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Prenatal Molecular Evaluation of Six Fetuses in Four Unrelated Korean Families with Ornithine Transcarbamylase Deficiency

Ornithine transcarbamylase (OTC) deficiency, an X-linked inborn error of the urea cycle, leads to the accumulation of ammonia, causing neurologic deficits. Clinical management for the patients with OTC deficiency is frustrating and requires a burdensome medical regimen, since they may have impairment and recurrent episodes of hyperammonemia in spite of intensive care. Therefore, prenatal diagnosis of the affected fetus is important in genetic counselling for the family at high risk. In this study, mutations in the OTC gene of three obligate heterozygotes and a proband have been identified in four unrelated families: R141Q, R320X, H214Y, M205T. Each mutation altered restriction recognition sites; *Taq*I for R141Q, *Nla*III for M205T, *Rsa*I for H214Y, *Bcl*I for R320X. Based on their molecular defects, prenatal diagnoses of 6 fetuses including one set of fraternal twins were successfully made at the ninth to eleventh week of gestation by polymerase chain reaction (PCR)-restriction digestion using genomic DNA from chorionic villus sampling (CVS). We predicted the outcome of all fetuses prenatally. Among six, four were females and two were males, which were determined by PCR amplification of the sex determining region of the Y chromosome (SRY) gene. Each carried a wild type allele for the corresponding mutant allele. They were also tested postnatally for the mutations to be unaffected.

Key Words : *Hereditary diseases, Ornithine carbamoyltransferase deficiency; Prenatal diagnosis; Mutation*

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INTRODUCTION

Ornithine transcarbamylase (OTC: EC 2.1.3.3) deficiency, the most common inborn error of the urea cycle, is inherited in X-linked semicodominant manner. Deficiency of this enzyme leads to hyperammonemia, hampering normal neurological functions. In affected hemizygote males, OTC deficiency manifests as hyperammonemic coma that often results in death in the newborn period, while those who recover from coma or develop late onset manifestations, may be crippled owing to mental retardation and cerebral palsy. Female heterozygotes may have milder clinical manifestations. Especially in the early onset (neonatal onset) type of male patients, the prognosis is guarded in spite of intensive clinical management. Most succumb in early infancy. Even those who survive are neurologically handicapped (1).

Approximately one third of all male patients have the neonatal onset type. Females who are heterozygous for mutant OTC allele may, as consequences of lyonization, have clinical manifestations similar to those of less seriously affected hemizygous males of late onset. When clinically suspected, the diagnosis of OTC deficiency can be made on the basis of low blood citrullin level and increased urinary excretion of orotic acid. However, prenatal diagnosis or detection of asymptomatic carriers has been problematic. Since the cDNA and genomic structure of the human OTC gene was elucidated (2-4), direct identification of molecular defects has been facilitated. A large variety of mutant genotypes have been reported, indicating genetic heterogeneity in OTC deficiency. Therefore it is important to investigate the molecular defect in each individual family. Once the molecular defect is defined, accurate prenatal molecular diagnosis of the fetus can be made in the family at high risk. Otherwise, intragenic RFLP markers can be utilized for prenatal diagnosis in the informative family.

In this study, we characterized molecular defects in the OTC gene of four unrelated Korean families with OTC deficient patients. We applied these results to monitor

the molecular status of fetuses in these families.

MATERIALS AND METHODS

Family history

Family #1: Previously, this mother had lost three sons consecutively a week after birth owing to hyperammonemia. We presumed that the mother is an obligate heterozygote for OTC deficiency. Protein loading (1 g/kg/body weight) test revealed elevated urinary orotic acid excretion. Her DNA obtained from peripheral leukocytes was analyzed to detect mutation in the OTC gene. The fourth pregnancy was monitored by DNA analysis of the fetus using chorionic villus sampling (CVS) at the tenth week of gestation.

