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RESEARCH ARTICLE

Ornithine Transcarbamylase Deficiency: Characterization of Gene Mutations and Polymorphisms

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We identified three new and three known mutations in male patients with OTC deficiency using PCR amplification of all the individual exons, including the adjacent intron sequences, followed by direct sequencing of the amplimers. Two mutations were found in males presenting with neonatal fatal hyperammonemia and no detectable enzyme activity in their livers. The H302Y mutation found in one patient affects the putative binding site for ornithine. The second patient had an R141X mutation, which is one of the few recurrent mutations in the OTC gene. Four different missense mutations were identified in male patients with late onset of the disease and residual OTC activities between 14% and 35%. The mutations are Y176C and P220A and the known mutations K88N and T343K, respectively. Four of the patients' mothers were identified as carriers. In two cases, the mutations had occurred spontaneously. In addition, the frequency of four polymorphisms of the OTC gene was studied. The K46R polymorphism in exon 2 and the Q270R polymorphism in exon 8 were found in 36% and 4% of screened alleles, respectively. Two questionable polymorphisms in exon 4, F101L and L111P, were not present in any of the screened alleles. © 1996 Wiley-Liss, Inc.

KEY WORDS: OTC deficiency, PCR amplification, Polymorphisms

INTRODUCTION

Ornithine transcarbamylase (OTC; ornithine carbamylphosphate transferase, EC 2.1.3.3) is a mitochondrial matrix enzyme that catalyzes the condensation of ornithine and carbamyl phosphate to citrulline in the second step of the urea cycle. The OTC gene is located on the short arm of the X chromosome (Xp21.1). It contains 10 exons coding for a precursor protein of 354 amino acids. A leader sequence consisting of 32 amino acids is cleaved off after transport of the protein into the mitochondria.

OTC deficiency is the most common inborn error of the urea cycle. Affected boys typically present during the neonatal period with hyperammonemic coma and death. Males with partial enzyme activity and heterozygous females present with late onset of symptoms and milder phenotypes resulting in episodic metabolic crises, behavioral abnormalities or mental retardation. These patients, however, are at risk to develop fatal hyperammonemia induced by catabolic stress, infections, and high protein intake.

OTC deficiency reflects a wide variety of genetic defects. Except for a few recurrent mutations (Tuchman, 1993; Tuchman et al., 1994, Matsuura et al., 1993, 1994a; Gilbert-Dussardier et al., 1994; Oppliger Leibundgut et al., 1995), the majority of mutations occurs in only one family. Therefore, it is important to identify the "private" molecular defect in each patient. This knowledge may then be used for carrier detection and prenatal diagnosis in affected families. This paper reports three previously unknown and three known mutations identified in six unrelated male patients. Several known mutations of the OTC gene have been reported that do not affect enzymatic activity, although they do cause amino acid substitu-

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tions, producing a normal phenotype. In this paper, we studied the frequency of two common polymorphisms in exon 2, K46R (Grompe et al., 1989; Petty et al., 1991), and in exon 8, Q270R (Tuchman et al., 1992, 1994, Oppliger Leibundgut et al., 1995), respectively. Two additional differences in the OTC sequences of Horwich et al. (1984) and Hata et al. (1988), F101L and L111P, were studied by determination of the allele frequency. The substitution of leucine for phenylalanine in codon 101 had been proposed to be a polymorphism by Hata et al. (1988). The proline instead for leucine in codon 111 in exon 4 originally found in the cDNA by Horwich et al. (1984) was later identified in a patient with very low OTC activity by Grompe et al. (1989), a fact that increases the likelihood for this exchange to be a deleterious mutation, rather than an innocent polymorphism.

PATIENTS

Patient 14, a 7-year-old boy, experienced repeated episodes of lethargy during the first 2 years of life. During one crisis, his blood ammonia level was 200 μmol/L, with undetectable citrulline. Urinary orotic acid was 242 μmol/mmol creatinine. OTC activity in a liver biopsy was 14% of the lower reference limit.

Patient 19 had developed several episodes of protracted vomiting in the first years of life. At the age of 2½ years, he was admitted to the hospital in a hyperammonemic coma. His blood ammonia was 1176 μmol/L, decreasing to normal within 12 hr after hemodialysis was instituted. Despite administration of sodium benzoate and arginine supplementation, the patient died of cardiac insufficiency. Elevated levels of orotic acid were found in his urine. Residual OTC activity in his liver was 35%. Family history revealed that four siblings of the patient's mother had died in early childhood of unknown cause. The patient's mother, who is totally asymptomatic, showed slightly elevated blood ammonia levels and high excretion of orotic acid under protein loading, suggesting that she is a carrier. A younger brother of the patient presented with several episodes of protracted vomiting and mildly elevated blood ammonia. Under protein loading, he developed hyperammonemia and orotic aciduria, while his blood citrulline was low. He now follows a low-protein diet with citrulline substitution.

