Histochemical and Molecular Genetic Study of MELAS and MERRF in Korean Patients

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episode (MELAS) and myoclonic epilepsy and ragged-red fibers (MERRF) are rare disorders caused by point mutation of the tRNA gene of the mitochondrial genome. To understand the pathogenetic mechanism of MELAS and MERRF, we studied four patients. Serially sectioned frozen muscle specimens with a battery of histochemical stains were reviewed under light microscope and ultrastructural changes were observed under electron microscope. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed and the tRNA genes were sequenced to confirm mutations. In two patients with MELAS, strongly succinyl dehydrogenase positive blood vessels (SSVs) and many cytochrome oxidase (COX) positive ragged-red fibers (RRFs) were observed, and A3243G mutations were found from the muscle samples. In two patients with MERRF, neither SSV nor COX positive RRFs were seen and A8344G mutations were found from both muscle and blood samples. In the two MERRF families, the identical mutation was observed among family members. The failure to detect the mutation in blood samples of the MELAS suggests a low mutant load in blood cells. The histochemical methods including COX stain are useful for the confirmation and differentiation of mitochondrial diseases. Also, molecular biological study using muscle sample seems essential for the confirmation of the mtDNA mutation.

Key Words : *MELAS Syndrome; MERRF Syndrome; Korea*

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INTRODUCTION

Human mitochondrial DNA (mtDNA) is a double-stranded, 16.6-kb circular genome that codes 37 genes, all of which are essential for the structural and functional maintenance of the mitochondrial respiratory chain (1). So far, various pathogenic mutations have been reported affecting mtDNA (2). Among them, mutations in tRNA gene produce translation defect and thus, produce global dysfunction in mitochondrial respiratory chain. Both mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episode (MELAS) and myoclonic epilepsy and ragged-red fibers (MERRF) are caused by such mechanism. In addition, they have common clinical features including high serum lactate level, ragged-red fibers (RRFs) on muscle biopsy, and overlapping neurological findings such as myopathy and seizure. In MELAS and MERRF, the mutations tend to cluster in some specific sites (3), which enables the polymerase chain reaction-restriction length polymorphism (PCR-RFLP) an effective strategy for

the molecular study. The main purpose of this study was to evaluate pathological and molecular genetic features, and thus, to approach its pathogenesis among Korean patients with MELAS and MERRF.

METHODS

Patients

Among 14 unrelated biopsy-proven patients with mitochondrial disease diagnosed by the authors, two MELAS and two MERRF cases were selected for the study. All showed typical clinical features of MELAS or MERRF with lactic acidosis and RRFs on muscle biopsy. Muscle phosphorus[32]-magnetic resonance spectroscopy was performed in 3 cases and all showed a decrease in phosphocreatine (PCr)/inorganic phosphate (Pi) ratio, which is typical of mitochondrial myopathies (4). Before beginning of the study, the purpose and

the extent of the experiment was explained to the patients and their family members, and written consents were obtained. Detailed clinical and laboratory features of the cases are shown in Table 1.

Pathological study

Muscle samples were obtained from biceps muscles by open biopsy, frozen immediately in liquid nitrogen-cooled isopentane, and stored at -70℃ until used. Fresh frozen blocks of biopsied muscle were sectioned at 10 μm thickness, processed for modified Gomori-trichrome (MGT), succinyl dehydrogenase (SDH), ATPase at pH 4.3, and cytochrome oxidase (COX), and were finally reviewed under light microscope. For electron microscopy, the samples were fixed with 2% glutaraldehyde and embedded in epoxy resin accord-

ing to the standard procedure. Semithin 1 μm thick resin sections were stained with toluidine blue for light microscopy. Ultrathin sections (60 nm thick) were cut with diamond knives on an ultramicrotome (REICHERT SUPER-NOVA™, Leica, Germany), double stained with uranyl acetate and lead citrate, and examined under electron microscope (GEM1200EX-2™, JEOL, Japan).

PCR-RFLP analysis

Since about 80% of the reported cases are caused by A3243G transition (5), 10% by T3271C transition (6) and rarely by T3291C (7) and A3260G (8) of the mtDNA, the PCR-RFLP analysis was designed to detect A3243G, T3271C, and T3291C in MELAS cases (Table 2, 3). In MERRF, more than 90% of the cases have A8344G muta-

Table 1. Clinical profile of the patients with MELAS and MERRF

Case	Gender/Age (yr)	Syndrome	Symptom/sign	Serum lactate/pyruvate (mg%)	Electro-myography	Muscle P^{32} MRS	Affected family
I	M/32	MELAS	stroke, seizure, myopathy, DM, S-N deafness	48.9/not done	mixed myopathic and neurogenic	decreased PCr/Pi	none; mother has DM
II	M/35	MELAS	stroke, myopathy, S-N deafness	21.9/not done	myopathic	decreased PCr/Pi	unknown
III	M/18	MERRF	myoclonic epilepsy, myopathy, ataxia, dysarthria	118.6 /not done	normal	decreased PCr/Pi	mother, brother, and mother's sister
IV	M/28	MERRF	myoclonic epilepsy, S-N deafness, ataxia, dysarthria	22.9/1.2	normal	not done	mother

MELAS; myopathy, encephalopathy, lactic acidosis and stroke-like episode, MERRF; myoclonic epilepsy with ragged red fibers, DM; diabetes mellitus, S-N; sensorineural, P^{32} MRS; phosphorus^{32} magnetic resonance spectroscopy, PCr; phosphocreatine, Pi; inorganic phosphate.

