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Tissue-Specific Involvement of Multiple Mitochondrial DNA Deletions in Familial Mitochondrial Myopathy

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It is still uncertain how deleted mitochondrial DNA (mtDNA) is distributed to each tissue during development, although deletions of mtDNA have been extensively observed in various pathologic conditions. This paper presents two Japanese siblings with progressive external ophthalmoplegia exhibiting multiple mtDNA deletions. In one patient, similar multiple mtDNA deletions were found in skeletal muscle specimens as well as in the spinal cord but not in the myocardium, liver or leukocytes. A similar deletion pattern was found in the skeletal muscle but not in the leukocytes of the other patient. The results suggest the complex mechanism to generate, expand and eliminate the deleted mtDNA in humans.

Multiple deletions of mtDNA have been detected in various pathologic conditions: autosomal dominant (1-4) and autosomal recessive (5)/ progressive external ophthalmoplegia (AD-PEO and AR-PEO); a mitochondrial multisystem disease with PEO, peripheral neuropathy, and intestinal pseudo-obstruction (MNGIE) (6,7); and a variety of disorders with diverse clinical manifestations (8,9). These multiple deletions are also present in other conditions such as sporadic inclusion body myositis (s-IBM) (10,11), a late-onset form of mitochondrial myopathy (LOMMP) (12), and even in tissues from healthy elderly people (13), although we do not know their exact pathogenic role. This paper reports two siblings with PEO who had similar patterns of multiple mtDNA deletions in their skeletal muscle specimens. In one patient, deletions were identified in the spinal cord as well as in the skeletal muscle but not in the myocardium, liver or leukocytes. A similar deletion pattern was present in the skeletal muscle but not in the leukocytes of the other patient. Based on these findings, we discuss the origin and expansion (or regulation) of multiple mtDNA deletions in the context of "tissue specificity" of mitochondrial DNA abnormality.

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MATERIALS AND METHODS

Patients. Two Japanese siblings with PEO, a male (Patient 1) and his younger sister (Patient 2), born of unrelated parents, are presented. FIG. 1 outlines the pedigree of the patients and designates them as II-6 and II-7, respectively.

Patient 1 had been well until the age 24, when he first noticed bilateral blepharoptosis. He remained untreated for the next decade because his symptoms had not significantly progressed. At age 39, dysarthria became evident, followed by dysphagia four years later. Soon afterward he was hospitalized due to ataxic gait. Physical examination found his height to be 152 cm and his weight 41 kg. Cardiorespiratory signs were normal. He had generalized muscle weakness and atrophy, including the facial and laryngopharyngeal muscles. Severe blepharoptosis and ophthalmoplegia with absolute limitation of eye movement were also observed. Serum creatine kinase (CK) was 336 IU/l (normal < 30), of which CK-MB comprised 4%. Serum lactate and pyruvate values at rest were 29 mg/dl (normal, 9-16) and 1.0 mg/dl (normal, 0.3-0.6), respectively. Electrocardiography and echocardiography revealed no cardiac dysfunction. Computed tomography of the brain revealed slight cerebral cortical and cerebellar atrophy. Muscle biopsy specimens from the quadriceps and gastrocnemius showed variation in muscle fiber size and, by Gomori-trichrome staining, scattered ragged-red fibers (RRFs). The cytochrome *c* oxidase stain revealed many fibers with deficient enzyme activity. The patient was diagnosed as having mitochondrial myopathy at age 45. Muscle weakness and atrophy, dysphonia, dysphagia and bilateral ophthalmoplegia gradually progressed over the next 10 years. At age 56, he died of pneumonia complicated by respiratory muscle weakness.

