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Mutation Analysis of the Ornithine Transcarbamylase (OTC) Gene in Five Japanese OTC Deficiency Patients Revealed Two Known and Three Novel Mutations Including a Deep Intronic Mutation

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Ornithine transcarbamylase (OTC) deficiency is the most common inborn error of the urea cycle. Although a combination of molecular methods have been used including DNA sequencing of all 10 exons and exon-intron boundaries of OTC gene, only ~80% of patients with OTC deficiency are found to have mutations. We report two known and three novel mutations of the OTC gene in five Japanese patients including two neonatal-onset, one late-onset, and two symptomatic female patients. Known nonsense mutations (c.578G>A and c.421C>T) were detected in a neonatal-onset male and a symptomatic female patient, respectively. Mutation analysis revealed two novel mutations including one splice site mutation (c.386+1G>C) in a symptomatic female patient and one missense mutation (c.515T>A) in a late-onset male patient. In the remaining case, which was a neonatal-onset male patient, no mutation was disclosed by direct sequencing of all 10 exons and their flanking intron sequences. Therefore, OTC mRNA in the liver was analyzed by RT-PCR, and remarkably, a 135-nt insertion was detected between exons 5 and 6. Genomic DNA analysis of intron sequences revealed a single nucleotide change at 265 bp downstream from the 3' end of exon 5, which created the novel splice acceptor site. Thereby, a 135-nt exon was created from the central part of an intron sequence. This is the first report of mutation deep in the intronic sequence in the OTC gene. Molecular analysis using genomic DNA and mRNA will increase the mutation detection ratio in the OTC gene.

Ornithine transcarbamylase (OTC) deficiency (OTCD; MIM#311250) is the most common inborn error of the urea cycle. OTC catalyzes the formation of citrulline from carbamyl phosphate and ornithine. The human OTC gene is located on the short arm of the X chromosome within band Xp21.1 [22]. The gene spans 73kb with an open reading frame of 1,062 nucleotides and contains 10 exons [13,15]. The OTC gene is expressed exclusively in the liver and small intestinal mucosa. It encodes a precursor OTC protein containing 354 amino acids and the amino terminus contains a leader sequence of 32 amino acids which is cleaved in two steps upon incorporation into the mitochondrial matrix [16].

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The phenotype of OTC deficiency is extremely heterogenous [28]. In most affected hemizygotic males, OTC deficiency manifests as hyperammonemic coma in the newborn period, whereas some develop late-onset manifestations. Females who are heterozygous for a mutant OTC allele as a consequence of skewed lyonization may have clinical symptoms similar to those of less seriously affected males.

Since the cloning of the human OTC gene, more than 341 mutations have been identified, which are largely private mutations [48]. Mutation analysis of the OTC gene has usually included Southern blotting for screening of deletion and single strand conformational polymorphism (SSCP) and/or DNA sequencing of all 10 exons and their flanking intron regions for small mutations. Nevertheless, only ~80% of patients with enzymatically proven OTC deficiency are found to have mutations in the OTC gene [48].

In this study, we report the mutation analysis of five Japanese patients with OTC deficiency, including two neonatal-onset male, one late-onset male, and two symptomatic female patients. Conventional direct DNA sequencing of all 10 exons and their flanking intron regions revealed the responsible mutations in four of five cases, whereas no mutation was disclosed in the remaining case. Remarkably, analysis of OTC mRNA in the liver revealed the incorporation of the central part of an intron sequence into mRNA, which was due to a deep intron mutation that created a new splice acceptor site. Among the five mutations reported in this study, three mutations were novel. Furthermore, this is the first report of a mutation deep in the intron sequence of the OTC gene.

PATIENTS AND METHODS

Patients

Case 1. The patient was a 24-year-old Japanese male. On the second day of life, he became lethargic and then comatose. His blood ammonia rose rapidly to >3000 μg/dl. Blood ammonia level was decreased by peritoneal dialysis and clinical symptoms were improved, but his neurological damage was severe. His OTC enzyme activity in a liver biopsy specimen was ~1% of the control. He was diagnosed with neonatal-onset OTC deficiency.