Family #2: The proband was the first male newborn to a healthy mother who had no suspected history of OTC deficiency in the family. On the 3rd day after birth, he developed a sudden onset of vomiting and lethargy. Blood ammonia level rose up to 586 μmol/L, plasma citrulline level was too low to be detectable. Urinary excretion of orotic acid increased. The DNA from the proband has been analyzed. The second pregnancy was prenatally evaluated by CVS from the fetus at the ninth week of gestation.

Family #3: This subject was a healthy asymptomatic mother of twin male babies, one of whom manifested severe hyperammonemia (790 μmol/L) requiring peritoneal dialysis at three days of age. Plasma amino acid analysis of her affected son showed markedly increased glutamine, alanine, and glycine, whereas plasma citrulline was undetectable. He died of hyperammonemia at 10 months of age despite protein restriction, peritoneal dialysis, and sodium benzoate administration. Since liver tissue or blood specimens for DNA analysis were not available from the affected, instead, the DNA of his mother was analyzed. She was pregnant with twin fetuses again. At the eleventh week of gestation, chorionic villi was sampled from each twin under the guidance of ultrasound.

Family #4: The proband was the second female born to healthy parents after an uneventful pregnancy and delivery. She was in good health until 9 months of age, when she presented with episodic vomiting, convulsions and lethargy. On the day of admission, her plasma ammonia was 494 μmol/L. Plasma citrulline was reduced (6.8 μmol/L), urinary orotic acid was markedly elevated (130 mmol/mol creatinine), and the diagnosis of OTC deficiency was entertained. Since then, she has suffered from recurrent hyperammonemia with lethargy. Her development has lagged behind developmental milestones appropriate for her age. Her elder brother and three uncles on the mother's side died of presumptive OTC deficiency about a week after birth. Her maternal aunt visited our clinic for the prenatal diagnosis of OTC deficiency at her first and second pregnancy.

Establishment of lymphoid cell lines

Peripheral blood samples were collected from the four unrelated probands and their family members after informed consent. Lymphoid cell lines were established using cyclosporin A and Epstein-Barr virus as previously described (5).

Genomic DNA isolation

Genomic DNA was isolated from peripheral blood leukocytes, lymphoid cell lines, or chorionic villi from the patient, fetus, and appropriate family members. After treatment with 0.5% sodium dodecyl sulphate and proteinase K, genomic DNA was extracted using phenol and chloroform and precipitated with cold ethanol according to standard procedures.

Amplification of the OTC gene and sex determining region of the Y chromosome (SRY) gene using genomic DNA from leukocytes or CVS

To determine the fetal sex, multiplex PCR of the DNA binding region of the SRY and repetitive DNA sequences (DYZ3) located at the centromeric region of the Y chromosome were carried out using two sets of primers. For the SRY gene, the 270 base-pair sequence representing the DNA binding domain of the SRY gene was amplified with primers: SRY-1F 5'-CAG TGT GAA ACG GGA GAA AAC AGT-3', SRY-1R 5'-CTT CCG ACG AGG TCG ATA CTT ATA-3'. At the same time, the 1.1 kilobase-pair covering centromeric repetitive sequences was amplified with primers: DYZ3-F 5'-TGA AAA CTA CAC AGA AGC TG-3', DYZ3-R 5'-ACA CAT CAC AAA GAA CTA TG-3' (6, 7). Amplification by PCR of all 10 exons of the OTC gene was performed using nine pairs of synthetic oligonucleotide primers designed to span all exons and their adjacent intronic regions. Exon 7 and 8 were amplified together using a single pair of primers. The nucleotide sequences of primers were as published by Matsuura and colleagues (8) and they were synthesized on an Applied Biosystems synthesizer (Model 380B, Foster City, CA, USA). After an initial denaturation of the template DNA, amplification was performed for 30 cycles and consisted of denaturation at 94℃ for 1 min, annealing at 48℃ (exon 7 and 8) or 50℃ (the other exons, SRY, and DYZ3) for 30 s,

and extension at 72℃ for 2 min. For each reaction, the 100 μL amplification mixture contained 100 ng of genomic DNA, 1 μmol/L each of sense and antisense primers, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.8, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 50 μmol/L dNTPs, and 2.5 units of *Taq* DNA polymerase (Promega Biotec, Madison, WI, USA). The reaction was run in a thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA).