From the autopsy, generalized atrophy of skeletal muscle was evident. Histologically, proliferation of muscle fiber nuclei, muscle fiber degeneration, and RRF were observed in the biceps, quadriceps, ilio-

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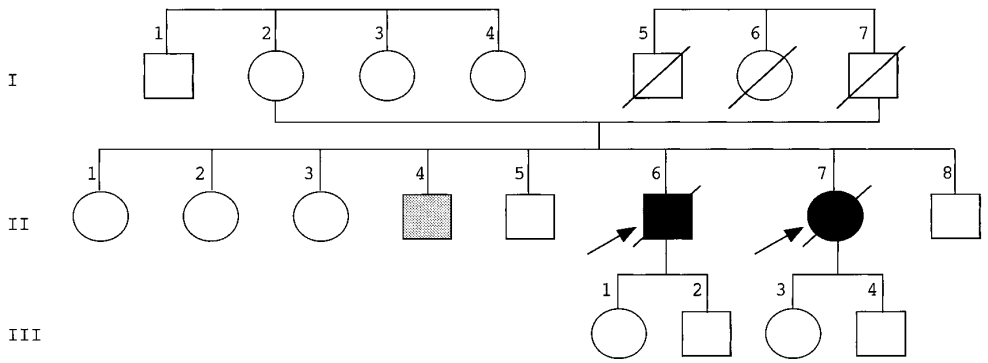

FIG. 1. Family pedigree. Solid symbols indicate symptomatic individuals. The gray-shaded symbol indicates a presymptomatic individual. Barred symbols indicate deceased individuals. Arrows indicate the probands.

psoas, gastrocnemius and pectoralis major muscles. Subsarcolemmal proliferation of abnormal mitochondria containing paracrystalline inclusions was observed from electron microscopy. These inclusions showed parallel lines variously oriented in terms of mitochondrial cristae. No atrophic muscle fibers or abnormal mitochondria were present in the myocardium. Autopsy of the brain was not permitted.

Patient 2 had been healthy until age 35, when ataxic gait developed and muscle weakness became evident in the lower extremities. Despite medication initiated at age 36, her symptoms did not significantly improve. At age 39, she noticed bilateral blepharoptosis. Muscle weakness progressed until she was hospitalized at age 40. On examination her height was 149.5 cm and her weight 37 kg. Vital signs were stable. Bilateral blepharoptosis and limitation of eye movement in all directions were observed. Muscle atrophy was generalized with proximal accentuation. Serum CK was 67 IU/l. Serum lactate and pyruvate levels at rest were 16.7 and 1.2 mg/dl, respectively. Morphologic studies of the biceps muscles showed quite similar findings to those of Patient 1. An electrocardiogram showed no signs of ischemia or arrhythmia. She was also diagnosed as having mitochondrial myopathy, and symptoms slowly progressed over the next decade. She died of pneumonia at age 50 after a clinical course similar to that of her brother. Autopsy was not permitted.

An older brother (II-4 in FIG. 1) had dysarthria, but no other members of the family revealed any neuromuscular disease.

DNA extraction. The muscle biopsy and autopsy samples were frozen and stored at −80° C until DNA extraction. DNA was isolated by a standard phenol/chloroform extraction procedure and resuspended in TE solutions.

Southern blot hybridization. Each 2 μg of total DNA was digested overnight at 37° C by a restriction enzyme *Sph*I (TaKaRa BIOMEDICALS), which cut mtDNA once at nucleotide position (nt) 2436 (14), and then electrophoresed on a 0.8% agarose gel at 30 volts for 24 hours. DNA was blotted onto a nylon membrane (Hybond-N+, Amersham) and fixed under a UV Crosslinker (Amersham) according to the manufacturers' protocol. To probe a DNA fragment, we used ECL Probe-Amp Labelling Reagents (Amersham) with a set of primers; P335: 5′-(531)TAACCCCATACCCCGAACCA(550)-3′ and P336: 5′-(718)TCACTGGAACGGGGATGCTT(699)-3′. Hybridization and detection reactions were carried out with ExpressHyb Hybridization Solution (Clontech) and *Gene Image* CDP-*Star* Detection Module (Amersham) according to the manufacturers' protocols. The proportion of deletions was estimated by means of ImageMaster (Pharmacia).

