGGTCAAGGGCTTGTTTTCCAGAGGG	2434–2405

[a]Numbering according to GenEMBL accession number U46692.

bp product. The mismatched primer generates an *Xcm*I endonuclease site only when the 2404ΔTC mutation is present, which can be detected by agarose gel electrophoresis and will be helpful for the development of a molecular diagnostic assay for progressive myoclonus epilepsy of Unverricht-Lundborg type.

MATERIALS AND METHODS

Subject

The patient studied here (EP6) is described in detail as U-L patient 3 in a 5-hydroxy-L-tryptophan treatment trial [Pranzatelli et al., 1995]. Clinical symptomatology and exclusion of other disorders are provided there. After obtaining consent from the patient, genomic DNA was isolated from peripheral blood using standard techniques [Baas et al., 1984]. The research reported here was approved by the University of Michigan Institutional Review Board.

Northern Blot Analysis

Total RNA was isolated from lymphoblastoid cell lines using TRIzol reagent (Gibco BRL, Grand Island, NY) based on the method of Chomczynski and Sacchi [1987]. Total RNA (34 μg) was denatured at 65°C for 10 min in 3-[N-morpholino]propanesulfonic acid (MOPS)/formamide/formaldehyde buffer and loaded on a 2% agarose/17.8% formaldehyde gel. After 15 hr of electrophoresis, RNA was transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and fixed by baking for 2 hr at 80°C. The blot was hybridized to a 0.8-kb ^{32}P-labeled cystatin B PCR fragment (primers F11 and R1; Table I) in ExpressHyb hybridization solution (Clontech, Palo Alto, CA), washed with 2 × SSC at room temperature for 40 min, then with 0.1 × SSC at 50°C twice for 20 min, and subjected to autoradiography. The membrane was stripped by incubation in sterile H_2O containing 0.5% SDS during 10 min at 90–100°C and rehybridized to a β-actin probe (Clontech, Palo Alto, CA) under similar conditions.

DNA Sequencing of PCR Products

The coding region and intron-exon junctions of cystatin B were PCR-amplified from genomic DNA with the primers pF2, 51814R1, F11, and R1 [Pennacchio et al., 1996] (Table I).

Amplification of the 5′ part of the gene (primers pF2 and 51814R1) was carried out in 50 μl with 1 × PC2 buffer (Ab Peptides, Saint Louis, MO), 250 μM dNTPs, 0.2 μM primers, 5% DMSO, 10 units KlenTaq1 (Ab Peptides), and 0.2 unit Pfu polymerase (Stratagene, La Jolla, CA) at 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 67°C for 1 min, and 4 min at 72°C, with a final incubation for 10 min at 72°C.

Amplification of the 3′ part of the gene (primers F11 and R1) was carried out using the same conditions, except that 1 × PC2 was replaced by 1 × PEK buffer [Ponce and Micol, 1992].

PCR products were subjected to automated sequencing in both directions with PCR primers and the internal primers pr194, pr585, pr38, and pr231 (Table I).

Diagnostic PCR Assay

A 204-bp DNA fragment containing the 2404ΔTC deletion was PCR-amplified from genomic DNA using primers pr231 and pr133 (Table I). Reaction volume was 25 μl with 1 × PEK buffer, 250 μM dNTPs, 0.2 μM primers, 5 units KlenTaq1, and 0.1 unit Pfu polymerase for 30 cycles of 94°C for 1 min, 67°C for 1 min, and 72°C for 2 min. PCR product (2.5 μl) was digested in a volume of 15 μl with *Xcm*I using the vendor's condi-