Patient 36 was healthy until the age of 2½ years when he became comatose and was admitted to the hospital with hyperammonemia and orotic aciduria. Despite therapy, the patient died 6 days later. Residual OTC activity in the patient's liver was 19%.

Patient 53 was healthy until the age of 4 years, when he began to experience episodic vomiting in association with lethargy and hyperammonemia. At 7 years of age, he was admitted to the hospital with sleepiness and protracted vomiting. His blood ammonia level was 215 μmol/L, and urinary orotic acid was 276 μmol/mmol creatinine. Aminotransferases were elevated. After administration of arginine all parameters were normalized. OTC activity in a liver biopsy was 23% of the lower reference limit. Family history revealed that an elder brother had died in a coma with endocrine hypertension at the age of 12 years, after a first crisis with protracted vomiting at the age of 9 years.

Patient 55 became irritable and then comatose on the second day of life. His blood ammonia level rose to 780 μmol/L. A metabolic acidosis was corrected by administration of bicarbonate and he required mechanical respiration. Despite protein restriction and arginine supplementation, the patient died on the 6th day of life. No OTC activity was detectable in the liver. The patient's asymptomatic mother had slightly elevated blood ammonia, suggesting carrier status.

Patient 63, the first child of healthy parents, presented with lethargy and poor feeding at the first day of life. Marked hyperammonemia (664 μmol/L) and high excretion of urinary orotic acid led to the diagnosis of OTC deficiency. Plasma alanine and glutamine levels were extremely elevated. OTC activity in the liver was undetectable. Despite a low-protein diet (1 g/kg body weight), the patient was in very poor condition and died in coma after 1 month. The mother's pedigree up to the fifth generation was without a history of abortion or male deaths. Nevertheless, a positive allopurinol loading test identified her as a carrier.

METHODS

Isolation of genomic DNA from blood leukocytes or cultured skin fibroblasts and polymerase chain reaction (PCR) amplification of all 10 exons of the OTC gene, including exon/intron boundaries were performed as described before (Oppliger Leibundgut et al., 1995), with the exception that one primer of each pair was biotinylated.

After binding of the PCR product to streptavidin-coated magnetic beads (Dynal, Oslo, Norway) the DNA was denatured and the single stranded DNA was sequenced using the Sequenase kit version 2.0 (US Biochemicals, Cleveland, OH).

Single-strand conformational polymorphism (SSCP) analysis of the amplified DNA fragments was modified and optimized for gel compositions (12% polyacrylamide gel, 7% glycerol), electrophoresis conditions (90', 350 V) and staining procedures (silver staining, 30'), so that 97% of known mutations in all 10 exons were detected.

RESULTS

Direct Sequencing

PCR amplification of all 10 exons of the OTC gene, including the intron/exon boundaries followed by direct sequencing, revealed five different mutations in five unrelated male patients with diagnosed OTC deficiency. Figure 1 shows the sequences around the mutation site of each patient and family members. These were the only changes found in the coding region of the OTC gene of these five patients. An A-to-T base exchange in exon 3, leading to a substitution of lysine by asparagine in codon 88, was identified in patient 53, as well as in his heterozygous mother. The same mutation has previously been described by Reish et al. (1993). An A-to-G transition in exon 5 was detected in patient 36, resulting in a substitution of cysteine for tyrosine in codon 176. Analysis of the mother's DNA showed that she was heterozygous. In patient 19, a C-to-G substitution in exon 6 was identified changing the code for proline to alanine at codon 220. This mutation was also present in the patient's affected brother as well as in the heterozygous asymptomatic mother and sister. A C-to-T base exchange in exon 9 was identified in patient 55, resulting in the replacement of histidine by tyrosine in codon 302. The patient's mother could be excluded as a carrier. In patient 14, a change of C to A in exon 10 resulted in the substitution of lysine for threonine in codon 343, a mutation that had been recently reported by Tuchman and Plante (1995). The mutation was not present in his mother, indicating that it had occurred spontaneously.

SSCP Analysis

The full gene was not sequenced in patient 63, but all 10 exons were screened for single-strand variants. Exon 5 showed an altered SSCP banding pattern (Fig. 2), and subsequent sequencing revealed a C-to-T transition changing arginine at position 141 into a stop codon. The SSCP pattern of the patient's mother indicated that she was heterozygous for the mutation.

