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MITOCHONDRIAL tRNA^{Ile} MUTATION IN FATAL CARDIOMYOPATHY[1]

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Summary: A patient with mitochondrial encephalomyopathy who died from progressive intractable cardiac failure at the age of 18 is reported. At the age of 4, he presented with short stature, but multiorgan disorders including deafness, focal glomerulosclerosis, epilepsy and dilated cardiomyopathy appeared later in his clinical course. Laboratory tests showed hyperlactatemia and hyperpyruvatemia. Histopathological findings demonstrated mitochondrial myopathy with ragged red fibers and focal cytochrome C oxidase-deficient fibers in skeletal and cardiac muscles. The activity of cytochrome C oxidase was 30% less than the control level in skeletal muscle. Sequencing of the entire mitochondrial tRNA genome revealed a novel point mutation in the tRNA^{Ile} region (nt 4269). This A-to-G substitution was found in none of the 30 controls by screening using mispairing PCR and Ssp I digestion methods, suggesting that this new mutation was pathogenic in our case.

A large deletion or various point mutations of the mitochondrial DNA (mtDNA) genomes are related to distinct clinical mitochondrial disorders. For example, most patients with chronic progressive external exophthalmoplegia (CPEO) have a large deletion of mitochondrial DNA (1), and about 80% of the

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patients with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episode (MELAS) have point mutations in the $tRNA^{Leu(UUR)}$ (nt 3243, 3271: nucleotide number designated according to the Cambridge sequence) gene (2, 3), while patients with myoclonic epilepsy with ragged-red fibers (MERRF) have a point mutation in the $tRNA^{Lys}$ (nt 8344) gene (4, 5). Besides, these clinically distinct syndromes, defects of the mitochondrial genome have recently been identified in association with various human diseases (6-8). Tanaka et al. identified a point mutation in the $tRNA^{Ile}$ gene (nt 4317) in fatal infantile cardiomyopathy (9). We also found a novel mutation in the $tRNA^{Ile}$ gene in a patient with mitochondrial encephalomyopathy who died from intractable fatal cardiomyopathy.

Case Report. The patient was born to healthy unrelated parents after an uneventful pregnancy and delivery. His neonatal period was also uneventful. He had growth failure (height and weight below -2SD) from the age of 4. At 10 years of age, mild to moderate proteinuria was recognized, and renal biopsy disclosed focal segmental glomerulosclerosis. From the age of 11, progressive neurogenic hearing loss appeared. At 13 years of age, he had a generalized tonic clonic seizure for the first time following episodes of nausea and transient loss of vision. Brain CT scan, echocardiography, and aminograms of the serum and urine were all normal. Tests of hypopituitary function including the loading tests of LH-RH, TRH, insulin, or arguinine were all normal. From the age of 18, progressive generalized fatigue appeared, and the second episode of generalized tonic-clonic convulsion occurred. On admission, chest roentgenography revealed cardiac enlargement, and echocardiography revealed dilated cardiomyopathy with pericardial effusion. Laboratory studies showed elevated levels of lactate (44 mg/dl) and creatinine kinase (252 IU/ml; normal, 30-160 IU/ml) in his serum. Except for mild mental retardation and neurogenic hearing loss, no neurological abnormalities were recognized. Muscle biopsy disclosed ragged red fibers and focal cytochrome c oxidase (CCO)-deficient fibers, and the enzymic activity of CCO was about 30% of the normal control level in the biopsied sample. Thus, the diagnosis of mitochondrial encephalomyopathy was made. He died from progressive intractable cardiac failure 6 months after admission. The autopsy findings will be reported in detail elsewhere. Briefly, there were many ragged red fibers without

CCO activity (up to 50% of the total fibers) in the ileopsoas muscle fibers and cardiac muscle fibers which showed extensive thickness of the endocardium.

MATERIALS AND METHODS

DNA preparation. Total DNA was extracted and purified from skeletal muscle, cardiac muscle or white blood cells by method of Old et al (10).