Table 2. Primers, PCR reactions, and the resultant size of the PCR products for PCR-RFLP

Type	Site		Primers	PCR reaction	PCR product
MELAS	A3243G	Forward;	5′-AGGACAAGAGAAATAAAGGC-3′	initiation at 94℃ for 1 min, 30 cycles of denaturation (94℃ for 15 sec), annealing (55℃ for 15 sec), and extension (72℃ for 30 sec), and final extension at 72℃ for 7 min.	294 bp
		Backward;	3′-ATGCGTTTCCGGGGTTGCAC-5′		
	T3271C	Forward;	5′-AGGACAAGAGAAATAAAGGC-3′		170 bp
		Backward;	3′-AATTCCAGTCTCCAAGTTAAGGAGAAGAAT-5′		
	T3291C	Forward;	5′-AACTTAAAACTTTACAGTCAGAGGTTGGAT-3′	initiation at 94℃ for 4 min, 35 cycles of denaturation (94℃ for 30 sec), annealing (45℃ for 30 sec), and extension (72℃ for 1 min), and final extension at 72℃ for 7 min.	309 bp
		Backward;	3′-TGGAATCGAGAGCGGTAGCG-5′		
MERRF	A8344G	Forward;	5′-AGCCCACTGTAAAGCTAACT-3′		83 bp
		Backward;	3′-GGTTGTGCCGAAATGTCACTTTACGGGGTT-5′		
	T8356C	Forward;	5′-AGCCCACTGTAAAGCTAACT-3′		97 bp
		Backward;	3′-ATCTCACTTTACGGGGTTGATTTATGATGGCA-5′		
	G8363A	Forward;	5′-AGATTAAGAGAACCAACACCTCTTTTACA-3′		87 bp
		Backward;	3′-CCGGGTGGTATTAATGGGGG-5′		

MELAS; mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episode, MERRF; myoclonic epilepsy with ragged-red fibers.

Table 3. Restriction enzyme digestion reactions used in the study

Type	Site	Restriction enzyme (cut sequence)	Composition (total volume; 10 μL)	Result
MELAS	A3243G	*Apa*I (GGGCC/C)	10 × RB (TOYOBO, L-buffer) 1 μL PCR product 8 μL *Apa* I (TOYOBO) 1 μL	Wild-type; unchanged (294 bp) Mutant; 183 bp+111 bp
	T3271C	*Afl*II (C/TTAAG)	10 × RB (TOYOBO, M-buffer) 1 μL PCR product 8 μL *Afl* II (TOYOBO) 1 μL	Wild-type; unchanged (170 bp) Mutant; 140 bp+30 bp
	T3291C	*Bam*HI (G/GATTC)	10 × RB (TOYOBO, H-buffer) 1 μL PCR product 8 μL *Bam* HI (TOYOBO) 1 μL	Wild-type; unchanged (309 bp) Mutant; 280 bp+29 bp
MERRF	A8344G	*Bgl*I (GCCNNNN/NGGC)	10 × RB (TOYOBO, H-buffer) 1 μL PCR product 8 μL *Bgl* I (TOYOBO) 1 μL	Wild-type; unchanged (83 bp) Mutant; 53 bp+30 bp
	T8356C	*Xba*I (T/CTAGA)	10 × RB (TaKaRa, M-buffer) 1 μL bovine serum albumin 1 μL PCR product 7 μL *Xba* I (TaKaRa) 1 μL	Wild-type; unchanged (97 bp) Mutant; 67 bp+30 bp
	G8363A	*Sna*BI (TAC/GTA)	10 × RB (BM, M-buffer) 1 μL PCR product 8 μL *Sna* BI (BM) 1 μL	Wild-type; unchanged (87 bp) Mutant; 57 bp+30 bp

MELAS; mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episode, MERRF; myoclonic epilepsy with ragged-red fibers, RB; reaction buffer. All restriction enzyme digestion reaction were performed overnight at 37℃.

tion (9) and other reported cases also cluster at tRNA^{Lys} gene (T8356C (10), G8363A (11)). So, in cases with MERRF, the PCR-RFLP was designed to detect A8344G, T8356C, and G8363A mutations (Table 2, 3).