Case 2. The patient was a Japanese male infant who was born to healthy parents. On the first day of life, he experienced convulsion and lethargy, and become comatose, then was referred to Kobe University Hospital, Japan. His blood ammonia rose rapidly to >400 μg/dl. Clinical response to repeated peritoneal dialysis was good, but the neurological damage was severe. Urinary orotic acid was elevated (140 μg/mg creatinine), and as was serum glutamine (5987 nmol/l), but citrulline was undetectable. His OTC enzyme activity in liver biopsy specimen was undetectable. He was diagnosed with neonatal-onset OTC deficiency. He died at 1 year of age.

Case 3. The patient was an 8-year-old Japanese girl. She developed normally until the age of 5 years when she became drowsy with hyperammonemia (311 μg/dl). Hyperammonemia was rapidly improved by glucose and arginine transfusion. Urinary orotic acid and uracil were elevated (230 and 75 μg/mg creatinine, respectively), as was serum glutamine (1674 nmol/l), but arginine was decreased (44 nmol/l). She was diagnosed with symptomatic female OTC deficiency.

Case 4. The patient was a 6-year-old Japanese girl. She developed normally until the age of 1 year when she became drowsy with hyperammonemia (598 μg/dl). Hyperammonemia was rapidly improved by glucose transfusion. Urinary orotic acid and uracil were elevated (83 and 41 μg/mg creatinine, respectively), as was serum glutamine

(848 nmol/l), but arginine was decreased (23 nmol/l). She was diagnosed with symptomatic female OTC deficiency.

Case 5. The patient was a 3-year-old Japanese boy. He developed normally until the age of 10 months, when he experienced repeat vomiting with hyperammonemia (194 μg/dl). Hyperammonemia was rapidly improved by glucose transfusion. Urinary orotic acid was elevated (15μg/mg creatinine), as was serum glutamine (930 nmol/l), but arginine was decreased (28 nmol/l) and citrulline was low normal (18 nmol/l). He was diagnosed with late-onset OTC deficiency.

Analysis of OTC genomic DNA

Genomic DNA was isolated from lymphocytes obtained from these OTC deficiency patients using a previously described method [23]. To examine the small mutations in the coding region of the OTC gene, all 10 exons and their flanking intron regions were amplified using PCR, and the nucleotide sequences of the amplified products were determined (Mitsubishi Kagaku BCL Co., Tokyo, Japan) as previously reported [25].

To determine the intron 5 sequence of case 2, PCR was performed using primers OTCex5F and OTCint5R, and primers OTCint5F and OTCex6R (Table 1, Fig. 3). The amplified products were subcloned into the pT7 vector (Novagen Inc., Madison, WI, USA) and the inserted DNA was sequenced using an automated DNA sequencer (Model 373A; Perkin-Elmer Applied Biosystems Inc., Norwalk, CT, USA).

Analysis of OTC transcripts

Total RNA was isolated from patient and control livers obtained at biopsy using the method of Chomczynski et al [6], and cDNA was prepared as previously described [39]. Full-length OTC cDNA was amplified as two separate fragments using primers OTCex1F and OTCex6R, and primers OTCex5F and OTCex10R (Table 1). The amplified products were subcloned into the pT7 vector, and the inserted DNA was sequenced using an automated DNA sequencer.

Table 1. Primer sequences.

Locatin	Forward primer	Reverse primer
exon 1	OTCex1F: GGCTAACTTGCTGTGGAGTT	
exon 5	OTCex5F: ACCATCCTATCCAGATCCTG	
intron 5	OTCint5F: GCATACAAAATGGACAAGCC	
intron 5		OTCint5R: CAGACCTGATGTCTATAGAA
exon 6		OTCex6R: GCAGGATATTGTTCCCATCC
exon 10		OTCex10R: CATACCACGTGTTAGGGATT

RESULTS

Identification of mutations in the OTC gene of cases 1, 3, 4 and 5

To detect the small mutations including nonsense, missense and splice site mutations, and deletions or insertions of a few nucleotides, all 10 exons and their flanking intron regions were amplified using PCR and the nucleotide sequences of the amplified products were determined.