Mitochondrial DNA (mtDNA) analysis. Screening for reported mutations in the mtDNA (nt 3243 and nt 8344) were examined by polymerase chain reaction methods (PCR) as described (2, 11), and deletion analysis was performed by Southern hybridization using whole mtDNA extracted and purified from human placenta (1, 12). For direct sequence analysis, a symmetrically-amplified DNA template was prepared by PCR using 9 sets of primers, which were synthesized according to the Cambridge sequence. These fragments encompass all the mitochondrial tRNA genes with the following nucleotide coordinates: 520-1722, 3130-4262, 4116-5283, 4469-5928, 7375-8410, 9912-10555, 12051-12410, 14553-15582, 15806-16310. The sets of sequencing primers were the same as described those by Goto et al. (3). The tRNA genes were sequenced directly using a Taq DyeDeoxy™ Terminator Cycle Sequencing Kit and DNA sequencer (Applied Biosystems) according to the manufacturers' manual.

Detection of the mutation at nt 4269. The mutation at nt 4269 was detected by the "mispairing PCR" method and restriction enzyme analysis. The sequence of the primers used in this study was Ile-For:5'-CTTATGAATTCGAACAGCAT-3' (nt 4116-4135) and Ile-Rev:5'-ACTCTATCAAAGTAACTCTTTTATCAGAaA-3' (nt 4299-4270, mispairing primer). The nucleotide in lower-case letter differs from the Cambridge sequence and create the new restriction site of SspI in only wild type mtDNA. Amplification of mtDNA was performed with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. The amplified 184-bp fragment that originated from wild type mtDNA was cleaved into 153 and 31 bp. The digestion products were analyzed on a 12% polyacrylamide gel and stained by either ethidium bromide or silver as described (13).

RESULTS AND DISCUSSION

Screenings for reported point mutations (nts 3243 and 8344) and deletions were negative. However, direct sequence analysis of the tRNA genomes of mtDNA disclosed an A to G base substitution in the $tRNA^{Ile}$ region (nt4269) (Fig. 1).

To confirm this mutation, mispairing PCR and SspI restriction analysis was performed. As described above, the

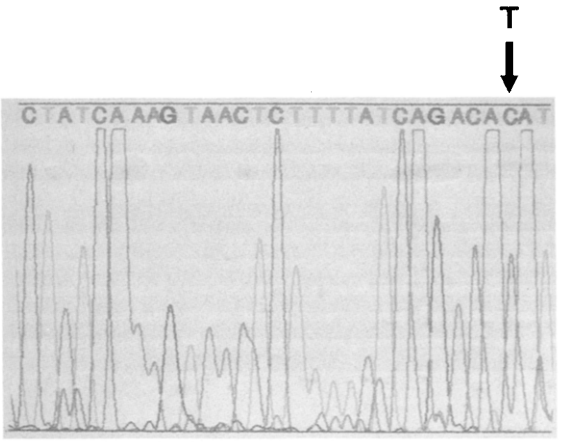

Fig. 1. Sequence analysis of the $tRNA^{Ile}$ region of the mtDNA by the DNA sequencer (ABI). Since sequence analysis was performed in the 3' to 5' direction, the base substitution of T to C (nt 4269) in our patient is the homologous A to G substitution in the Cambridge sequence.

184-bp amplified fragment was only cleaved into 153 and 31 bp in wild type mtDNA (Fig. 2, lane 5-8). In our patient, only a 184-bp band was detected in the mtDNA from skeletal muscle and blood, and a faint band of 153 bp was also visible in the mtDNA from cardiac muscle (Fig. 2, lane 2-4). Thus our patient was heteroplasmic for the mutation where mutated mtDNA was exclusively dominant. His mother was also revealed to be heteroplasmic, showing maternal transmission of the mutant DNA. However, in his mother, the amount of the mutant mtDNA was much less than wild type mtDNA. The mutant DNA was detected in none of the 30 controls (Fig. 2).

