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A NOVEL POINT MUTATION IN THE MITOCHONDRIAL tRNA$^{Ser(UCN)}$ GENE DETECTED IN A FAMILY WITH MERRF/MELAS OVERLAP SYNDROME

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We found a new point mutation in the mitochondrial tRNA$^{Ser(UCN)}$ gene in a family with MERRF/MELAS overlap syndrome by screening for heteroplasmy by means of chemical cleavage of mismatch (CCM). Our strategy was based on the previous observations that most pathogenic mtDNA mutations in mitochondrial encephalomyopathies are heteroplasmic, whereas almost all neutral mitochondrial polymorphisms are homoplasmic. CCM followed by nucleotide sequencing of the corresponding region of the mitochondrial genome revealed a heteroplasmic mutation at nt 7512 in the tRNA$^{Ser(UCN)}$ gene. The 7512 (T to C) mutation disrupts a highly conserved base pair in the acceptor stem, and this mutation was not found in any of 120 normal controls, or in 43 patients with mitochondrial diseases. The proportion of the mutant mtDNA was 93% in muscle, 76 and 87% in the blood of the patients. A family member without apparent neuromuscular symptoms carried less mutant mtDNA. These findings support the view that this mutation is pathogenic in this family. Detection of heteroplasmy by CCM is an efficient means of screening pathogenic mtDNA point mutations.

In the past few years, mtDNA mutations have been found in a broad spectrum of human diseases. The list of pathogenic mtDNA mutations is expanding rapidly and it includes both large-scale deletions or duplications and point mutations in tRNA genes, protein coding regions, and in one of the rRNA genes. Nevertheless, in many patients suspected of having a mitochondrial disorder, mutations have not been detected.

A conspicuous feature of mtDNA mutations in mitochondrial encephalomyopathies is that many of them are heteroplasmic. Mutant and wild type mtDNA coexist in a patient, in a cell, or possibly in a mitochondrion (1-3). The proportion of the mutant mtDNA is the major factor that determines the severity of defective mitochondrial translation and respiratory chain activity (1, 3, 4). A small proportion of wild-type mtDNA seems necessary and is often sufficient to protect cells and patients from lethal mitochondrial failure.

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In contrast to pathogenic mtDNA mutations, neutral polymorphisms are almost always homoplasmic in individuals (5, 6). There are only a few reports describing silent heteroplasmy in humans (7-9). On the basis of these observations, we considered that screening for heteroplasmy could be an efficient means of uncovering pathogenic mitochondrial mutations.

The chemical cleavage of mismatch (CCM) can detect any type of point mutation, including small insertions or deletions (10-12). CCM is based on the fact that mismatched C and T bases in heteroduplexes are more reactive with hydroxylamine and osmium tetroxide, respectively, than matched bases. The C or T residues modified by these chemicals are subsequently cleaved with piperidine, and the presence and the position of mutations can be derived from the length of the cleavage fragments. The advantage of CCM over other screening methods such as single strand conformation polymorphism (SSCP) or degenerating gradient gel electrophoresis (DGGE) is that it can detect all sequence changes (13) and it can be used to screen DNA fragments up to 1000 base pairs.

We applied CCM to screen heteroplasmy in a family with MERRF/MELAS overlap syndrome, and found a novel point mutation in the mitochondrial $tRNA^{Ser(UCN)}$ gene.

MATERIALS AND METHODS

Patients (Fig. 1A): Patient II-3 (proband) was a mentally retarded 26 year old female (described as Patient 2 in ref 14) who has had epileptic attacks since the age of 15. On admission to our hospital at age 20, myoclonic seizures, generalized tonic-clonic seizures and paroxysmal hearing disturbance lasting for a few seconds were documented, and mental deterioration, muscle atrophy, weakness and truncal ataxia were found. Lactate levels in both blood and cerebrospinal fluid were elevated. A brain CT scan revealed cerebral atrophy and calcification of the bilateral basal ganglia. Muscle biopsy showed many ragged red fibers and abnormal mitochondria with concentric cristae. At age 26, muscle atrophy and weakness progressed with mental deterioration, and she developed myoclonic jerks, asterixis, incoordination of hands and a mild hearing disturbance.

