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Identification of a Mitochondrial DNA Mutation in Paraffin-Embedded Muscle Tissues

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We investigated feasibility of using the formalin-fixed and paraffin-embedded tissue to study mitochondrial mutations in the case that fresh or frozen tissue, or blood samples are not available. Four paraffin blocks of muscle biopsies in Korean MELAS (*mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes*) patients were chosen. Total DNA was extracted from these blocks for PCR/RFLP analysis, and sequencing was performed to study the most common mutation, A to G transition at nucleotide position 3243 underlying MELAS in the mitochondrial $tRNA^{Leu(UUR)}$ gene. We could identify the A to G mutation at nt.3243 in three MELAS patients. Our results show that the mitochondrial genome of our paraffin blocks is presumably in good condition. Our results are in accordance with the previous findings by other investigators that PCR allows molecular genetic analysis of paraffin-embedded tissues stored in most histopathology laboratories.

Key words – paraffin-embedded tissue, PCR/RFLP analysis, sequencing, mitochondrial DNA mutation, MELAS

Human mitochondrial DNA (mtDNA) is a small, circular, double-stranded molecule of 16,569 base pairs (bp) containing two ribosomal genes, 22 transfer RNAs, and 13 polypeptide genes, all of which are subunits of components of the respiratory chain complexes. A number of point mutations in both mitochondrial protein coding genes and tRNA genes have recently been found associated with diseases like LHON (*Leber's hereditary optic neuropathy*), NARP (*Neurogenic weakness ataxia and retinis pigmentosa*), MERRF (*Myoclonic epilepsy and ragged red fibers*) and MELAS (*Mitochondrial encephalopathy lactic acidosis and stroke like episodes*), *etc.*[4].

Among them, A to G transition (A>G) mutation at nucleotide position (nt.) 3243 in mitochondrial $tRNA^{Leu(UUR)}$ has been demonstrated in a high proportion of patients with clinical features of MELAS. MELAS is a maternally inherited, multisystem disorder characterized by lactic acidosis, episodic vomiting, seizures, migrainelike headaches, short stature, and recurrent cerebral insults resembling strokes and causing hemiparesis, hemianopia, or cortical blindness. At least nine point mutations have been associated with this disorder : a G>A mutation at nt. 1642 ; A>G mutation at 3243 (in about 80% of MELAS cases); A>G mutation at 3252; A>G mutation at 3260; T>C mutation at 3271; T>C mutation at 3291; A>G mutation at 5814; T>C mutation at 9957; and G>A mutation at 13513 [5-7]. The nucleotide positions correspond to the published mtDNA sequence[1]. In this disorder, the population of wild-type and mutant-type mtDNA molecules coexists, a situation known as *heteroplasmy*. There are several lines of investigation that support the pathogenic role of these mtDNA mutations in MELAS. It was known that the 3243 (A to G) mutation alters an evolutionarily highly conserved position in the tRNA molecule and has been further implicated in the disease by in-vitro and in-vivo studies [1,2].

Paraffin-embedded tissues are a valuable source of DNA for molecular genetic analysis, when fresh or frozen tissue or blood samples are not available. Determination of mutations of mtDNA in paraffin-embedded tissues by PCR-based methods is possible, if the amplified fragments are small. We have previously shown the feasibility of using PCR to amplify fragments of DNA from paraffin sections from patients with *Kearns Sayre syndrome* (KSS) and *chronic progressive external ophthalmoplegia* (CPEO). We could demonstrate the so-called "4977 bp common deletion" on these patients by amplifying the size of 123 and 152 bp fragments[9,10].

In an attempt to study the molecular nature of Korean MELAS patients and also to study further feasibility of using formalin-fixed and paraffin-embedded tissue to study mitochondrial DNA mutations, especially point mtDNA

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mutations, PCR (*polymerase chain reaction*)/RFLP (*restriction fragment length polymorphism*) analysis and mtDNA sequencing were performed. We could identify the 3243 (A to G) point mutation in three paraffin-embedded tissues of MELAS patients.

Methods and Materials

Pathological analysis

The diagnosis of MELAS patients was made by clinical presentations, neurological examinations combined with enzyme histochemistry and characteristic electron microscopic findings (data not shown).