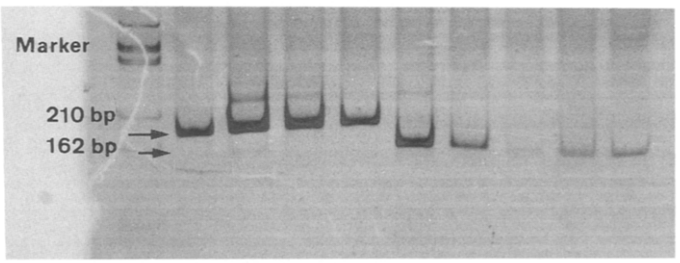

Fig. 2. Restriction enzyme analysis of the $tRNA^{Ile}$ gene fragments amplified by mispairing PCR. Lanes 1-2:patient's cardiac muscle (1:undigested and 2:digested by SspI), lane 3:patient's skeletal muscle, lane4:patient's lymphocytes, lanes 5-9: lymphocytes (5:mother, 6:father, and 7-9:normal controls). Two arrows indicate 184 bp (upper) and 153 bp (lower) fragments.

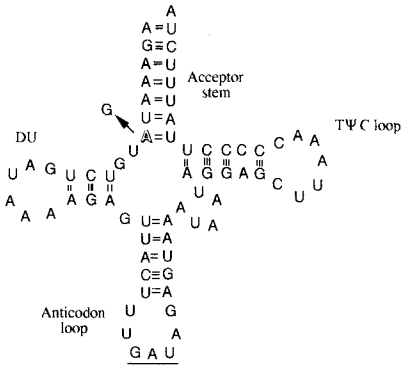

Fig. 3. Tertiary cloverleaf structure of the mitochondrial $tRNA^{Ile}$ gene. The base substitution occurred at the root of the acceptor stem (arrow).

Recent studies showed that mutations in the tRNA genes of mtDNA could cause a mitochondrial encephalomyopathy due to the decreased synthesis of mitochondrial coded proteins (14, 15, 16). Our patient was consistent with the diagnosis of mitochondrial encephalomyopathy by both clinical symptoms and laboratory data, and present the histological characteristics of focal CCO deficiency usually recognized in the established syndromes of abnormal mtDNA tRNA genes (MELAS and MERRF) (4, 11) . The basic defect in our patient was strongly suggested to be located in the mtDNA $tRNA^{Ile}$ genes (A to G substitution at nt 4269) for the following reasons: 1. This mutation was recognized in none of the 30 normal controls (Fig. 2); 2. The base concerned is at the root of the acceptor stem, and it creates tight hydrogen bond between the corresponding base. Thus its substitution should cause the aberrant tertiary cloverleaf structure (Fig. 3), leading to the deficient translation of mitochondrial-coded protein; 3. The described mutation is in the well-conserved nucleotides of the $tRNA^{Ile}$ gene (Fig. 4). However, a mutation in mitochondrial DNA at the widely conserved residue does not warrant that the mutation is the cause of the mitochondrial disease. To overcome the enormous polymorphism, the comparison of the mtDNA sequence of the normal cell with the mutant cell obtained from the same patient was important (17), but the percentage of the mutant mtDNA was almost 100% in this case, resulting in the difficulty of isolating cell lines.

The relatively late onset of the symptoms in this patient may be due to the high threshold of the mutation to cause the

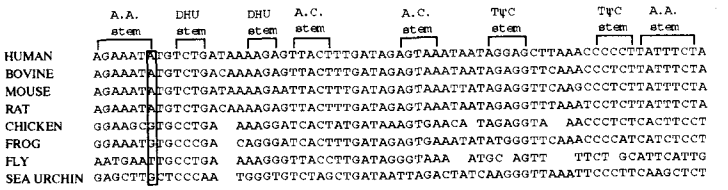

Fig. 4 Homology of the mitochondrial $tRNA^{Ile}$ gene from different species. The boxed nucleotides indicate the position of the mutation observed in our patient.

disease and slow propagation of the mutant DNA to almost all cells. However, these are only speculations and further investigation is required. To date, mutations in the $tRNA^{Leu(UUR)}$ and $tRNA^{Ile}$ genes have been reported in association with fatal cardiomyopathy (2, 8, 9). Protein synthesis in mitochondria may be especially important for the dynamic energy production of the heart muscles.

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