Polymorphisms

Two known polymorphisms in the OTC gene were studied by sequence analysis of the respective exon in unrelated male and female probands with suspected or proven OTC deficiency. Screening for the K46R polymorphism in exon 2 yielded 45 of 70 alleles (64,3%) containing the AAA codon for lysine and 25 alleles (35,7%) with the AGA codon for arginine (Table 1). In exon 8, the Q270R polymorphism was detected in three out of 71 alleles, resulting in an overall allele frequency of 4,2%. No linkage disequilibrium was found between the polymorphic alleles and the disease. Furthermore, we screened for two possible polymorphisms in exon 4. Neither the A-to-T substitution in codon 101 changing phenylalanine to leucine nor the substitution of leucine by proline in codon 111 could be detected in 67 alleles.

DISCUSSION

Three new and three known mutations were identified in the OTC gene of six unrelated boys (Table 2). Two of these mutations were found in patients with neonatal onset of the disease and fatal outcome. In both patients, no OTC activity was detectable in a liver sample. The C-to-T mutation identified in patient 63 changes the highly conserved arginine 141 to a stop codon. This nonsense mutation represents one of the few recurrent mutations in the OTC gene. It causes neonatal onset of disease in males and was associated with very low residual activity in a female patient (Maddalena et al., 1988; Hata et al., 1989; Grompe et al., 1991) A different mutation in the same codon, R141Q, was described in several patients with neonatal hyperammonemia (Maddalena et al., 1988; Suess et al., 1992; Matsuura et al., 1994b).

The H302Y mutation in exon 9 of patient 55 is likely to be deleterious because it changes the putative ornithine binding site (Horwich et al., 1984), a sequence that is highly conserved across species (Kraus et al., 1985; Huygen et al., 1987; Veres et al., 1987) from Phe-Leu-*His*-Cys-Leu-Pro to Phe-Leu-*Tyr*-Cys-Leu-Pro. Another mutation, L304F, affecting this catalytic domain was reported previously by Tuchman et al. (1992) and Hoshide et al. (1993). Interestingly, this latter mutation caused only mild symptoms and late onset deficiency, but was associated with very low residual enzyme activity in a male patient (Tuchman et al., 1992). The replacement of the basic amino acid residue by a neutral one may explain the more severe phenotype in our patient.