DNA extraction from paraffin-embedded muscle tissues and fresh muscles

Total DNA was extracted from paraffin blocks with xylene by a modified method of Love *et al.* (1993). Small amounts of tissues were cut from paraffin blocks using a sterile scalpel blade and incubated with 400 μl xylene and 500 μl mineral oil for 2 h at room temperature under gentle agitation. After centrifugation (5 min, 12,000×*g*), the paraffin-containing supernatant was discarded, and the extraction was repeated with 400 μl xylene. In order to remove remaining xylene, the pellet was extracted with 400 μl ethanol. After solubilization in 140 μl PCR-TE buffer (10 mM EDTA, 200 mM Tris-HCl, pH 8.0), 20 μl 10% SDS and 80 μl 1% proteinase K were added and incubated overnight at 55℃. DNA was further purified by phenol/chloroform extraction, and the resulting pellets were resuspended in 50 μl PCR-TE buffer and used for PCR. Total DNA was isolated from fresh muscles (a healthy individual and a MELAS patient) using standard protocols.

Polymerase chain reaction (PCR)

With six DNAs, PCR amplifications were carried out in a volume of 100 μl using 20 μM of each dNTP, 2 units *Taq* DNA polymerase, 10 X *Taq* buffer, 30 pM of each primer (a forward primer, 3116-3134 and a reverse primer, 3353-3333) and 50 ng of template DNA. After a single predenaturation step (10 min, at 95°C), PCR was done by 1 min. denaturation at 94°C, 1 min. annealing at 55°C and 1 min. elongation at 72°C for 30 cycles in a Perkin-Elmer PCR-Thermal Cycler. PCR products were electrophoresed in a 1% agarose gel and the amplified fragments were visualized by ethidium bromide staining.

Restriction fragment length polymorphism (RFLP) analysis and sequencing

Half microliter (50 μCi) ^{32}P-*alpha*-dATP was added on 90 μl PCR products and additional 1-3 same PCR was done as above. If one uses radioactive dNTPs in the PCR reaction, the digested products can be visualized on an autoradiogram of the gel, and even as little as 1% of a mutant population of mtDNAs can be observed. Ten μl of radio-labeled PCR products was digested with diagnostic enzyme (HaeIII) overnight. The restriction digests were electrophoresed in a 12% polyacrylamide gel, the gel was put in cassete and developed with X-ray film overnight. The 4 PCR-amplified fragments (one normal and three patients) encompassing the 3243 (A to G) mutation were sequenced in an ABI Prism 310 Genetic analyzer using Big Dye Terminator Cycle Sequencing Reaction Kits to confirm the PCR/RFLP results and also, to check other mutations in this region.

Results and Discussions

Since the 3243 A to G mutation is common in MELAS, we decided to screen this mutation first. To screen it, the DNA was PCR-amplified using two primers : a forward primer (5' to 3') at nt. 3116-3134 and a reverse primer (5' to 3') at nt. 3353-3333 to amplify a 238 bp fragment which contains the 3,243 mutation. After PCR, the 10 μl of each sample reaction tube was analyzed on a 1% agarose gel and we could find the PCR-amplified 238-bp fragments in all of six samples (Fig. 1).

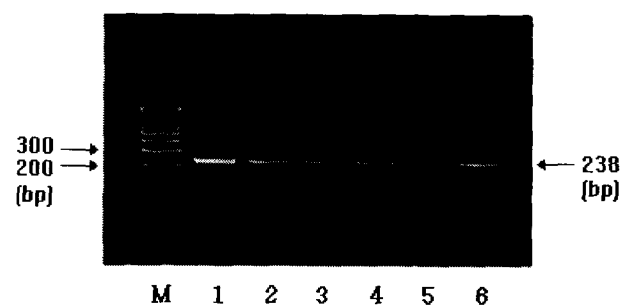

Fig. 1. Agarose gel electrophoretic analysis of the PCR products.
PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. M denotes molecular weight marker (Bioladder TM100). Lane 1 and 2 show 238 bp PCR products from a healthy person (fresh muscle) and from a MELAS patient (MELAS control, fresh muscle), respectively. Lane 3-6 show PCR products from four MELAS patients (paraffin- tissues); cs 96-1606, cs 96-1863, cs 95-3263 and cs 94-10142.