The DNA was extracted from the whole blood and muscle samples as described previously (12, 13). Eppendorf Mastercycler 5330 (Eppendorf Co., U.S.A.) machine was used for PCR. The total volume of PCR reaction was 25.0 μL, which consists of 2.5 μL of × 10 reaction buffer, 2.0 μL of 10 mN dNTPs, 2.5 μL of each primers, 0.2 μL of Ex *Taq* polymerase (TaKaRa, Japan), 1 μL of DNA template, and 14.3 μL of distilled water. The primers, thermal cycling conditions and resultant size of the PCR products are summarized in Table 2 and 3. The electrophoresis was performed on 4% 1:3 NuSieve agarose gel (FMC, U.S.A.) with an addition of 10 μL of ethidium bromide (EtBr) in 0.5 × TAE buffer solution (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). The 3 μL of PCR-RFLP product was loaded with the 2 μL of loading buffer and run at 100 V for 30 min.

DNA sequencing and analysis

Our strategy of DNA sequencing for MELAS was first to analyze the sequence of tRNA$^{Leu(UUR)}$ gene, and then shift to other areas reported to be associated with MELAS (tRNA^{Phe}, tRNA^{Val}, tRNA^{Cys}, COX III and ND5 (14-18)). In case of MERRF, sequence analysis was first performed for tRNA^{Lys} gene.

The primers for amplification of tRNA$^{Leu(UUR)}$ in MELAS cases were 5′-CCAGGTCGGTTTCTATCTAC-3′ and 5′-AG AGTTTTATGGCGTCAGCG-3′. The primers for tRNA^{Lys} in MEERF cases were 5′-AACCAAACCACTTTCACCGC-3′ and 5′-ATGGGCTTTGGTGAGGGAGG-3′. The total volume of reaction was 50 μL, consisting of 5.0 μL of × 10 reaction buffer, 4.0 μL of 10 mM dNTPs, 5 μL of each primers, 0.8 μL of Ex *Taq* polymerase (TaKaRa, Japan), 5 μL of DNA template, and 24.2 μL of distilled water. The thermal cycling conditions were initiation at 94℃ for 1 min, 35 cycles of denaturation at 94℃ for 15 sec, anealing at 55℃ for 15 sec, and extension at 72℃ for 1 min. Final extension at 72℃ for 7 min was added at the end of the cycle.

The PCR product was then purified with MicroSpin™ S-400 HR Column (Amersham Pharmacia Biotech, U.K.), and the sequencing reaction was performed using BigDye™ Terminator Cycle sequencing kit (PE Applied Biosystems, U.S.A.). The total volume of the reaction was 20 μL, which includes 8 μL of BigDye Terminator (BDT), 2 μL of primers, and 8 μL of purified PCR product. The thermal cycling conditions were initiation at 96℃ for 10 sec and 25 cycles of denaturation at 96℃ for 10 sec, annealing at 50℃ for 5 sec, and extension at 60℃ for 4 min. Then, the product was purified with MicroSpin™ S-200 column (Amersham Pharmacia Biotech, U.K.) and analyzed by electrophoresis in 1% agarose. The automated sequencing was performed with ABI Prism 377 (PE Applied Biosystems, U.S.A.). Two microliters of loading buffer was added to the product, denatured at 95℃

for 2 min, and 1.5 μL of the solution was loaded on 4.25% polyacrylamide (acrylamide: bis=29:1), 6 M urea gel. The electrophoresis was performed for 10 hr with 'Seq Run 48E-1200' Run module using 48 cm WTR plate. Then, Gel Image file was reviewed with minimizing the interference between samples. Finally, sequence data were analyzed with GENETYX-MAC program, comparing with the Anderson's standard mtDNA sequence (1) and the suspected site of point mutation was confirmed by manual inspection of the electropherogram.