Patient I-2, the mother of Patient II-3, was a 55 year old female. She had myoclonic jerks of the arms and generalized seizures since the age of 37. At age 47, she was moderately demented. Muscle weakness or ataxia were not apparent. A brain CT scan revealed calcification of the bilateral basal ganglia and bilateral occipital lobe atrophy. At age 55, she developed blindness after an episode of generalized seizure, and since then she has been bed-ridden and severely demented. T_2-weighted MR images showed transient high intensity area in the right occipital lobe, followed by severe atrophy. Blood lactate and CK levels were elevated. Muscle biopsy revealed marked fiber size variation, ragged-red fibers, which were cytochrome c oxidase negative, and many paracrystalline inclusions in the mitochondria. Strongly SDH-reactive blood vessels (15) were not recognized.

Subject II-1, was a premature female infant who died within a week of birth.

Subject II-2, was a 31 year old male. Although he had no history of epilepsy or myoclonus, he was regarded as having an abnormal personality due to eccentric behavior, and had not been able to work.

Patients II-3 had features of myoclonus epilepsy with ragged-red fibers (MERRF), but Patient I-2 had features of both MERRF and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). We therefore diagnosed both patients as having MERRF/MELAS overlap syndrome according to Zeviani et al. (16).

Methods: Total DNA was extracted from muscle biopsy samples or frozen blood containing EDTA. Mutations of 3243(A to G), 3271(T to C), 3291(T to C), 8344(A to G), 8356(T to C) and 8993(T to G), which are associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), MERRF, neuropathy, ataxia, and retinitis

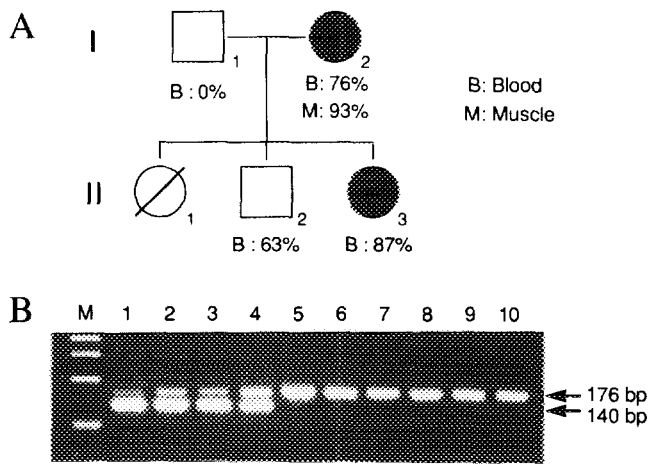

Fig. 1. (A) Pedigree of the family. Solid symbols denote clinically affected individuals. The numbers below the symbols represent percentages of the 7512 mutation in blood(B) or muscle(M). (B) PCR-RFLP analysis of the 7512 mutation. PCR amplified fragments of 176bp are spliced into 140 and 36bp after XhoI digestion when they harbor the mutation. Muscle (lane 1) and blood (lane 2) of Patient I-2, blood of Patient II-3 (lane 3), II-2 (lane 4), I-1(lane 5) and normal controls (lane 6-10). M is a 100bp ladder as a size marker.

pigmentosa (NARP) or Leigh's syndrome, were tested by PCR-RFLP. Southern blotting was performed as described (17).

Screening of heteroplasmy by CCM: When heteroplasmy is present in template mtDNA, heteroduplex products are formed after PCR amplification. We screened for heteroduplex DNA by CCM.

The entire mitochondrial genome was amplified by PCR using 17 sets of 28mer-primers. They were designed to generate 1200-1300bp fragments with overlaps of 200-300bp. PCR was performed with 75ng of template DNA, 0.5μM of each primer, 200μM dNTPs, 20mM Tris-HCl pH8.8, 10mM KCl, 2.0mM $MgCl_2$, 6mM $(NH_4)SO_4$, 0.1% Triton X-100 and 0.1mg/ml BSA, in a volume of 75μl. The hot start PCR conditions were as follows: 1.85U of PFU polymerase (StrataGene) was added to the medium after the reaction tube was heated to 85°C. An initial 3 minute denaturation at 94°C was followed by 33 cycles of 30 sec at 94°C, 1 min at 68°C, 2 min at 72°C, and a terminal extension for 5 min. The PCR products were ethanol precipitated, and the pellets were resuspended in 20μl of $TE_{0.1}$ buffer (10mM Tris-HCl, pH 8.0, 0.1mM EDTA).