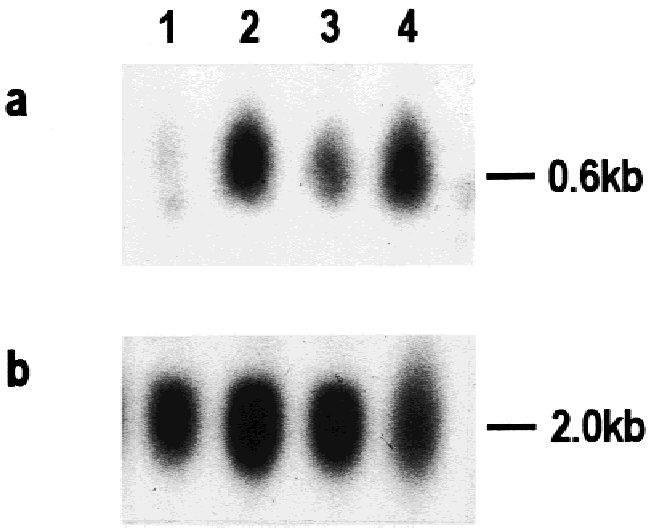

Fig. 1. Northern blot analysis of cystatin B total RNA in cell lines of one U-L affected (**lane 1**) and three U-L unaffected (**lanes 2–4**) individuals. **a:** Hybridization with 3′ end of cystatin B gene. U-L patient shows lower level of cystatin B expression than controls. **b:** Hybridization of the same blot with human β-actin probe. Size of the RNA transcripts is indicated.

tions (NEB, Beverly, MA). Digests were subjected to gel electrophoresis in 2.5% agarose and photographed.

RESULTS AND DISCUSSION

We examined the level of cystatin B in the immortalized lymphoblastoid cells derived from U-L patient EP6 and from 3 unaffected individuals. The 3′ end of the cystatin B gene and the β-actin control probe were used to probe total RNA on a Northern blot (Fig. 1). Hybridization with cystatin B revealed a 0.6-kb transcript corresponding to the fully processed transcript of cystatin B [Ritonja et al., 1985]. The cystatin B mRNA level of patient EP6 was markedly reduced compared to the control samples (Fig. 1a), consistent with previous observations that U-L patients have decreased cystatin B mRNA levels [Pennacchio et al., 1996]. Probing for β-actin confirmed that similar amounts of RNA were present in all lanes (Fig. 1b). Reduced mRNA level for cystatin B can have three causes: a defect in the promoter region for the cystatin B gene, a mutation causing coding or splicing abnormalities which reduce the amount or stability of mRNA, or a large deletion or insertion, which is unlikely to be present in the region of the cystatin B gene between nucleotides 1651–2822, since no alterations were detected by Southern blot analysis of *Bfa*I genomic DNA digests hybridized to a PCR fragment amplified with primers F11 and R1 (data not shown). The former two cases have been observed: the stop and splice mutations were shown to reduce the mRNA amount, but patients of Finnish origin and haplotype, in which no coding or splice mutations were found, also showed reduced mRNA amounts [Pennacchio et al., 1996] presumed to have regulatory mutations, for instance in the promoter.

For cystatin B mutation analysis of patient EP6 we screened the coding sequence of the gene and intron-exon splice junctions using PCR combined with automated sequencing. For sequencing of PCR products, previously published and newly synthesized internal oligonucleotide primers were used (Table I). Sequence comparison did not reveal any of the previously described mutations in this patient: G-to-C transversion at the last nucleotide of intron 1 and change CGA to TGA, generating a translation stop codon at amino acid position 68 [Pennacchio et al., 1996]. Sequence comparison did, however, identify a new mutation, 2404ΔTC, in the cystatin B gene of patient EP6: a deletion of two nucleotides in exon 3 (Fig. 2A). This mutation, 2404ΔTC, causes a frameshift and a truncated