RESULTS

Histochemical study

The proportion of RRFs judged by SDH stain was 15% and 12% in cases with MELAS (cases I and II) and 5% and 1% in cases with MERRF (cases III and IV). In each case with MELAS, strongly SDH-positive blood vessels (SSVs) were observed (Fig. 1). In the cases with MELAS, some RRFs had focally increased COX activities (20% among RRFs in
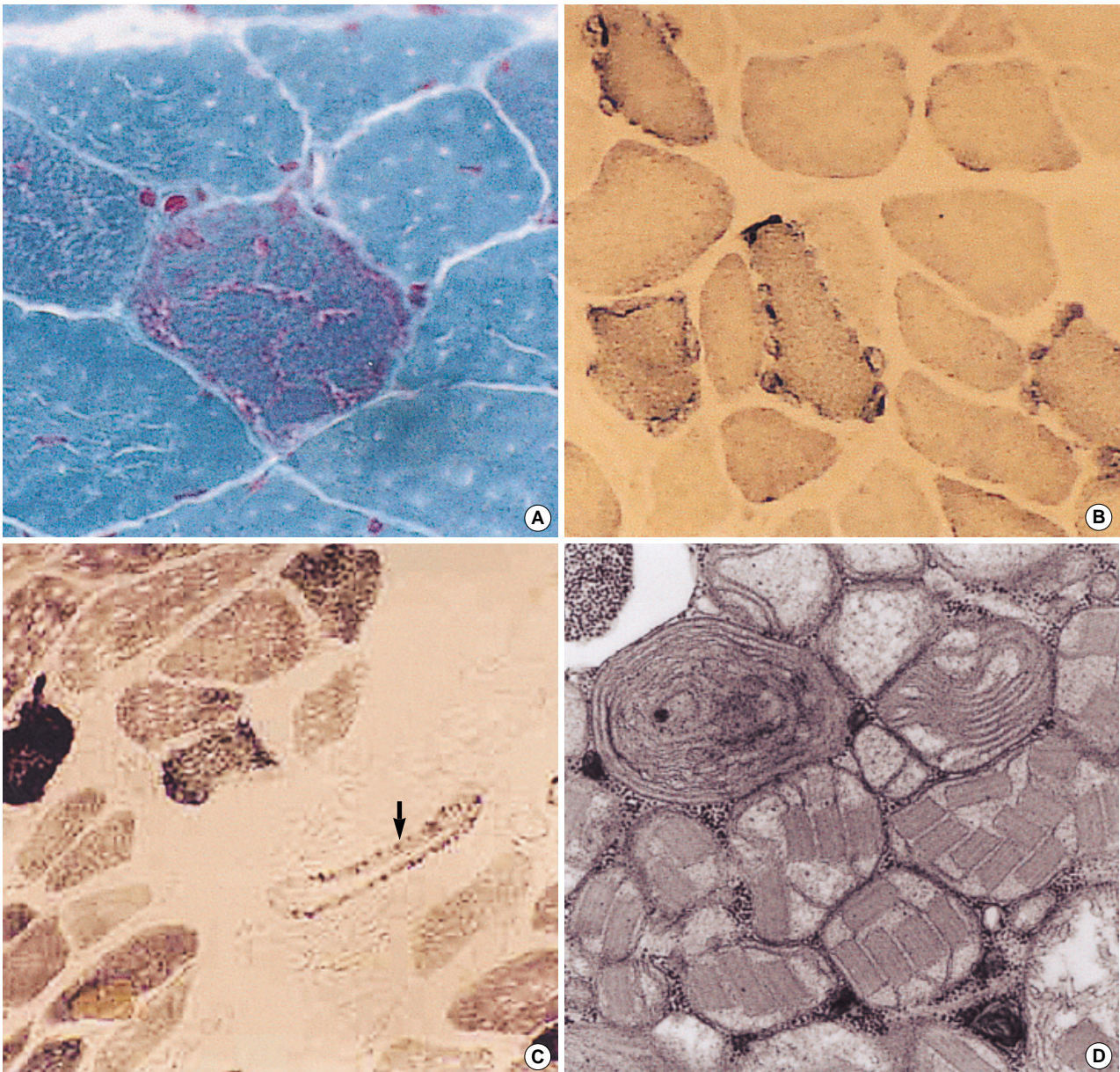