RFLP analysis was done with these 238 bp fragments. Two Hae III enzyme sites are present in these 238 bp fragments. As shown schematically in Fig. 2, the A to G mutation at 3243 will create a new Hae III restriction enzyme site which is diagnostic for the 3243 MELAS mutation. The 238 bp fragments are fragmented into 169, 37 and 32 bp fragments in the case of wild-type molecules. The 169 bp fragment which contains the region of 3243 is further digested into 97 and 72 bp fragments (the characteristic fragments of MELAS) in MELAS. Therefore, we can expect 169, 97, 72, 37 and 32 bp fragment in MELAS patients due to the heteroplasmic nature of this mitochondrial disorder. As shown in Fig. 3, we could find 97 and 72 bp fragments in three MELAS patients (No.3-5), but in a MELAS patient (cs 94-10142) these fragments were not shown (No.6). The results of the PCR/RFLP analysis was further confirmed by sequencing. As shown in Fig. 4, the MELAS 3243 (A to G) mutation was found in all three MELAS patients. We could not find this 3243 mutation in the patient (cs 94-10142) and no other sequence abnormalities were noted in this patient. At the moment, we are studying other tRNA regions of the mtDNA of this patient, because tRNA genes seem to be hot spots for point mutations to cause mitochondrial disorders. Many pathogenic point mutations have been reported in the tRNA regions of mtDNA[4,5,7].

Another notable finding in this study is that we could

Forward Primer (3116-3134) **5'-CCTCCCTGTACGAAAGGAC-3'**
Reverse Primer (3353-3333) **5'-GTAACATGGGTAAGATTA-3'**

Size of the PCR-amplified fragment : 238 bp

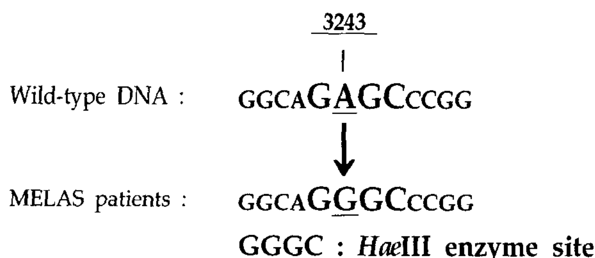

Fig. 2. Schematic representation of the PCR amplification strategy to study the 3243 mutation in MELAS.
The A to G transition mutation at 3243 will create a new Hae III restriction enzyme site which is diagnostic for the 3243 MELAS mutation. The 238 bp fragments are fragmented into 169, 37 and 32 bp fragments in the case of wild-type molecules. The 169 bp fragment which contains the region of 3243 is further digested into 97 and 72 bp fragments (the characteristic fragments of MELAS) in MELAS.

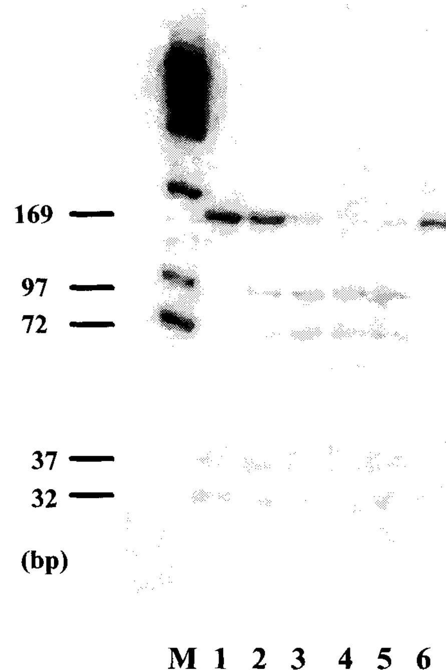

Fig. 3. RFLP analysis of the 3243 MELAS mutation.
PCR products was digested with diagnostic enzyme (HaeIII) overnight and electrophoresed in a 12% polyacrylamide gel. The gel was put in cassete and developed with X-ray film overnight. M denotes molecular weight marker and lane 1,2 show HaeIII-digested 238 bp PCR products from a healthy person (fresh muscle) and from a MELAS patient (MELAS control, fresh muscle), respectively. Lane 3,4,5,6 show HaeIII-digested 238 bp PCR products from four MELAS patients (paraffin-tissues); cs 96-1606, cs 96-1863, cs 95-3263 and cs 94-10142. Five fragments of 169, 97, 72, 37 and 32 bp are shown in three MELAS patients due to the heteroplasmic nature of this mitochondrial disorder (lane 3,4,5).