Direct sequencing of double stranded PCR product

PCR product (10 μL of each) was electrophoresed on 1.8% agarose gel to examine whether the targeted template was specifically amplified. When a single specific PCR product was amplified, double stranded DNA sequencing of each exon and its intron-exon boundaries was performed directly without purification using sense and antisense primers, and a Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, USA) according to manufacturer's instructions with the following modifications. The original amount of template for individual sequencing reaction ranged from 5 to 10 μL of each PCR product. Sequenase DNA polymerase and inorganic pyrophosphatase were mixed together (1 volume of each) and diluted with 6 volumes of glycerol enzyme dilution buffer (20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L dithiothreitol, 0.1 mmol/L EDTA, 50% glycerol). The labelling reaction using [α-^{35}S] dATP was extended to 30 min at room temperature. The reaction mixture was electrophoresed on 8% denaturing polyacrylamide gel in 1X glycerol tolerant gel buffer (20X buffer is 216 g Tris base, 72 g taurine, 4 g $Na_2EDTA.2H_2O$ in 1 liter H_2O)

Restriction enzyme analysis

The amplified PCR product of exon 5 was digested with *Taq*I enzyme at 65℃, the exon 6 product with *Rsa*I or *Nla*III at 37℃, and exon 9 product with *Bcl*I at 50℃ for 2 hrs to monitor the inheritance of mutations in fetuses in the appropriate family.

RESULTS

Family #1:

We found a G to A transition in a codon 141 in exon 5, causing a substitution of a glutamine for an arginine (R141Q) in a female obligate heterozygote whose previous three sons succumbed to presumed OTC deficiency in the neonatal period (Fig. 1). This mutation eliminated *Taq*I recognition site. The fourth pregnancy was monitored by DNA analysis of the fetus using CVS at the tenth week of gestational age. The genomic DNA covering exon 5 was amplified and digested with *Taq*I. The pattern of restriction fragment lengths indicated that the fetus was unaffected since the 269 base-pair PCR product was completely digested, generating 184 and 85 base-pair fragments (Fig. 1). PCR amplification for the SRY gene was positive (data not shown). Therefore, the fetus

