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MERRF/MELAS overlap syndrome: A double pathogenic mutation in mitochondrial tRNA genes

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ABSTRACT

Background: Myoclonic epilepsy with ragged-red fibers (MERRF) and mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) are established phenotypes of mitochondrial encephalomyopathy. The m.8356T>C transition in the mitochondrial $tRNA^{Lys}$ gene is a pathogenic mutations of MERRF. The m.3243A>G transition in the mitochondrial $tRNA^{Leu}$ gene is detected in most MELAS patients. Although previous analyses of double mutations in mitochondrial DNA (mtDNA) were useful for discussing their nature, many unsolved questions remain.

Objective: To describe the clinical and genetic features of a family with the above mtDNA double point mutations, and discuss the role of double mtDNA mutations in diverse clinical features in the family.

Patients and Methods: The proband was a 23-year-old female with MERRF harboring m.8356T>C and m.3243A>G transitions in mitochondrial tRNA genes. We assessed her clinical aspects of her and those of her three relatives, and performed mutation analyses on their mtDNA.

Results: Phenotypes of the four patients were MERRF, MERRF/MELAS overlap syndrome, and asymptomatic carrier. We hypothesize that the course of the phenotype of this family begins with MERRF and is followed by MELAS. This double mutation was heteroplasmic in

blood of all four patients, but with different rates in each patient, while m.8356T>C appeared homoplasmic, and m.3243A>G was heteroplasmic in muscle of the two examined cases. No other mutations were detected in the total mtDNA sequence in this family.

Conclusions: This is the first reported case of a double point mutation in mtDNA, both of which were heteroplasmic and pathogenic for the established phenotypes.

The concept of myoclonic epilepsy with ragged-red fibers (MERRF) was first proposed in 1980, and has been established as one of the phenotypes of mitochondrial encephalomyopathy [1]. It is maternally inherited and patients express myoclonus, myoclonic or generalized convulsions, cerebellar ataxia, and myopathy with ragged-red fibers [2]. In 1990, the most common pathogenic mutation, m.8344A>G transition in the mitochondrial $tRNA^{Lys}$ gene was reported [3], and report of a second pathogenic mutation, m.8356T>C transition in the mitochondrial $tRNA^{Lys}$ gene followed in 1992 [4]. The first report of the m.8356T>C transition indicated a pure MERRF expression [4], however, subsequent reports revealed that patients, with or without MERRF symptoms, of the families with this mutation, frequently showed migraine, stroke-like episodes, and strongly succinate dehygrogenase-reactive vessels (SSVs) in muscle pathology, which are typical for another phenotype of mitochondrial encephalomyopathy; mitochondrial mayopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) [5, 6]. It was speculated that the m.8356T>C transition might occur in patients with some of the expressions of MELAS, such as migraine, stroke-like episodes, and SSVs; however, the mechanism by which various expressions typical for two distinct phenotypes might occur in patients with only this point mutation in mitochondrial DNA (mtDNA) has not been clarified [5, 6].

The concept of MELAS was proposed in 1984, also as one of the phenotypes of

mitochondrial encephalomyopathy [7]. This entity was maternally inherited and characterized by stroke-like episodes, episodic headache and vomiting, seizures, dementia, lactic acidosis, skeletal myopathy, short stature and as being with or without MERRF symptoms [7]. The first pathogenic mutation, the m.3243A>G transition in the mitochondrial $tRNA^{Leu}$ gene, was reported in 1990 [8]. Moreover, it was revealed that most of the patients with MELAS had this mutation as a primary cause of the disease [9]. On the other hand, there were several reports of patients with an m.3243A>G transition, who also expressed features typical of phenotypes of mitochondrial encephalmyopathy other than MELAS, such as MERRF [10, 11], MERRF/MELAS overlap syndrome [12, 13], progressive external ophthalmoplegia (PEO), and MERRF/PEO overlap syndrome [14], suggesting that the m.3243A>G transition was potentially indicative of a broad expressive spectrum. However, what determined the range of expression in each patient had not been resolved [10-14].

There are reports of rare families with mitochondrial encephalomyopathy, possessing double point mutations in mtDNA [15-22]. Some of these consist of two mutations reported to separately express the same [15, 18, 20, 22] or different [17, 19, 21] distinct phenotypes, and others consist of one mutation of a well-known pathogenesis and one mutation of unknown effect [16]. It is speculated that in some families only one of the mutations expresses a phenotype [16, 17], that each mutation expresses different phenotypes in a given family [19],

and both mutations act synergistically to express one phenotype in other families [18, 21, 22]. These double mtDNA mutations might be the keys to resolving the mechanisms underlying the formation of threshold effects. However, these issues have yet to be resolved, presumably due in part to the various combinations of established phenotypes and mutational expressions in each of the families mentioned above.