In case 1 (neonatal-onset male), nucleotide sequences of the amplified region encompassing exon 6 revealed a single nucleotide substitution at 38 bp downstream from the 5' end of exon 6 (c.578G>A) (Fig. 1a, b). The nucleotide change converted a TGG codon, which encodes tryptophan at the 193 amino acid position, to a stop TAG codon (p.193W>X).

This nonsense mutation has been reported in a previous case of neonatal-onset OTC deficiency [34], which is consistent with our case. In case 3 (symptomatic female), a heterozygous nonsense mutation (c.421C>C/T) was detected in exon 5 after direct sequencing (Fig. 1c, d), which has been reported in a male patient of neonatal-onset OTC deficiency [12]. In case 4 (symptomatic female), direct sequencing of the region encompassing exon 4 of the genomic DNA revealed the heterozygous peaks of G and C at the first nucleotide of intron 4 (c.386+1G>C) (Fig. 1e, f), which was a novel mutation. This nucleotide change disrupted a highly conserved GT dinucleotide sequence at the splice donor site. In case 5 (late-onset male), a single nucleotide change from T to A was discovered in the amplified region comprising exon 5 (c.515T>A) (Fig. 1g, h). The nucleotide change resulted in a missense mutation (p.172I>N), which has not previously been reported.

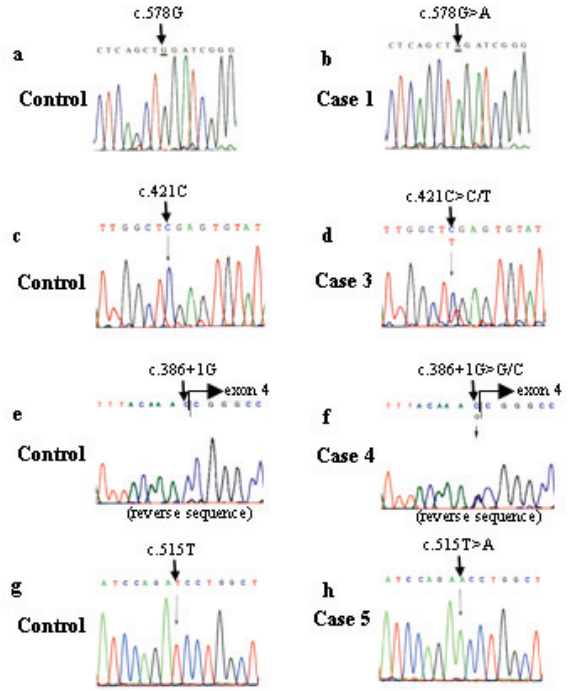

Fig. 1. Mutation analysis of OTC genomic DNA in cases 1, 3, 4 and 5. (a, b) PCR amplification of a fragment comprising exon 6 from lymphocyte genomic DNA. Sequences of exon 6 from a control subject (a) and case 1 (b) are shown. A single nucleotide (c.578G) located on exon 6 was changed to A (c.578G>A) in case 1, which resulted in the creation of a stop codon (p.193W>X). (c, d) PCR amplification of a fragment comprising exon 5 from lymphocyte genomic DNA. Sequences of exon 5 from a control subject (c) and case 3 (d) are shown. Heterozygous C and T nucleotides were identified at nucleotide 421 in case 3, which represents a nonsense mutation in one of the two alleles (p.141R>X). (e, f) Nucleotide sequences of the boundary of exon 4 and intron 4 from a control subject and case 4. Reverse sequences are shown. The first nucleotide of intron 4 (c.386+1G) was changed to heterozygous C and G in case 4, which represents the destruction of highly conserved GT dinucleotide at splice donor site in one of the two alleles. Horizontal arrows indicate the 3' end of exon 4. (g. h) Nucleotide sequences of exon 5 from a control and case 5. A single nucleotide (c.515T) located on exon 5 was changed to A (c.515T >A) in case 5, which resulted in a missense mutation (p.172I>N).