The PCR products were then submitted to CCM as described (18) with some modifications. Six μl of the PCR products were modified with hydroxylamine or osmium tetroxide for 60 and 5 minutes at 37°C, respectively. The reaction was terminated and DNA was precipitated by adding 800μl of Stop/Precipitate (200μl of 0.3M sodium acetate, 600μl of 100% ethanol). As a non-modified control, a reaction without hydroxylamine, but processed identically was included. The precipitated DNA fragments were treated with 1M piperidine at 90°C for 30 minutes, ethanol precipitated, and the pellets were then air dried and subsequently resuspended in 6μl of distilled water.

An equal volume of loading dye (95% formamide, 20mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue) was added to the samples, which were heated to 95°C for 2min, then quenched on ice. The DNA fragments were resolved by electrophoresis on polyacrylamide gels (70 x 100 x 0.75mm) containing 7M urea at 60°C. Single strand DNAs were visualized by silver staining (19).

Sequence analysis: Asymmetrically PCR-amplified fragments, or PCR products cloned into pUC118, served as sequencing templates. The sequencing primer was 5'-end-labeled

with [γ-^{32}P]ATP by T_4-polynucleotide kinase, and the fragments were sequenced using a TTH sequencing Kit (TOYOBO).

PCR-RFLP analysis and quantitation of the 7512(T to C) mutation: As a simple analysis for the 7512(T to C) mutation, we developed a PCR-RFLP using a mismatched primer. We designed the reverse primer 5'-GACAAAGTTATGAAATGGTTTTTCTAATACC-TTCTCGA (nt 7550-7513), with two mismatches (underlined) to create a recognition site for XhoI in the presence of the mutation. This primer was combined with a forward primer (nt 7375-7396). The amplified mutant fragment of 176bp was cleaved into subfragments of 140 and 36bp after XhoI digestion, whereas those of the wild type were not. The digestion products were electrophoresed through a 2.5% agarose gel containing ethidium bromide and photographed on a UV-transilluminator.

The mutation was quantified by last cycle hot PCR (20).

RESULTS

Muscle and blood samples from Patient I-2 and blood from Patient II-3, I-1 and II-2 were analyzed. None of the 3243(A to G), 3271(T to C), 3291(T to C), 8344(A to G), 8356(T to C), 8993(T to G) mutations, or large-scale deletions were found in the mtDNA from these patients. Next, we searched for heteroplasmy by means of CCM along the entire length of the mitochondrial genome of muscle and blood from Patient I-2. CCM revealed two of heteroplasmy, one in the fragment between nt 7339-8637 (Fig. 2A), and the other between nt 16020-709 (data not shown).

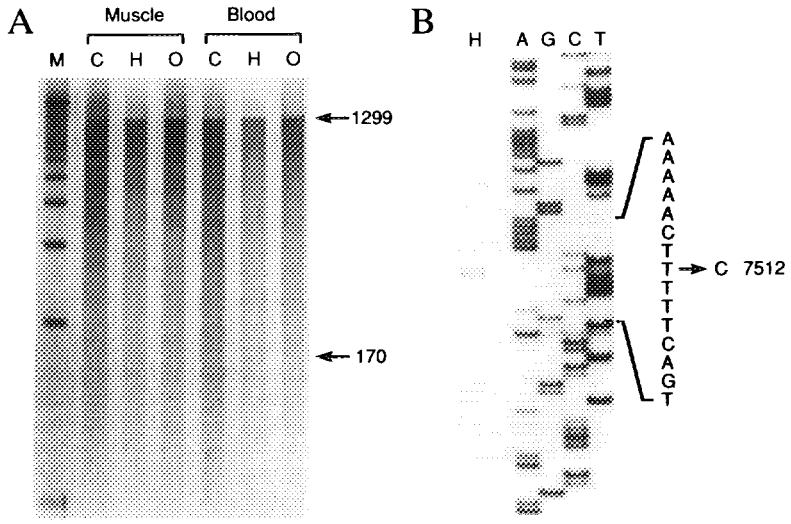

Fig. 2. (A) Detection of heteroplasmy in Patient I-2 by CCM. PCR products between nt 7339-8637 (1299bp) were analyzed by CCM. M, 100 base ladder as size marker; C, control; H, hydroxylamine reaction; O, osmium tetroxide reaction A cleavage band of 170 bases was revealed by the hydroxylamine reaction, and very faintly by that of osmium tetroxide in the blood. The rest of the cleavage band (1130 bases) that appears as the silver stain develops, is not visible in this figure. (B) Direct sequencing of the region encompassing the heteroplasmic nucleotide indicated by CCM. The cleavage band in hydroxylamine reaction(H) shows the position where it is one base shorter than the heteroplasmic transition(T to C) at nt 7512.