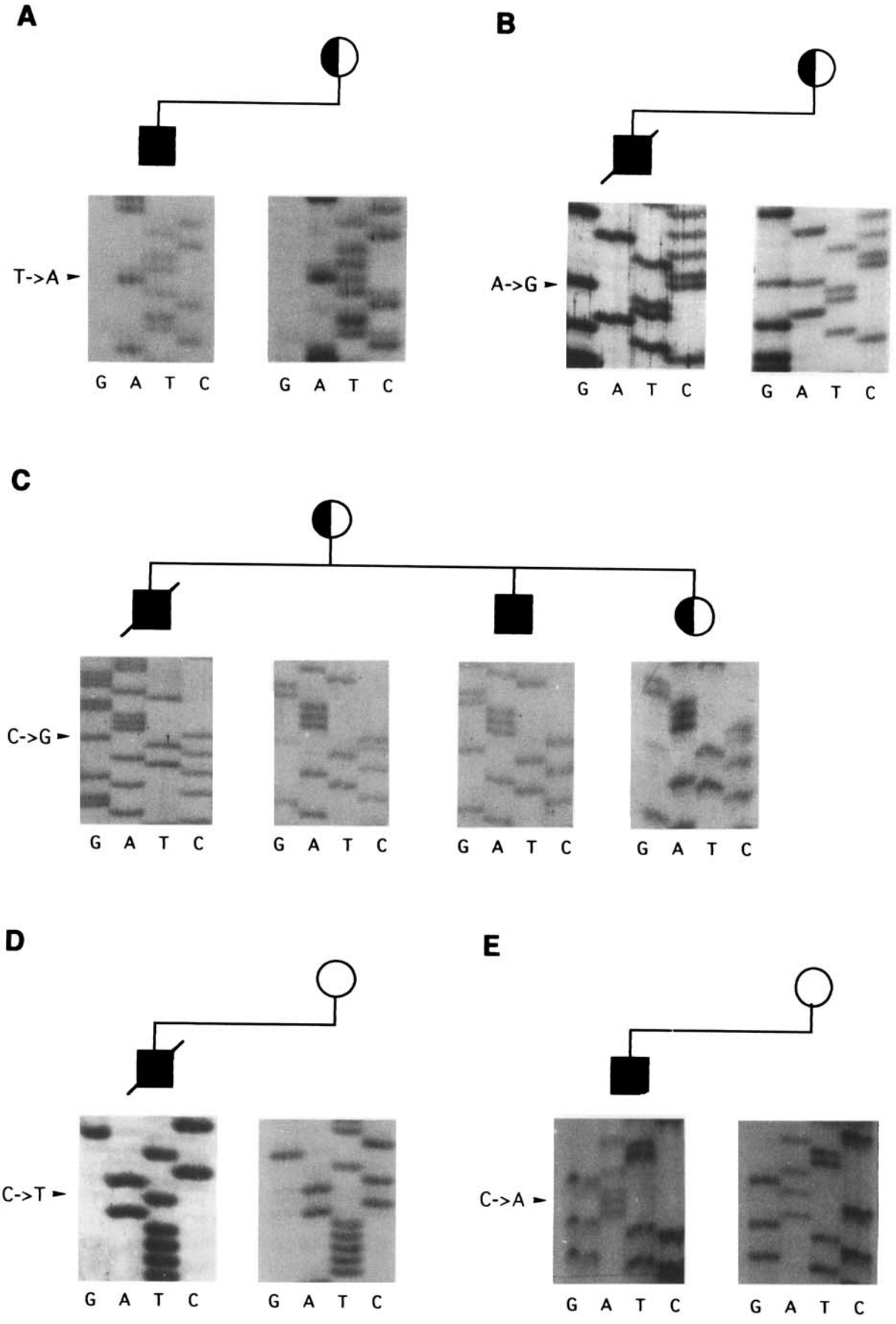

FIGURE 1. Sequences of PCR-amplified DNA around the mutation sites of five male patients and family members. *Arrow*, mutations. A: T-to-A substitution (antisense) at nucleotide 264 in exon 3 detected in patient 53 and his mother. B: A-to-G mutation at nucleotide 527 in exon 5 detected in patient 36 and his mother. C: C-to-G exchange at nucleotide 658 in exon 6 detected in patient 19, his affected brother, and in his mother and sister. D: C-to-T substitution at nucleotide 904 in exon 9 detected in patient 55, but not in his mother. E: C-to-A mutation at nucleotide 1028 in exon 10 detected in patient 14, but not in his mother.

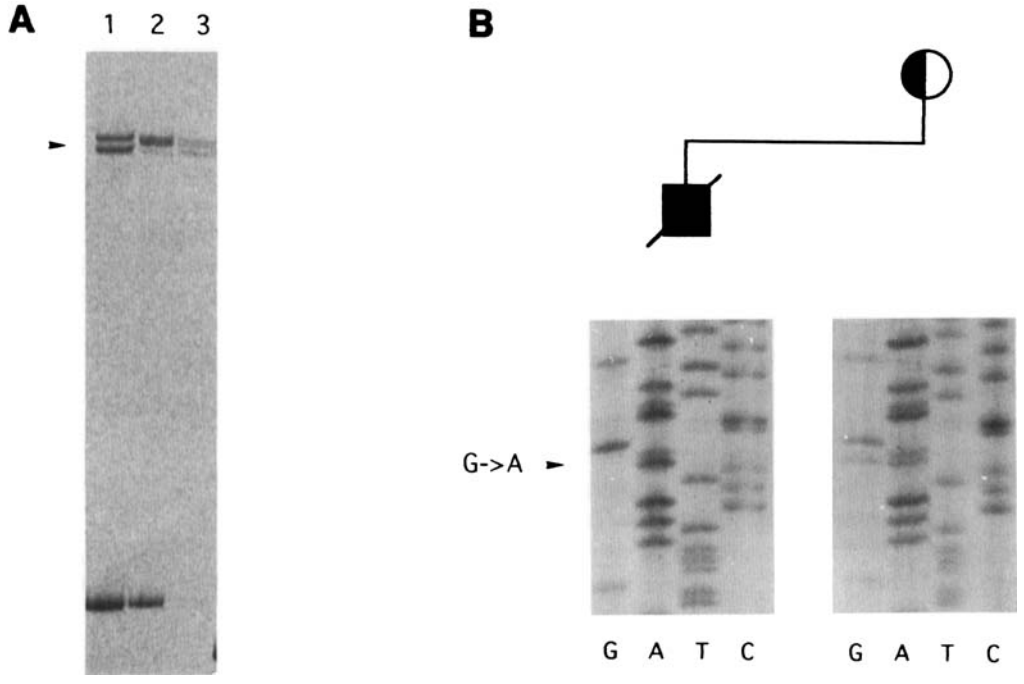

FIGURE 2. **A:** Single-strand conformational polymorphism (SSCP) shows an abnormal migration pattern of amplified exon 5 of patient 63 (*lane 2*) and his heterozygous mother (*lane 3*). *Lane 1*, normal pattern of control DNA. The bands at the bottom of the gel represent double-stranded DNA. **B:** Partial sequence of exon 5 from patient 63 and his mother. The G-to-A mutation (antisense) at nucleotide 421 was detected in the patient, as well as in his mother.