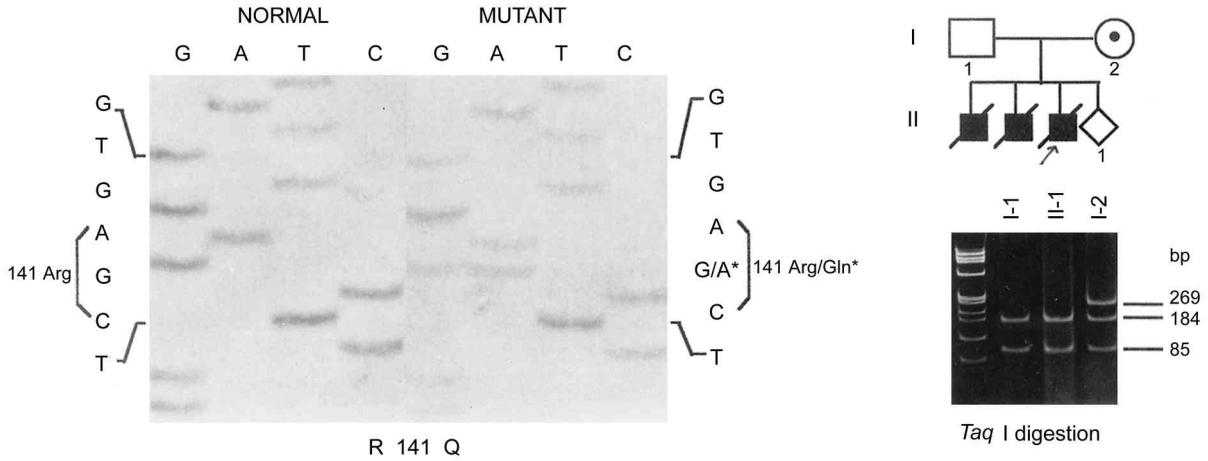

Fig. 1. Partial nucleotide sequences of PCR-amplified genomic DNAs for the OTC gene exon 5 in the family #1 (left panel) When compared with normal sequence, the mother (I-2) was heterozygous for a G to A transition in codon 141 in exon 5, causing substitution of a glutamine for an arginine (R141Q). The mutated base or amino acid residue is indicated by an asterisk. Prenatal monitoring of the fetus for the presence of the mutation by *Taq*I digestion analysis in the family #1 (right panel) The mother (I-2) was heterozygous for the mutant allele, the fetus (II-1) was unaffected since the normal allele was digested, resulting in 184 and 85 bp fragments, whereas the mutant allele remained undigested. The ϕx174 DNA, digested with *Hae*III was used as a size marker.

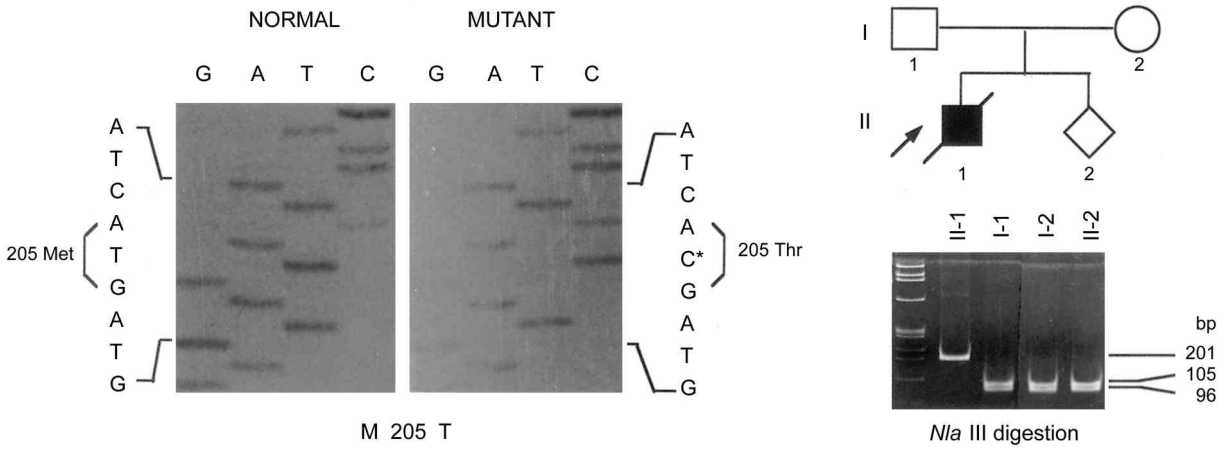

Fig. 2. Partial nucleotide sequences of PCR amplified genomic DNAs for the OTC gene exon 6 in the family #2 (left panel) When compared with the normal allele, the proband (II-1) was hemizygous for a T to C transition in codon 205 in exon 6, substituting a threonine for a methionine (M205T). the mutated base or amino acid residue is indicated by an asterisk. Prenatal evaluation of the fetus for the presence of the M205T mutation by *Nla*III digestion analysis in the family #2 (right panel) The fetus (II-2) was unaffected as father (I-1) or mother (I-2), since the 201 bp PCR product from exon 6 was completely digested with *Nla*III, resulting in 105 and 96 bp fragments. Only the mutant allele in the proband (II-1) was resistant to *Nla*III digestion, therefore this mutation was thought to be de novo. The ϕx 174 DNA, digested with *Hae*III was used as a size marker.

was a male hemizygote for the normal allele. He was born and tested for the mutation using his leukocytes which were found to be normal.