When the PCR product of the blood DNA using the forward primer, F8000 (nt 7339-7366) and the reverse primer, R8000 (nt 8637-8610) was analyzed by CCM, cleaved bands of 170 and 1100 bases were identified by the hydroxylamine reaction and very faintly by the osmium reaction (Fig. 2A). These bands were not identified in the reaction of muscle DNA, suggesting that the heteroplasmic mutation in blood is almost homoplasmic in muscle. To determine the position of the mismatch, one of the primers was 5'-labeled with ^{32}P, and hydroxylamine-generated CCM products of the blood of Patient I-2 were visualized by autoradiography. This assay revealed a band of 170 bases when F8000 was labeled. Therefore, the mismatch was presumed to be located about 170 bases from the 5'-end of F8000 (near nt 7510).

We sequenced the muscle DNA of Patient I-2 focusing on this position using ^{32}P-labeled F8000 as the sequencing primer. The CCM product described above was run parallel to the sequence ladder. Because the same primer (F8000) was labeled, the CCM band indicated the position of the heteroplasmy (one base shorter because the mismatched nucleotide is excised), and we found a T to C transition at nt 7512 in the $tRNA^{Ser(UCN)}$ gene (Fig. 2B). No other mutations were detected in this gene.

To assess the functional significance of the 7512 mutation, we compared the mitochondrial $tRNA^{Ser(UCN)}$ gene of various species according to the model proposed by Yokogawa et al. (21) (Fig. 3). Although the base at this site was variable, a base pair in the acceptor stem was strictly conserved through evolution.

The 7512(T to C) mutation was found in Patient I-2, Patient II-3, II-2, but not in I-1 nor in 120 normal controls or 43 patients with mitochondrial diseases (12 with MERRF; 24 with MELAS; 5 with chronic progressive external ophthalmoplegia; 2 with unclassified

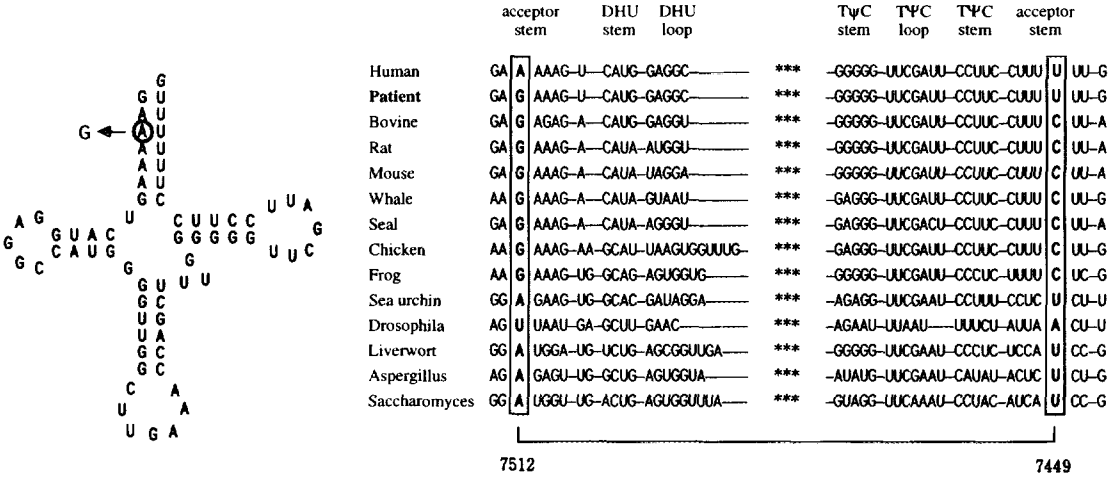

Fig. 3. Location of the 7512 mutation in the secondary structure of $tRNA^{Ser(UCN)}$ according to Yokogawa's model, and a comparison of the $tRNA^{Ser(UCN)}$ among different species. Those of bovine and Saccharomyces cerevisiae were determined at the RNA level, whereas others were deduced from the mtDNA sequences. The boxes show highly conserved base pairs in the acceptor stem.

mitochondrial myopathy: None of them had mutations of MERRF or MELAS) by PCR-RFLP method (Fig. 1B). II-2 carried the mutation, but less abundantly than his clinically affected sister II-3 (Figs. 1A and B).