TABLE 1. Polymorphisms in the Human OTC Gene and Gene Frequencies

Exon	Codon	Codon sequence by Horwich et al (1984)	Frequency	Codon sequence by Hata et al (1988)	Frequency	No. of alleles
2	46	AAA (Lys)	0.643	AGA (Arg)	0.357	70
4	101	TTT (Phe)	1.000	TTA (Leu)	0.000	67
4	111	CCT (Pro)	0.000	CTT (Leu)	1.000	67
8	270	CGA (Arg)	0.042	CAA (Gln)	0.958	71

TABLE 2. Clinical Data and Characterization of Gene Mutations of Six Male Patients With OTC Deficiency

Patient	Exon	Codon	Mutation	Amino acid substitution	OTC activity in liver (%)	Onset of symptoms
53	3	88	AAA>AAT	Lys>Asn	23	Late
36	5	176	TAC>TGC	Tyr>Cys	19	Late
63	5	141	CGA>TGA	Arg>stop	0	Neonatal
19	6	220	CCA>GCA	Pro>Ala	35	Late
55	9	302	CAC>TAC	His>Tyr	0	Neonatal
14	10	343	ACA>AAA	Thr>Lys	14	Late

Four different point mutations were found in four patients with late onset disease and relatively high residual OTC activities ranging from 14% to 35% of the lower reference limit. The K88N mutation in exon 3 found in patient 53 was previously described by Reish et al. (1993). The residual OTC activity of 23% and the mild clinical course in our patient confirm the finding of Reish et al. (1993) that the conversion of the highly conserved lysine into asparagine, although in close proximity to the putative carbamyl phosphate binding site (Horwich et al., 1984), causes only partial OTC

activity associated with relatively mild disease and favorable outcome.

In patient 36, the substitution of cysteine for tyrosine in codon 176 affects a residue that is conserved in the rat, but not in yeast and bacteria (Huygen et al., 1987). In the absence of information of the three-dimensional structure of the OTC protein, the effect of this mutation on enzymatic activity is difficult to explain.

The P220A mutation found in patient 19 affects a proline residue that is highly conserved across species. Nevertheless, the residual enzymatic activity of 35% of the lower reference value indicates that the proline is not absolutely essential for a functional enzyme. Despite residual enzymatic activities of 19% and 35%, respectively, patients 36 and 19 died at 2½ years of age, while an affected brother of one of the patients is doing well on a protein-restricted diet. These cases confirm the well-known variability of symptoms and poor correlation of enzymatic activity in vitro with the clinical course of the disease.

The substitution of lysine for threonine in codon 343 in exon 10, identified in patient 14, had recently been published by Tuchman and Plante (1995). This amino acid exchange introduces an additional positive charge and may affect the proper folding of the peptide chain although the substantial residual enzyme activity found in our patient suggests that this part of the OTC protein tolerates certain changes. Carrier detection in all six families revealed that four of the patient's mothers as well as one sister and an affected brother carried a mutant allele. In families 14 and 55, the patient's mother could be excluded as a carrier. Thus, these two mutations seem to have occurred spontaneously in the mother's germ cells.

In addition to the many mutations associated with reduced enzymatic activity, several mutations have been identified that apparently do not affect the enzyme activity. We could confirm two polymorphisms in exon 2, K46R, and in exon 8, Q270R, respectively. We found the K46R polymorphism in 36% of 70 alleles in good agreement with 32% in 56 chromosomes reported by Petty et al. (1991). The Q270R polymorphism was present in 4% of the alleles, again in good agreement with previous findings (Tuchman et al., 1994). Two additional differences in the OTC sequences reported by Horwich et al. (1984) and Hata et al. (1988), respectively, were originally observed. In this study we could detect neither the substitution of leucine for phenylalanine at codon 101 nor the substitution of proline for leucine at codon 111. The absence of leucine 101 in the cDNA sequence of Hata et al. (1988) in any of the screened alleles suggests the presence of a sequencing error rather than a polymorphism. The proline at codon 111, originally found in the cDNA by Horwich et al. (1984), was later identified in a patient with very low OTC activity by Grompe et al. (1989). It remains to be defined by further studies whether this substitution represents an extremely rare innocent polymorphism or a deleterious mutation.

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