Family #2:

The DNA analysis of the OTC gene in the proband revealed a T to C transition in exon 6, abolishing *Nla*III restriction site, substituting a threonine for a methionine in the codon 205 (M205T) (Fig. 2). This mutation was thought to be a de novo mutation since it was found only in the patient, not in his asymptomatic mother by nucleotide sequencing or restriction digestion. The second pregnancy was evaluated using genomic DNA isolated from CVS at the ninth week of gestation. The 201 base-pair PCR product originated from exon 6 of the OTC gene was completely cleaved with *Nla*III, resulting in 105 and 96 base-pair fragments, while the PCR product from the proband remained uncleaved (Fig. 2). Also the PCR product of the SRY gene was not detected (data not shown). The fetus was diagnosed as an unaffected female based on these findings.

Family #3:

In this family, an asymptomatic female whose son (proband) died of acute neonatal onset OTC deficiency, turned out to be a heterozygote for a C to T transition in codon 214 in exon 6, substituting a tyrosine for a histidine (H214Y) (Fig. 3). This base substitution creates a new *Rsa*I recognition site in exon 6. The pattern of *Rsa*I digestion using PCR product of exon 6 in a heterozygous mother, showed an undigested 201 base-pair fragment for the normal allele, digested 132 and 69 base-pair fragments for the mutant allele. She was pregnant with twin fetuses. At 11 weeks of gestational age, chorionic villi was sampled from each twin under the guidance of ultrasound. The pre-procedure ultrasound showed dichorionic placentation. The 201 base-pair PCR product of exon 6 from each fetus was resistant to digestion with *Rsa*I enzyme, indicating both were unaffected (Fig. 3). They were positive for the SRY gene amplification (data not shown). After they were born, their genomic DNA from leukocytes were tested as normal for the mutation.

Family #4:

A C to T transition was found in codon 320 (encoding an arginine residue in exon 9), which creates a *Bcl*I recognition site in exon 9 with generation of a stop codon resulting in premature termination in the enzyme (R320X) (Fig. 4). This nonsense mutation was identified in a symptomatic female proband and her asymptomatic mother, whose son died of OTC deficiency during the neonatal period. By cleaving 274 bp amplified genomic DNA with *Bcl*I restriction endonuclease, the proband, mother, and her aunt on the maternal side were confirmed to be heterozygous for the mutant allele since they showed an uncleaved 274 base-pair fragment derived from the normal allele, the cleaved 138 and 136 base-pair doublet from the mutant allele. The hetero-