Long polymerase chain reaction (PCR) and sequencing of the deletion junction. We modified the method described previously (15). The extracted DNA was amplified by *TaKaRa Ex Taq* (TaKaRa BIOMEDICALS). We used two sets of primers from original sequences (14) as follows; P1001: 5′-(5205)TCCACCCTCCTCTCCCTAGG-(5224)-3′ and P1004: 5′-(16422)ATTGATTTCACGGAGGATGG-(16403)-3′ for a larger portion, and P329: 5′-(15756)GAATCGGAG-GACAACCAGTA(15775)-3′ and P318: 5′-(5739)GCGGGAGAAG-TAGATTGAAG(5720)-3′ for a smaller portion of the mtDNA between O_H and O_L. The PCR conditions were 30 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 8 minutes followed by an additional extension at 72°C for 10 minutes. Amplified DNA fragments were electrophoretically checked on a 1.0% agarose gel (15). A major type of deletion was subjected to the previously described sequencing procedures for determination of the deletion junction.

RESULTS

Southern blot analysis demonstrated multiple mtDNA deletions in the skeletal muscles and spinal cord of Patient 1 and the biopsied skeletal muscle of Patient 2. The bands of 16.6 kb and 9.0 kb corresponded to the full-length and the major deleted mtDNA, respectively, indicating that the size of deletion is 7.6 kb (FIG. 2A). The proportion of deleted mtDNA was 60.7% in quadriceps, 63.1% in gastrocnemius, 63.6% in pectoralis major, 48.3% in trapezius, 54.0 % in iliopsoas muscle of patient 1 and 55.8 % in biceps muscle of patient 2. Deletions were not present in the myocardium, liver or leukocytes of Patient 1 nor in the leukocytes of patient 2; this was confirmed by long PCR for a larger portion of the mtDNA between OH and OL.

The PCR products of 11.2 kb and 3.6 kb corresponded to the normal length and the major detected mtDNA, respectively, indicating that the size of deletion is 7.6 kb (FIG. 2B). No deletion was detected by long PCR for a smaller portion (data not shown). The deletion breakpoint of the major band was identified at nt 6331 to 13993, which was different from the so-called hot spot region.

Bands represented as deletions on Southern blot included the major one and other minor bands. It should be noted that a deletion pattern, which was dependent on the species number and the amount of each deletion, was similar among several muscles of patient 1, and even muscle of patient 2. This similarity was reproduced by means of long PCR analysis although the