We also analyzed the heteroplasmy between nt 16020-709 by the same strategy as for the 7512 mutation, and found length heteroplasmy in the CSB-II region, which has been described previously (7). It was also found in normal controls, and therefore it is unlikely to be pathogenic.

DISCUSSION

We described here a novel point mutation in the mitochondrial $tRNA^{Ser(UCN)}$ gene in a family with MERRF/MELAS overlap syndrome. We suggest that the 7512(T to C) mutation is pathogenic for the following reasons: (1)The mutation disrupts a highly conserved base pair in the acceptor stem of a tRNA. (2)The mutation was not found in controls. (3)The mutation was heteroplasmic and affected individuals carried more mutant mtDNA than an unaffected family member. The 7512 mutation may be considered as another mutation associated with MERRF/MELAS overlap syndrome (16). To confirm its pathogenicity and phenotypic association, we should identify the mutation in unrelated patients with MERRF/MELAS overlap syndrome.

Recently, Yokogawa et al. purified bovine mitochondrial $tRNA^{Ser(UCN)}$ and determined its sequence and secondary structure (21). They showed that the formerly supposed $tRNA^{Ser(UCN)}$ gene was incorrect. The tRNA starts from the third nucleotide of the putative $tRNA^{Ser(UCN)}$ gene sequence and possesses an unusual secondary structure. This novel structure suggests a more stable acceptor stem. The $tRNA^{Ser(UCN)}$ of humans and other mammals can be rearranged in a similar manner (Fig. 3) (21, 22). According to Yokogawa's model, the base pair between nt 7512 and nt 7449 in the acceptor stem is highly conserved either as A-U or G-C pairs. The 7512(T to C) mutation alters it to weak G-U bond. It may decrease the stability of the acceptor stem and affect the function of the tRNA (23). Examples of pathogenic mitochondrial tRNA point mutations which replace A-U or G-C pairs to G-U pair in acceptor stems include nt 3302(A to G) mutation in the $tRNA^{Leu(UUR)}$ gene (24) and nt 4269(A to G) mutation in the $tRNA^{Ile}$ gene (25, 26). The former is associated with abnormal RNA processing, and the latter with decreased level of the tRNA and reduced mtDNA-encoded polypeptide synthesis. The 7512 mutation may have similar mechanism to cause cellular dysfunction.

The mtDNA of patients suspected of having a mitochondrial disorder can be extensively sequenced. Nevertheless, the determination of pathogenic mtDNA point mutations is not straightforward. The major reason for this is the high number of polymorphisms in the mtDNA of normal individuals. As many as forty base changes from the standard sequence are encountered in any individual. To identify a possible pathogenic mutation from these polymorphisms, each base change must be inspected according to minimal criteria: the base (or codon) change is absent in unaffected individuals, and the base (or codon) change is in an evolutionarily conserved region (27). However, inter-species comparisons of mtDNA are not absolute and they may be misleading as noted above.

Screening mitochondrial point mutations by CCM has advantages. CCM ignores neutral polymorphisms, which are generally homoplasmic, and a base identified by CCM is likely to be a pathogenic mutation, which is mostly heteroplasmic.

One drawback of CCM as a screening method of mtDNA point mutations is that it cannot detect homoplasmic or nearly homoplasmic mutations. In this study, we detected the 7512 mutation in blood which contained 76% mutant DNA, but not in muscle, which contained 93%. Mitochondrial mutations found in patients with Leber's hereditary optic neuropathy or mildly deleterious mtDNA mutations are homoplasmic (28), and the 8344(A to G) mutation is nearly homoplasmic in some patients with MERRF (29). CCM may miss these mutations. In such situations, however, screening of the mitochondrial base differences between patients and healthy maternal relatives by CCM may reveal the mutation.

In conclusion, the detection of heteroplasmy by CCM is an efficient means of screening pathogenic mtDNA point mutations.

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