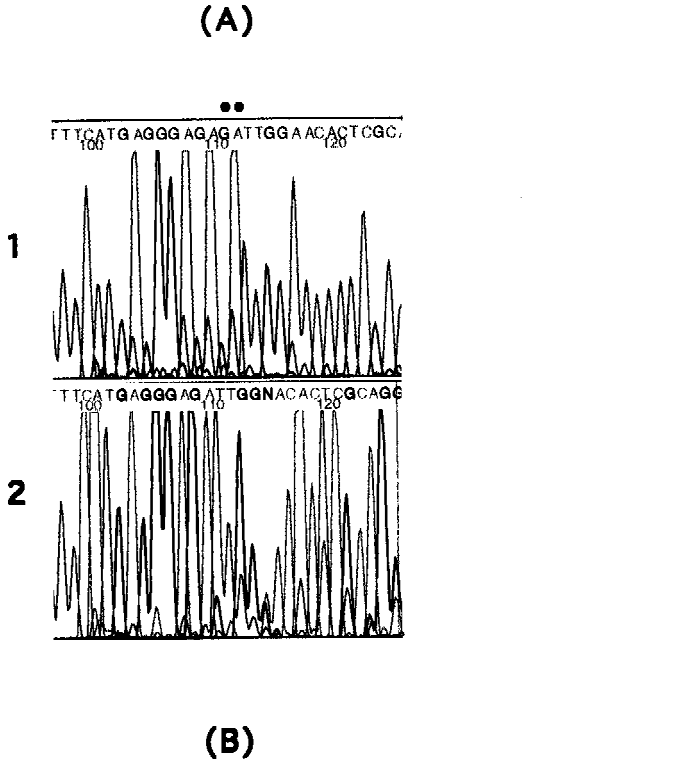

Fig. 2. **A:** Detection of a two-base pair (bp) deletion 2404ΔTC in the coding region of the cystatin B gene. Sections of chromatograms show the nucleotide sequences of cystatin B (position 2414–2385, reverse) of a control (1) and of patient EP6 (2). Nucleotides designated by points in the sequence of the unaffected person are deleted in Ep6. The heterozygous deletion causes the sequences of the two alleles to be staggered by 2 bp in the chromatogram. **B:** Comparison of the predicted protein sequences of exon 3 in control (1) and mutant (2) alleles. Asterisks show positions of stop codons.

74-amino acid protein by subsequent termination of protein synthesis at the new codon TGA, 24 amino acids before the normal stop codon (Fig. 2B). No other alterations were detected in the coding region and splice junctions in the patient cystatin B gene of patient EP6.

Patient EP6 was found to be heterozygous for the mutation 2404ΔTC: sequence analysis of the PCR products demonstrated a normal sequence in addition to a 2-bp-shorter product (Fig. 2A). The other allele had no mutation in the gene regions analyzed, and thus may represent a mutation in parts of the promoter that have not yet been cloned.

Since Unverricht-Lundborg disease is usually diagnosed by clinical criteria in conjunction with exclusion of similar disorders [Pranzatelli et al., 1995], molecular diagnosis might be useful in the future. The earlier-described 3′ splice site mutation destroys the recognition site for the restriction enzyme *Bfa*I and the stop codon mutation creates a recognition site for *Bsa*AI, providing a simple test for screening affected individuals [Pennacchio et al., 1996]. To establish a simple method for detecting the mutation 2404ΔTC in the coding region of suspected EPM1 patients, a PCR assay was developed using primers surrounding the region of deletion (primers 231 and 133, Table I). Since a commercial enzyme for direct detection of the 2404ΔTC mutation was not found, primers were designed in such a way that a restriction site is created in PCR fragments of affected individuals. Primer 133 contains two internal mismatches: substitution of two nucleotides on positions 2410A → T and 2411T →G for generation of a restriction site for the endonuclease *Xcm*I only in mutant alleles (Fig. 3A). When an allele with the 2404ΔTC mutation is amplified, the product is cleavable with *Xcm*I, in contrast to normal alleles. DNAs from patient EP6 and 3 unaffected individuals were assayed. All PCRs resulted in the expected 204-bp fragment (data not shown). After digestion with *Xcm*I, only patient EP6 showed an additional band of 175 bp, indicating heterozygosity for the 2404ΔTC mutation (Fig. 3B).

2404ΔTC is the third mutation in the cystatin B gene identified to date. Haplotype analysis of specific populations [Lehesjoki et al., 1992; Malafosse et al., 1992; Pennacchio et al., 1996] suggests that there will be only a limited number of deleterious mutations in the majority of U-L patients. Therefore, it may be hoped that molecular diagnosis by simple PCR may be possible in the future.