Fig. 1. Important pathological findings of the cases. (A) Ragged-red fiber in case I. Modified Gomori-trichrome stain, ×200. (B) Ragged-red fibers in case II. Succinyl dehydrogenase (SDH) stain, ×40. (C) Strongly succinyl dehydrogenase-positive blood vessel (SSV) in case I, ×100, arrow. (D) Electron microscopic finding. Abnormal proliferation of mitochondria with paracristaline inclusions in case III, ×20,000.
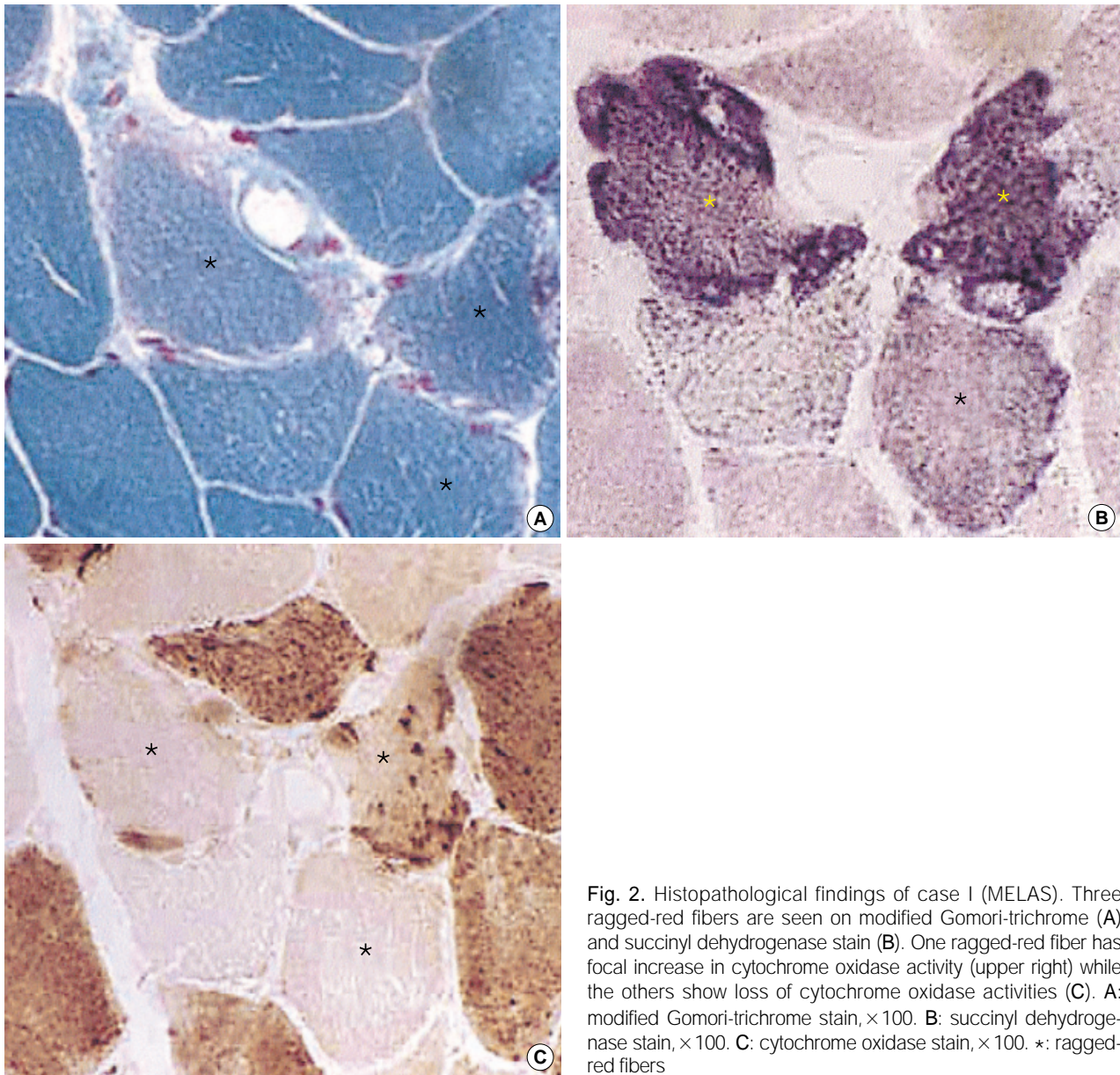
Fig. 2. Histopathological findings of case I (MELAS). Three ragged-red fibers are seen on modified Gomori-trichrome (**A**) and succinyl dehydrogenase stain (**B**). One ragged-red fiber has focal increase in cytochrome oxidase activity (upper right) while the others show loss of cytochrome oxidase activities (**C**). **A**: modified Gomori-trichrome stain, ×100. **B**: succinyl dehydrogenase stain, ×100. **C**: cytochrome oxidase stain, ×100. *: ragged-red fibers

case I and 15% in case II), while many other RRFs showed decreas-ed or normal COX activities (Fig. 2). In contrast, all RRFs showed either decreased or normal COX activities in the cases with MERRF, and no RRF with a focal increase in COX activity was observed (Fig. 3).

PCR-RFLP and sequence analysis of MELAS
(cases I and II)

In DNA form the blood, no mutation was found in PCR-RFLP for A3243G, T3271C, and T3291C in each case. Also, automated sequencing for tRNA$^{Leu(UUR)}$, tRNA^{Phe}, tRNA^{Val}, tRNA^{Cys}, COX III, ND5, and tRNA^{Ser} genes revealed normal sequences. However, in DNA from the muscle, treatment with the restriction enzyme *Apa*I showed an abnormal digestion pattern on PCR-RFLP and proved to have A3243G mutation on subsequent DNA sequencing (Fig. 4, 5).

PCR-RFLP and sequence analysis of MERRF
(cases III and IV)