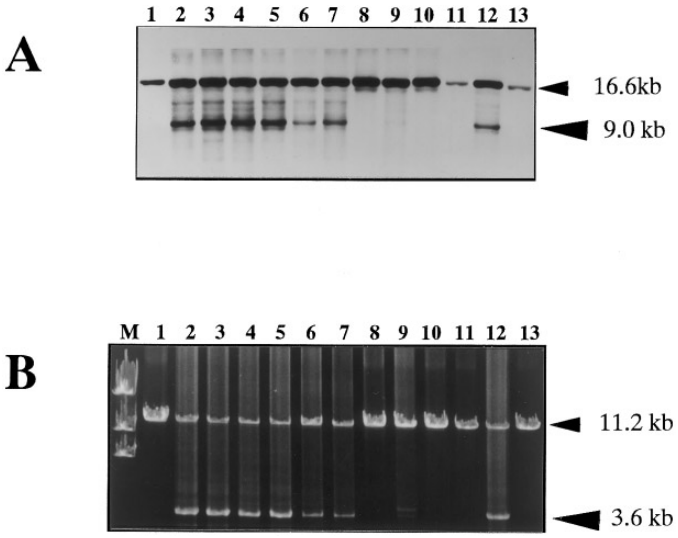

FIG. 2. (A) Southern blot analysis. Lane 1, normal control; The total DNA from Patient 1, lane 2, quadriceps muscle; lane 3, biceps muscle; lane 4, gastrocnemius muscle; lane 5, pectoralis major muscle; lane 6, trapezius muscle; lane 7, iliopsoas muscle; lane 8, heart muscle; lane 9, spinal cord; lane 10, liver; lane 11, leukocyte. Lane 12, skeletal muscle and lane 13, leukocyte from Patient 2. The bands of 16.6 kb (small arrow) and 9.0 kb (large arrow) correspond to the full-length and the major deleted mtDNA, respectively, indicating that the size of deletion is 7.6 kb. (B) Detection of deletions by long PCR for a larger portion. The bands of 11.2 kb (small arrow) and 3.6 kb (large arrow) correspond to the normal length and the major detected mtDNA, respectively, indicating that the size of deletion is 7.6 kb. The numbers at the top correspond (A), M; size marker (λ/ Hind III digest).

method might not necessarily reflect the original relative proportion of deletions.

DISCUSSION

Mitochondria, including mtDNA, are exclusively transmitted through maternal lineage. Very low amounts of deleted mtDNA were detected in all the "normal" oocytes examined (16), but mtDNA deletions are usually detected in sporadic cases with PEO. Furthermore, Larsson et al. (17) demonstrated the absence of deleted mtDNA transmission from a woman with Kearns-Sayre syndrome to her child. These findings suggest the possible existence of mechanisms to eliminate abnormal mtDNA or to control its expansion. In contrast, duplication of mtDNA, as similar to rearranged DNA structure as deletion, was reportedly transmitted through maternal inheritance in several families (18). The present patients had no duplicated mtDNA (data not shown).

Two facts are noteworthy in our study. First, multiple deletions in the restricted tissues are present in the studied family. There have been several reports in which multiple mtDNA deletions were detected in skeletal muscles but not in leukocytes (1-4,8) and two reports in which multiple deletions were detected in both tissues (19,20). Multiple deletions were almost always detected at variable levels in other tissues (4,20), but our patient was the first case where multiple deletions were not found in the myocardium, as confirmed by PCR analysis. Second, the multiple deletions among muscle specimens have similar deletion patterns in the two siblings. Previous reports demonstrated with Southern blot that a similar deletion pattern was observed among several skeletal muscles (3,4,8,20,21) and between muscles of affected members in a family (2,4,5,22).

Some possible mechanisms may provide an explanation for these phenomena of tissue-specific distribution and deletion pattern similarities. Clonal expansion that increases the amount of deleted mtDNA in a muscle cell was described in a patient with AD-PEO (23). In relation to this, one can speculate that frequent *de novo* deletions in each mitochondrion have occurred in each muscle cell of the patients. If this factor is solely responsible for multiple mtDNA deletions, deletions must occur randomly, resulting in more diverse deletion patterns than those seen in our patients. Therefore, the patients could share an non-random origin or certain effect to cause the similar mitochondrial deletions during development.

Another possibility is that a tissue-specific or cell-specific mechanism changes the efficiency to expand one genotype of mtDNA rather than the other(s). Jenuth et al. (24) recently demonstrated tissue-specific selection for different mtDNA genotypes in mice. Although they did not mention muscle tissues, two different mtDNA genotypes were segregated in opposite directions in blood/spleen vs. liver/kidney. Therefore, it can be speculated that a certain tissue-specific selection mechanism is present. If quite a low level of inherited or *de novo* mtDNA deletions in oocytes are present and the selective direction is altered due to a deficiency of the mechanism, this could explain the similarity of deletion patterns observed in our patients.

Multiple mtDNA deletions have been detected not only in such familial patients with AD-PEO and AR-PEO but also in aged tissues or in other diseases. In addition, no other sibling of the studied patients was clinically defined as having mitochondrial myopathy, and the patients exhibited a quite similar mtDNA deletion pattern in the restricted tissues. These findings suggest that putative nuclear encoded gene(s) plays some role(s) in controlling deleted mtDNA distribution. Because more than two loci have already been mapped in AD-PEO families (25,26), identification of the responsible genes would help to clarify the pathogenesis and, moreover, would probably be associated with the mechanisms mentioned above.

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