identify PCR-amplified fragments on DNAs extracted from the paraffin-embedded samples and we can use paraffin-blocks for molecular genetic study. Most laboratories prefer to use fresh muscle samples than paraffin blocks, because DNA in paraffin blocks is partially degraded into smaller fragments due to the extensive and possibly deleterious interaction between fixative and nucleic acids. Determination of mutations of mtDNA in paraffin-embedded tissues by PCR-based methods is possible, if the amplified fragments are small. As to the size of the DNA target amplified, a fragment size of approximately 300 bp or smaller was found to be optimal, although target sequences exceeding 500 bp could be amplified in many instances[13].

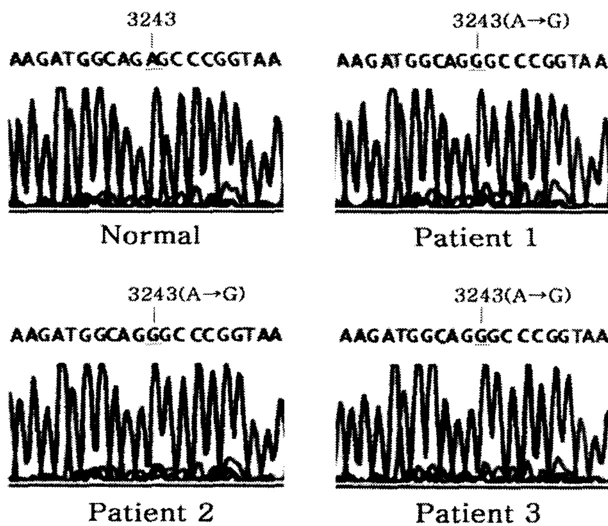

Fig. 4. Nucleotide sequences in the area near the 3243 (A to G) MELAS mutation.
Chromatograms represent nucleotide sequences of normal, wild-type (healthy person, Normal) and mutated (A to G at 3243) DNA fragments (Patient 1,2,3).

It is also noteworthy to mention that in our experiments, DNA was successfully amplified from archival material, although PCR inhibitors may be present in our DNA. The existence of an as-yet-unidentified PCR inhibitor which is intrinsic to paraffin-embedded tissue has been inferred from the results of many studies. It was reported that although PCR may be successfully performed directly on pieces of tissue simply scraped off a paraffin block or a stained histological tissue section, only standardized DNA extraction allows reproducible quantitative preparation of DNA and thus "diluting out" of PCR inhibitors[2,3,8,16]. In our hands, the tissues were extracted twice with xylene to remove paraffin and further, with ethanol to remove remaining xylene before phenol/chloroform extraction.

Our results are in accordance with the findings that PCR allows detailed genetic analysis of formalin-fixed paraffin-sections and the archives of paraffin-embedded tissue stored in most histopathology departments represent a valuable repository of genetic material for future investigation. Many systematic analysis are being done to assess the suitability of archival material for molecular genetic studies[14,18].

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초록 : 파라핀조직을 이용한 미토콘드리아 DNA 돌연변이 확인

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환자의 생조직, 얼린조직 혹은 혈액이 없는 경우에, formalin으로 고정된 파라핀조직을 이용하여 미토콘드리아 돌연변이를 확인할 수 있는지를 조사하였다. MELAS 환자 4명의 파라핀조직을 택해 이들 조직으로부터 DNA를 추출하여 대부분의 MELAS 환자 미토콘드리아 DNA의 $tRNA^{Leu(UUR)}$ gene의 3243 지역에서 발견되는 Adenine의 Guanine으로의 염기치환을 확인하고자 하였다. 실험결과 3명의 환자에게서 이 점 돌연변이를 확인할 수 있어 이들 파라핀조직의 상태가 좋은 것으로 여겨져 미토콘드리아 DNA 돌연변이 연구에 파라핀조직을 활용할 수 있을것으로 보인다.