In each case, PCR-RFLP analysis with both blood and muscle DNA samples revealed an abnormal *Bgl*I digestion pattern and were finally confirmed to have an A8344G mutation (Fig. 6, 7). The abnormal bands from muscle samples were clearer than those from venous blood on electrophoresis, suggesting there is a heavier mutant load in the muscle than in the blood. Among family members of case IV, an
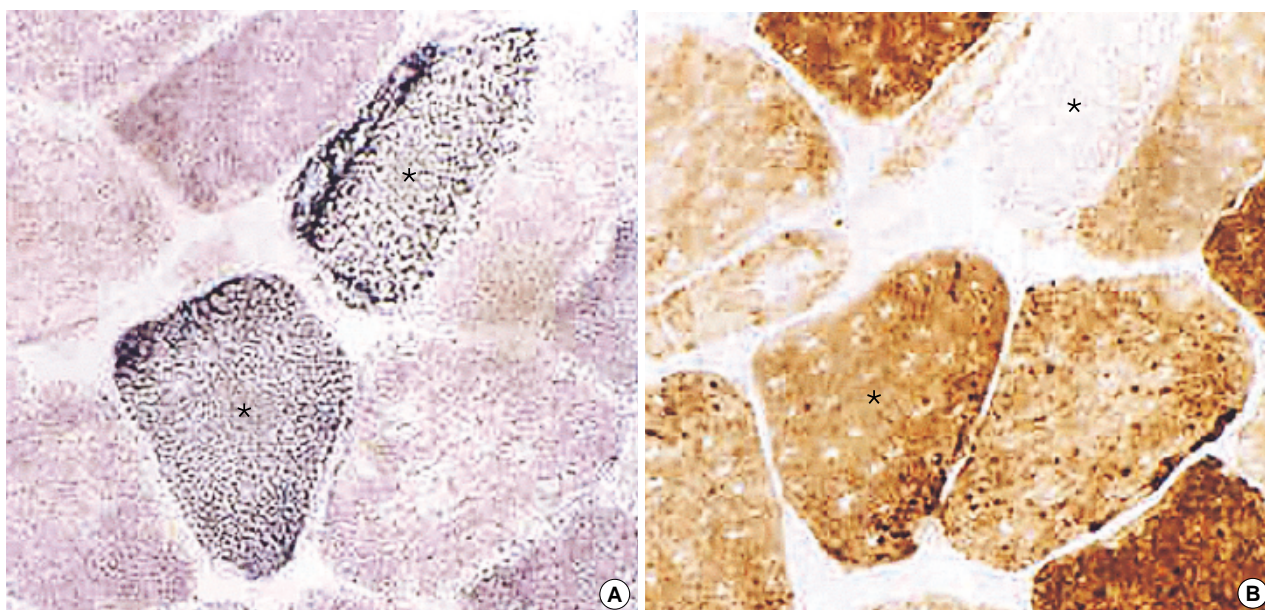
Fig. 3. Histopathological finding in case III (MERRF). Two ragged-red fibers are seen in succinyl dehydrogenase stain (**A**). On cytochrome oxidase stain, one of the ragged-red fibers show complete loss of cytochrome oxidase activity (**B**, upper right). **A**: succinyl dehydrogenase stain, ×200. **B**: cytochrome oxidase stain, ×200. ∗: ragged-red fiber.
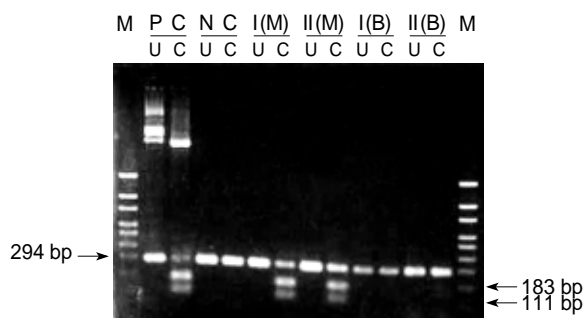

Fig. 4. The results of RFLP analysis using restriction enzyme *Apa* I for the detection of A→G transition at nucleotide position 3243 in cases I and II (MELAS). In mutants, treatment with the restriction enzyme *Apa* I produce two small fragments (183 bp and 111 bp) instead of single 294 bp band. In both cases, abnormal digestion pattern was identified only in muscle (M), but not in blood (B). M; molecular marker, PC; positive control, NC; negative control, U; uncut (undigested) PCR product, C; cut (digested) PCR product.

asymptomatic sister proved to have the same mutation on PCR-RFLP with restriction enzyme *Bgl*I (Fig. 6).

DISCUSSION

The RRF is a pathological marker for the mitochondrial disease, which is most readily identified by SDH stain on frozen muscle biopsy, and represents proliferation of the abnormal mitochondria in muscle fibers. It is well known that the amount of the mutant mtDNA in RRF is bigger than that of non-RRF muscle fibers in patients with mitochondrial myopathies. However, the exact mechanism that determines the amount of the mutant load in each muscle fiber is not known (19, 20).