Mutation analysis of case 2

As mentioned above, the direct sequencing of amplified products comprising all 10 exons and their flanking intron regions revealed the responsible mutations in cases 1, 3, 4 and 5, whereas no mutation was seen in case 2. To clarify the splicing abnormality in case 2, OTC transcript expressed in liver was analyzed using RT-PCR. Expected-size products were amplified in the normal liver after RT-PCR amplification of cDNA encompassing exons 1-6 and exons 5-10 as shown in Fig. 2a and b (lane C). Remarkably, the larger sized products were amplified from exons 1-6 and exons 5-10 in the patient liver cDNA (Fig. 2a, b lane P), although the normal sized bands were also slightly amplified. Analysis of nucleotide sequences revealed that the large-sized product contained the same exons as the normal-sized one, but an unidentified 135-nt sequence was found inserted between exons 5 and 6 (Fig. 2c).

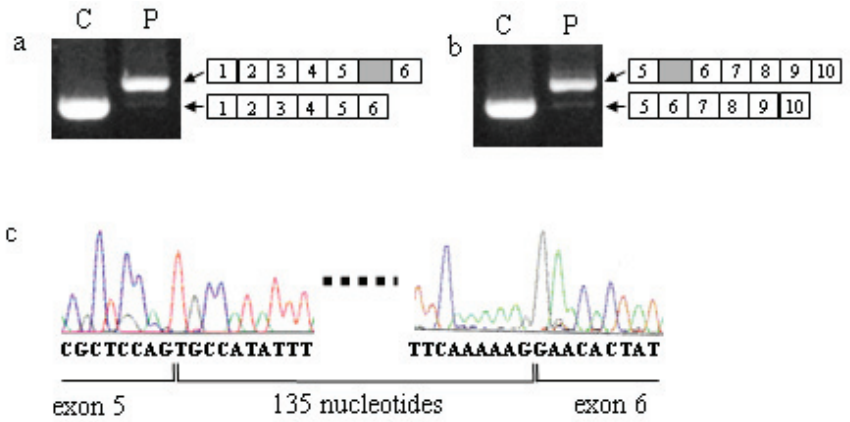

Fig. 2. OTC mRNA analysis of case 2. (a, b) RT-PCR products of a fragment comprising exons 1-6, and exons 5-10 are shown in panels a and b, respectively. C and P refer to the liver mRNA from a control subject and case 2, respectively. A faint band of the expected size and a larger dense band were obtained from case 2 (lane P). A schematic representation of the exon organization of the amplified products is shown on the right. Open and shaded boxes represent the authentic exons and 135-nt inserted sequence, respectively. (c) Partial nucleotide sequence of OTC cDNA from case 2. The 135-nt sequence of intron 5 was inserted between exons 5 and 6, corresponding to the large-sized band in panels a and b.

The fact that the 135-nt sequence was precisely inserted between exons 5 and 6 led us to speculate that this sequence could be from retained introns or unknown exons. The novel 135-nt inserted sequence did not match the sequence flanking either exon 5 or 6. A BLAST search of the 135-bp sequence revealed an identical sequence in the 5' region of intron 5 (bases 38145892-38146026 of RefSeq: NC_000023). The 135-nt sequence was located 267 bp downstream from exon 5 and 1789 bp upstream of exon 6 (Fig. 3). Taking the inserted sequence as an exon, consensus sequences for splicing sites were examined at either end of the insert in the wild-type sequence (RefSeq: NC_000023). GT dinucleotide as a candidate for the splicing donor site was found next to the 3' end of the inserted sequence. Shapiro's probability score for splicing donor sites is 0.90 [32]. However, the score for the splicing acceptor site was 0.74, far below the average for authentic exons. This excluded the possibility that the inserted sequence was functioning as an exon.