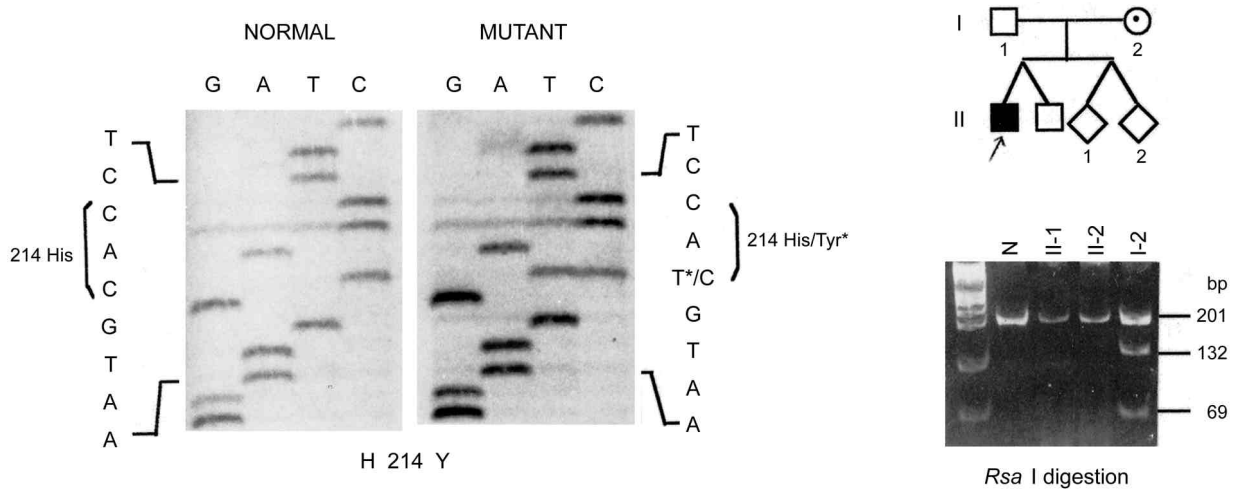

Fig. 3. Partial nucleotide sequences of PCR-amplified genomic DNAs for the OTC gene exon6 in the family #3 (left panel) When compared with the normal sequence, the mother (I-2) was heterozygous for a C to T transition in codon 214 in exon 6 substituting a tyrosine for a histidine (H214Y). The mutated base or amino acid is indicated by an asterisk. Prenatal evaluation of twin fetuses for the presence of the H214Y mutation by *Rsa*I digestion analysis of the family #3 (right panel) The mother (I-2) was heterozygous for the mutant allele, generating an uncleaved 201 bp, and cleaved 132 and 69 bp fragments, while fetuses (II-1,2) were unaffected as normal individual (N). The ϕx 174 DNA, digested with *Hae*III was used as a size marker.

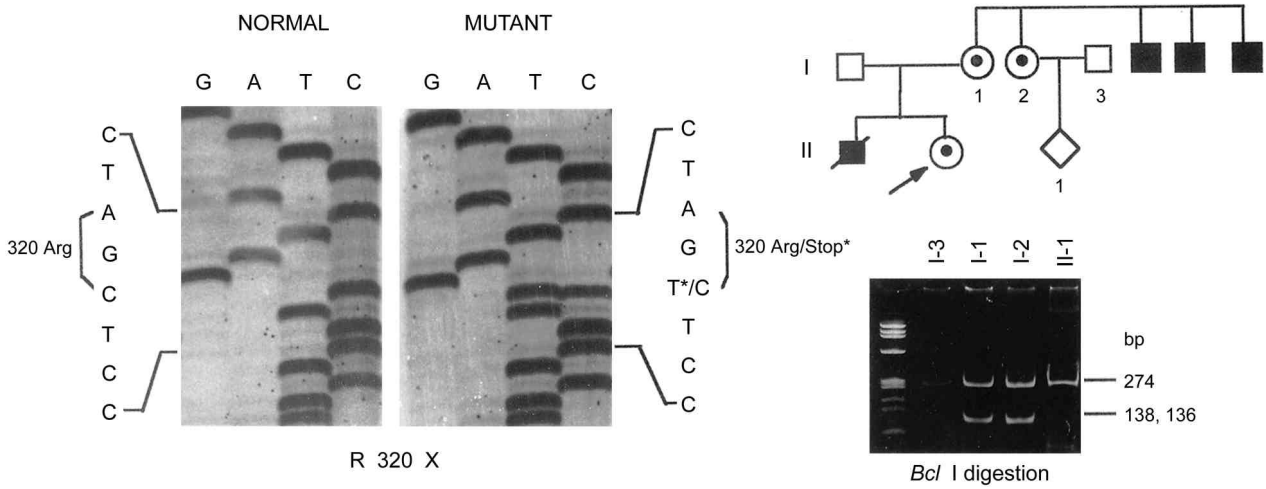

Fig. 4. Partial nucleotide sequences of PCR-amplified genomic DNAs for the OTC gene exon 9 in the family #4 (left panel) When compared with the normal allele, the proband was heterozygous for a C to T transition in codon 320, encoding an arginine residue in exon 9 with generation of a stop codon. Prenatal monitoring of the fetus for the presence of the R320X mutation by *Bcl*I digestion analysis in the family #4 (right panel) The proband's aunt (I-2) was heterozygous for the mutant allele as her sister (I-1) since they showed an uncleaved 274 bp fragment derived from the normal allele, the cleaved 138 and 136 bp doublet from the mutant allele. The fetus (II-1) was unaffected showing an uncleaved 274 bp fragment as its father(I-3). The ϕx 174 DNA, digested with *Hae*III was used as a size marker.