The COX activity in muscle fibers is determined by the functional expression of the COX subunits in the mitochondrial inner membrane and thus, roughly correlates to the amount of the normal mtDNA. Because type I muscle fibers normally have more mitochondria than type II fibers, they show relatively increased COX activity than type II fibers on histochemical stain and appear as dark brown color while type II fibers stain light brown. Although the RRFs in mitochondrial myopathies have increased number of mitochondria, they are functionally abnormal harboring mutant mtDNA. Thus many of RRFs show decreased or loss of COX activities depending on the amount of mutant mtDNA they have (21). Interestingly, the COX activity of RRFs in MELAS is known to be different from that of MERRF, and our study also showed differential staining patterns of MELAS and MERRF (22). This finding is most likely caused by the differences in the expression of the COX subunit, and could be regarded as the secondary change resulting from a different pathogenetic mechanism between MELAS and MERRF. One of the important facts on COX immunohistochemistry is that the reduced or loss of COX activity does not necessarily mean the presence of mtDNA mutation. Because COX is encoded by three mitochondrial (COX I, II, and III) and nine nuclear genes (COX IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII), its activity also can be altered by the

mutations of the nuclear DNA encoding the COX subunits, and other nuclear regulatory genes for COX (ex. *SURF1*) (23, 24). In this regard, the results of a recent study on the immunohistochemistry using COX subunits is noteworthy, where they found selective loss or reduction of COX I and II subunit activities in patients with known mtDNA mutations, while reduced staining of all subunits were observed in patients with probable nuclear DNA defects (25). Thus, this technique would be useful for the differentiation of mitochondrial disease either into mtDNA defect or nuclear DNA defect, although further study seems to be needed in order to confirm its usefulness.

The SSV is another important histological finding uniquely seen in MELAS (26). The presence of SSV represents the affection of the vascular smooth muscle cells in MELAS and thought to be responsible for the stroke-like episode in this disease (26).

The A3243G mtDNA mutation in MELAS was first reported by Goto et al. in 1990 (5), and is responsible for about 80% of the cases. Until now, most of the known MELAS mutations are concentrated on the tRNA^{Leu}(UUR) gene, even though there are some case reports affecting other genes in mtDNA (14-18). Same frequencies of common MELAS mutations also seem to apply among Korean patients with

MELAS, because 7 patients with A3243G mutation and one with T3271C out of 10 patients with MELAS had been identified in a study using blood sample (27).

In our cases of MELAS, the mutation was not found from

Fig. 6. Results of RFLP analysis using the restriction enzyme *Bgl* I for the detection of A→G transition at nucleotide position 8344 in MERRF families (families A and B). In mutants, treatment with the restriction enzyme *Bgl* I produce two small fragments (53 bp and 30 bp) instead of single 83 bp band. The dark circles and squares represent affected individuals. The probands are III-1 in family A (case III) and II-1 in family B (case IV). In probands, both whole blood (B) and muscle (M) show abnormal digestion pattern. In the mother of proband in family A (II-5), who also has myoclonic epilepsy and muscle weakness, shows same digestion pattern as in the proband. In family B, asymptomatic brother (II-2) and sister (II-3) of the proband (II-1) were tested and the sister shows same digestion pattern as in the proband. M; muscle, B; blood, P; positive control, N; negative control.
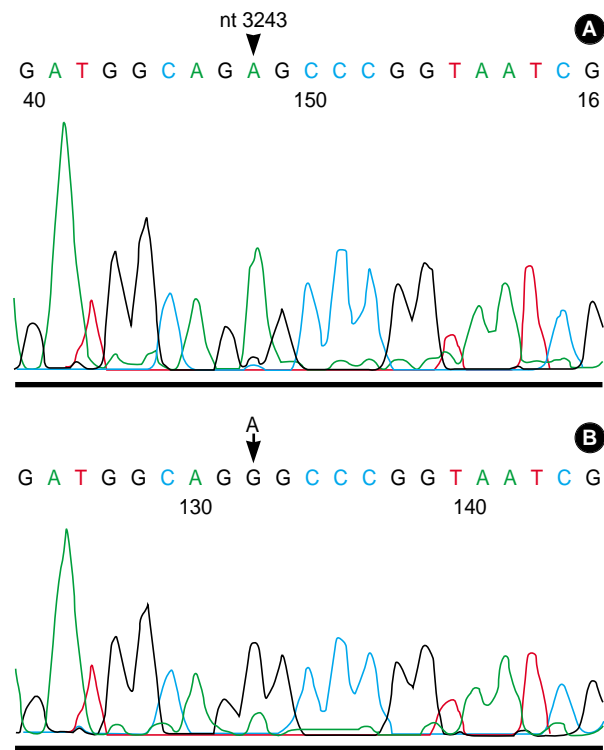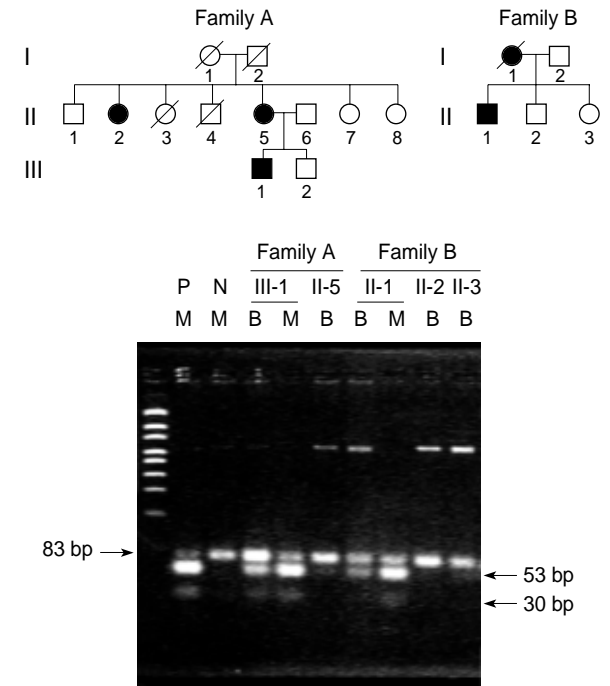