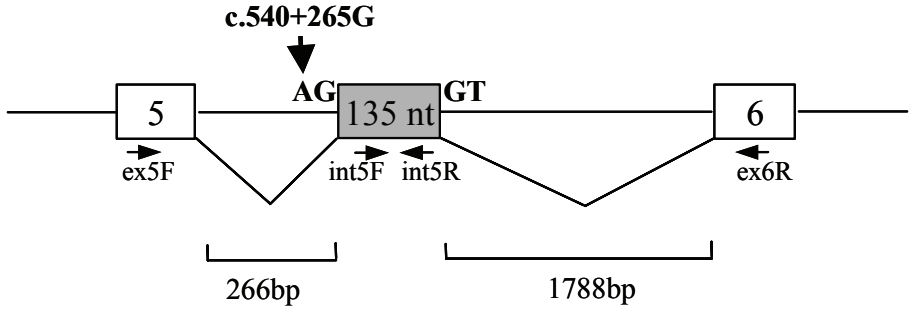

Fig. 3. Genomic structure of exons 5 and 6, and the 135-nt inserted sequence. The 135-nt sequence was located 267 bp downstream from exon 5 and 1789 bp upstream from exon 6. Open and shaded boxes represent the authentic exons and 135-nt inserted sequence, respectively, and lines indicate introns. The diagonal solid lines below the boxes represent the splicing events observed in the liver of case 2. Highly conserved dinucleotides of splice acceptor (AG) and donor (GT) sites observed in case 2 are shown as 5' and 3' flanking regions of the 135-nt insertion, respectively. In case 2, G nucleotide at the second position upstream from the 5' end of the inserted sequence was mutated to A (c.540+265G>A), which resulted in the creation of AG dinucleotide at the splice acceptor site (Fig. 4). Arrows under the boxes show the location and direction of primers for genomic DNA analysis. The figure is not drawn to scale.

To clarify the mechanism promoting the incorporation of the intron sequence into mRNA, the nucleotide sequences of the flanking regions of the inserted sequence were analyzed using the genomic DNA from case 2. Remarkably, sequencing of the amplified fragment disclosed a single nucleotide change from G to A at the second nucleotide upstream from the 5' end of the inserted sequence (Fig. 4), but all other sequences were completely identical to those of the wild-type sequence. The mutation created an AG dinucleotide consensus sequence for a splicing acceptor site instead of the wild-type GG dinucleotide. As a result, Shapiro's probability score for the splice acceptor site was increased from 0.74 to 0.91. Therefore, it was concluded that the novel splice acceptor site produced a novel exon structure from the intron sequence together with downstream cryptic splicing donor sites, and subsequently, that a novel exon structure was incorporated into OTC mRNA.

The 135-nt inserted sequence included an in-frame stop codon, which indicated disruption of the production of OTC protein.

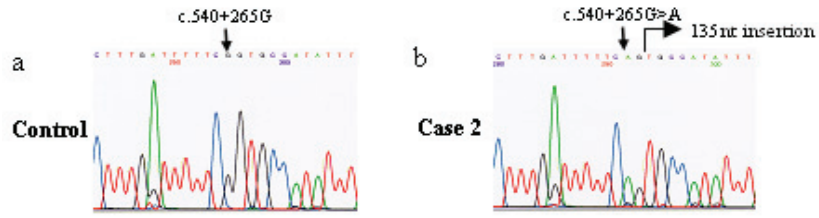

Fig. 4. Genomic sequences of the boundary between intron 5 and the inserted sequence. In case 2, the G nucleotide at the second position upstream from the 5' end of the inserted sequence was mutated to A (c.540+265G>A). Horizontal arrow indicates the 5' end of the 135-nt inserted sequence.

DISCUSSION

Molecular defects in OTC deficiency have been increasingly identified since the structure and organization of the human OTC gene has been clarified [13,15]. The mutations in the human OTC gene have been summarized by Yamaguchi et al. and a total 341 different mutations have been listed [48]. The mutations found in our study consisted of two nonsense, one missense and two splice site mutations, including a deep intronic mutation that created a new splice acceptor site. Furthermore, three of these five mutations were novel.

Nonsense mutations were detected in cases 1 (c.578G>A) and 3 (c.421C>T), and both mutations have been previously reported in neonatal-onset OTC deficiency [12,34]. Case 1 showed the typical clinical course for neonatal-onset OTC deficiency, which was consistent with previous report. On the other hand, the other nonsense mutation was detected in a female patient (case 3). More than 80% of heterozygous females are asymptomatic, whereas the remainder shows clinical severity similar to that in males with partial deficiency as a consequence of skewed lionization [41]. In this study, case 3 also showed the late-onset phenotype.