Since submission of this paper, the mutation described here has also been found by Lalioti et al. [1997]

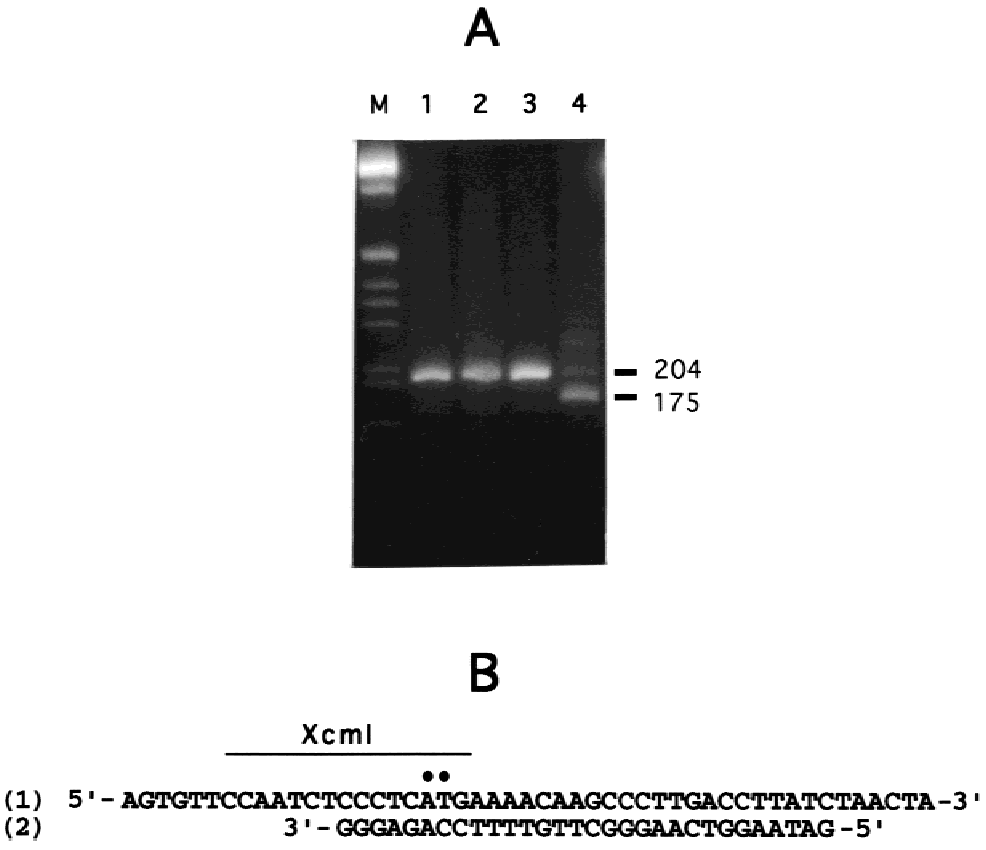

Fig. 3. Screening assay for the 2404ΔTC mutation by PCR followed by restriction digest. **A:** Genomic DNA from unaffected **(lanes 1–3)** and affected **(lane 4)** individuals was used for PCR amplification with primers 231 and 133, followed by restriction digest with *Xcm*I. The PCR product of EP6 (lane 4) could be digested partially with *Xcm*I (175-bp product) in contrast to control samples (lanes 1–3) in which the 204-bp product remained undigested. The additional bands of apparent higher molecular weight in lane 4 are likely the result of heteroduplex formation between the two alleles. Size marker **(lane M):** 1-kb DNA ladder. **B:** Creation of the *Xcm*I restriction site with mismatched primer: (1) genomic sequence, (2) primer 133 sequence. Asterisks show mismatched nucleotides in the template.

and Lafreniere et al. [1997]. Neither study describes a convenient PCR assay for this mutation. Lafreniere et al. [1997] have shown an increased expansion in the 5′ end of the gene detectable by Southern blot analysis in the majority of patients, and we could now show that the second mutation in this patient also has this expansion.

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