Fig. 5. The result of the automated sequencing in the case II (MELAS). A: The blood sample showed normal sequence at nucleotide position 3243 (arrowhead). B: The muscle sample of the same patient shows A to G transition at the same site (arrow) in the tRNA^{Leu} gene.
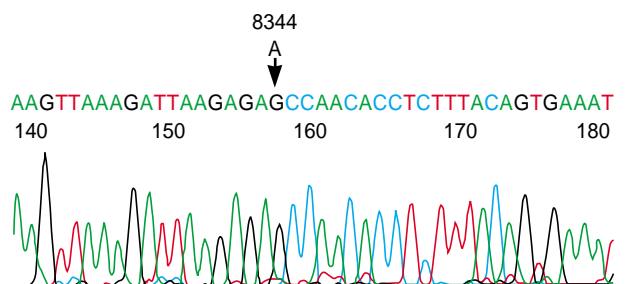

Fig. 7. The result of the automated sequencing in the case IV (MERRF). The electropherogram shows A to G transition at the nucleotide position 8344 (arrow) in the tRNA^{Lys} gene.

the blood DNA samples, but from the muscle samples. Thus, it is assumed that an exclusion of the MELAS mutation with a blood sample in clinically suspected cases could mislead the diagnosis. According to a report, the A3243G MELAS mutation can be found in about 50% of the cases when blood samples are used for PCR-RFLP (28). This is thought to be the result of the rapidly replicating ability of the blood cells, which will gradually eliminate the leukocytes with a higher mutation load (28). Interestingly, the A3243G mutations also have been found among different clinical phenotypes, including maternally inherited diabetes mellitus and deafness (MIDD) (29), progressive external ophthalmoplegia (PEO) (30), mitochondrial myopathy (31), and Leigh syndrome (32). Also, there is a report documenting the different mutant mtDNA ratio among different phenotypes of the A3243G mutation, suggesting the clinical phenotypes may be determined by the mutant load (33).

The mtDNA mutations causing MERRF are known to be more homogenous than in MELAS. The A8344G mutation, first reported by Shoffner et al. (9), is responsible for more than 90% of the cases of MERRF, and the T8356C (10) and G8363A (11) mutations were found in the rest of the cases. Also, in Korea, all of the molecular genetically confirmed cases had A8344G mutations (34, 35). Thus, the MERRF mutations are concentrated exclusively on the tRNA^{Lys} gene so far. Both of our MERRF cases clearly had a family history of maternal inheritance pattern typical of mitochondrial disorders, and the T8344C mutations were found both in PCR-RFLP and automated sequencing targeted for the tRNA^{Lys} gene. Also, the mutations were readily identifiable in blood samples although the amount of the mutant DNA is less in blood samples than in muscle samples. Unlike the MELAS, there is a clear relationship in the amount of mutation between muscle tissue and blood cells in MERRF and this may represent the different pathogenetic mechanism between them (36, 37). In case IV, PCR-RFLP for family members revealed the same mutation in an asymptomatic sister. According to Larsson et al., the same mtDNA mutations are found in a half of the siblings when the amount of the mutant DNA of the mother is 10-33%, and all the siblings will have the mutation when the amount of maternal mutant DNA is 43-73% in MERRF (36). The mother of case IV reportedly was unambulatory because of a profound muscle weakness when she was alive, and so, it is assumed that the amount of mutant DNA should be big enough to inherit the disease to some of her siblings, according to the "bottle neck hypothesis" in mtDNA segregation (37, 38). The A8344G MERRF mutation is located at the TψC loop of the tRNA^{Lys} gene, and different phenotypes have been reported as in A3243G MELAS mutation (39, 40). The A8344G mutation in the tRNA^{Lys} gene affects the translation of lysine, and thus, produce the defect in protein synthesis (ex. ND5) (41). Following the gene dosage effect, the symptoms tend to be more severe when there is a heavier mutant load in MERRF (42).

In conclusion, both MELAS and MERRF are caused by point mutations of the tRNA gene of the mitochondrial genome and have some common clinical findings despite many different aspects. Careful histochemical evaluation using frozen muscle tissue is important for the confirmation, differentiation and characterization of the mitochondrial diseases. In addition, the muscle tissue is essential for the molecular biological diagnosis and research of the mitochondrial diseases.

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