zygous aunt has been pregnant twice, the fetuses were examined for the mutation using CVS at the tenth week of gestation. Both were unaffected females since their PCR products across exon 9 region were uncleaved (Fig. 4) (data for the second pregnancy not shown). They were all negative for the SRY gene amplification (data not shown). Their genomic DNA have been analyzed after birth for the presence of mutation but were normal.

DISCUSSION

In this report, we have described four different mutations in four unrelated families (R141Q, M205T, H214Y,

R320X). These mutations were all associated with alterations of restriction recognition sites (*Taq*I, *Nla*III, *Rsa*I, *Bcl*I). Based on alterations of these sites, prediction of the specific mutation of the individual family has been successfully carried out in 6 fetuses.

The R141Q, H214Y, R320X mutations have been reported previously (9, 10). The M205T mutation is a new mutation, which was identified de novo in a proband only. Although it will remain uncertain whether these amino acid changes are responsible for causing OTC deficiency until expression studies in these families using mutant gene transfection is undertaken, polymorphisms are unlikely since none of the 100 normal alleles tested showed these base changes and these nucleotides are well conserved across species (11, 12). Each mutation has been associated with an alteration of restriction enzyme recognition site, which can be utilized for prenatal molecular evaluation of fetuses at risk for the OTC deficiency. The R141Q mutation is known to account for 10% of OTC deficiency together with the R141X (arginine to stop codon), both of which eliminate the *Taq*I recognition site (13, 14).

Matsuura and colleagues (8) maintained that DNA analysis of the OTC gene with *Taq*I digestion seemed to be the first choice for a prenatal monitoring in a family at risk since they found the frequency of *Taq*I site alteration was potentially high (20%) in the OTC deficient patient. For most families affected, linkage analysis using RFLP can be employed for both carrier assessment and for prenatal diagnosis. To date, more than 4 intragenic RFLP have been detected at the OTC locus (15). We developed a PCR-based RFLP assay for the TTA (Leu)/TTT (Phe) (L101F) using *Dde*I enzyme previously (10). However, these families are not informative for these RFLP markers.

A large variety of mutant genotypes have been recognized in OTC deficiency, including intragenic deletions and point mutations without predominance of a single particular genotype (9, 16, 17). Since genetic heterogeneity is considerable in this disorder, it is important to investigate the molecular defect in each individual family. In this study, we characterized each mutation in each individual family. Although this approach is labor-intensive, involving numerous sequencing, the direct detection of the nucleotide sequence alteration becomes technically feasible on a routine basis by automation of nucleotide sequencing or PCR-single strand conformation polymorphism (SSCP) screening.

In family #3, accurate CVS could have been technically difficult for a twin pregnancy. Fortunately, they were dichorionic twins, each chorionic villi was easily sampled under the guidance of an ultrasonogram. The risk of misdiagnosis due to maternal tissue contamination can be minimized by using informative polymorphic markers specific for paternal X chromosome, epecially in SRY-negative fetuses. In family #2, the mother was not thought to be a carrier by pedigree analysis, allopurinol loading test or mutational analysis. Though prenatal diagnosis may not be justified in this family, germinal mosaicism cannot be ruled out. Instead of CVS at the first trimester of gestation, preimplantation molecular diagnosis could be made, offering an alternative to terminating pregnancy at much later stages.

This study shows prenatal molecular evaluation has been successfully undertaken by direct investigation of specific mutations in fetuses at high risk whose families were not informative of intragenic RFLP markers.

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