Case 4, a heterozygote for the novel splice site mutation (c.386+1G>C), was also female. Although the c.386+1G>C mutation has not been previously reported, c.386+1G>T and c.386+1G>A have been found in the patients with OTC deficiency [48]. Furthermore, this nucleotide change disrupted a highly conserved GT dinucleotide sequence at the splice donor site, which indicated that the c.386+1G>C mutation was the responsible mutation in case 4. The mild phenotype of case 4 was also due to heterozygosity for a severe mutation and skewed X-chromosome inactivation.

Mutation analysis of case 5, who was male and showed a mild phenotype, disclosed a novel missense mutation (c.515T>A). The nucleotide change resulted in an amino acid change from isoleucine to asparagine at amino acid position 172. The reason why this missense mutation was responsible for OTC deficiency is outlined below. First, although this amino acid change has not previously been reported, amino acid changes from isoleucine to phenylalanine and from isoleucine to methionine at the same position have been found in cases of female and neonatal-onset male OTC deficiency, respectively [8,24]. Second, in the OTC structure, I172 is within alpha helix 5, which is at the core of the OTC monomer and participates in the active center in the region of carbamoyl phosphate binding [33]. Third, in the alignment of the known OTC sequences from different species, I172 is conserved from humans to rats [42]. Finally, this nucleotide change (c.515T>A) has not been reported as a polymorphism and has not been detected in more than 50 unrelated individuals. These facts indicate that this missense mutation was responsible for the mild OTC deficiency phenotype.

For mutation analysis of the OTC gene, a combination of molecular methods has been used, including southern blotting, SSCP and DNA sequencing of all 10 exons and exon-intron boundaries. Nevertheless, only ~80% of patients with enzymatically proven OTC deficiency are found to have mutations in the OTC gene [48]. In this report, we detected a single nucleotide change deep in the intron sequence of the OTC gene, possibly for the first time. In case 2, the majority of OTC transcripts included the central part of intron 5 sequence, which resulted in the creation of a premature termination codon, which was responsible for the neonatal-onset phenotype. In the genomic DNA, a single nucleotide substitution at 265 bp downstream from the 3' end of exon 5 was detected and this substitution created a novel splice acceptor site. The creation of a splice acceptor site acts as a mechanism to form an extra exon from the intron, together with a cryptic splice donor site.

Splicing mutations have been identified in a number of genes and have been estimated to account for 15% of all disease-causing mutations [21]. Those mutations have been shown to have a variety of consequences, including exon skipping and intron retention [26]. Most of them appear to affect canonical splice sites or their surrounding consensus sequences [36]. The creation of additional splice sites producing extra exons in the middle of an intron is considered to be a rare event [26], and only a few examples have been reported as far as we know, such as beta-globin [40], factor VIII [3], CFTR [5,14], PHEX [7], dihydropteridine reductase [17], alpha-galactosidase [19], TSC2 [26], ATM [27], NF1 [2,31], ornithine delta-aminotransferase [29], estrogen receptor [45], beta-glucuronidase [44], and dystrophin [4,9,18,43,47].

For detection of the nucleotide changes deep in intron sequences, it is important to screen using OTC cDNA, as shown here, whereas OTC mRNA is expressed exclusively in the liver and small intestinal mucosa. Illegitimate transcription in blood cells has been reported as the source for OTC mRNA analysis [10]. The utility of illegitimate transcription in blood cells for mRNA analysis has also been reported for other genes. We have used lymphocytes as a source of the dystrophin transcript which is expressed exclusively in muscle, and have detected splicing abnormalities using RT-nested PCR [1,11,20,30,35,37,38,46,47].

In conclusion, we report five OTC gene mutations in the Japanese patients with OTC deficiency. Among these, three are novel mutations, including the deep intronic mutation. Molecular analysis using genomic DNA and mRNA will increase the mutation detection ratio in the OTC gene.

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