We encountered a family with the double mutation, m.8356T>C and m.3243A>G. Individual family members expressed MERRF, MERRF/MELAS overlap syndrome, and other minor complications compatible with symptoms derived from mitochondrial dysfunctions. Because the various family members express potential phenotypes known to be caused by these two mtDNA mutations they were considered advantageous for ~~to~~ discussing the relationship between phenotypes and genetic effects. We had a chance to examine four members of this family, and tried to clarify the expression and the role of mtDNA point mutations.

PATIENTS and METHODS

Patients

The pedigree of the family we will discuss is shown in Figure 1. We first met patient III-1 (proband). She was introduced to us from another institution, diagnosed with myoclonic epilepsy. An interview of her and her mother (II-1) revealed that they had a familial history of mitochondrial encephalomyopathy (II-2; maternal aunt of the proband). We subsequently discovered that one of our colleagues had examined II-2 and had identified a mtDNA double point mutation (m.8356T>C and m.3243A>G) by a commercial-based (SRL Inc.) analysis of common mtDNA mutations, and that her muscle specimen had been preserved in our institution. Another patient of this family (III-6; cousin of the proband) was introduced to us from another institution, also diagnosed with myoclonic epilepsy. Thus, the main subjects of this study are these four cases; II-1 (mother), II-2 (maternal aunt), III-1 (proband), and III-6 (cousin). This pedigree contains other cases with hearing loss (I-1), hearing loss and mental retardation (III-5), and sudden death at one year of age (III-7). However, as we did not encounter these family members, we have no further information about them.

We conducted clinical (physical, neurological, laboratory, and radiological) examinations of II-1, III-1, and III-6 as far as we could. Clinical information of II-2 was gained from medical records. Histochemical analysis of muscle was conducted for II-2 and III-1. Genetic analyses

of mtDNA from blood and / or muscle was conducted in all four cases.

Report of cases

III-1; The proband

A 17 year-old female with bilateral sensorineural deafness developed a generalized clonic convulsion; she was diagnosed with myoclonic epilepsy one year later. Sodium valproate had been effective for preventing relapses of epileptic seizures. She was introduced to us at age 23 because her maternal aunt (II-2) was was diagnosed with MELAS. She exhibited a slightly short stature (154 cm) and definitely low body weight (36 kg). She did not complain of either episodic headache or nausea. Neurological examinations revealed deafness, slurred speech, weakness of bilateral quadriceps, and limb and truncal ataxia. By conversation, we were impressed that although she was somewhat childish for her age, her cognitive function was preserved. Her visual acuity and field were not disturbed. External ophthalmoplegia was not observed. Serum chemistry examinations detected no elevations in lactate, creatine kinase, transaminases, lactate dehydrogenase, amylase, hemoglobin A_{1c}, and creatinine. Blood hormonal evaluations of pituitary gland (ACTH, TSH, LH, FSH, GH, and prolactin), thyroid (fT3, fT4), adrenal cortex (cortisol), and genitals (estradiol) were normal. Elevated lactate was

detected in cerebrospinal fluid (35.3 mg/dL; normal <20.0 mg/dL). A CT scan of the trunk showed no abnormal forms of the organs in chest and abdomen. Transthoracic ultrasonic cardiography did not reveal cardiomyopathy. A brain MRI scan showed diffuse mild brain atrophy without focal lesions. Electroencephalography (EEG) showed bilateral slow waves. A muscle biopsy of the left *rectus femoris* revealed fiber size variability, scattered ragged-red fibers (RRFs), and vessels strongly stained with succinate dehydrogenase (SSVs) (Figure 2A). Cytochrome c oxidase defects were present in occasional fibers (focal COX deficiency), also among RRFs (Figure 2B). Most of the SSVs (approximately 80%) possessed COX activity.

II-2; Maternal aunt of III-1

The proband's maternal aunt had bilateral sensorineural deafness and progressive myoclonic epilepsy when she was 26 years old. At age 38, she developed stroke-like episodes with headache, truncal instability, and convulsion; she exhibited emaciation (stature: 157 cm, body weight 38 kg). Neurological examinations revealed aphasia, right hemianopsia, and cerebellar ataxia. A brain MRI scan showed a large lesion in the left cerebral hemisphere (Figure 3). Elevated lactate (34.6 mg/dL; normal <16.0 mg/dL) and impaired glucose tolerance (data not shown) were detected in serum. A muscle biopsy of the left *rectus femoris* showed fiber size variability, RRFs, focal COX deficiency, and SSVs (Figure 2C, E). Some

SSVs (approximately 40%) lacked COX activity (Figure 2C-F), and occasional RRFs also lacked COX activities. A stroke-like episode relapsed with disturbed consciousness at age 45, and the patient died of renal and heart failure one year later.

III-6; Cousin of III-1

A female cousin of the proband developed slurred speech, dysmetria of all four extremities, and truncal instability at age 18. After a few months, myoclonic jerks of the upper extremities often appeared, followed by generalized clonic convulsions. Her stature and body weight were in the normal range. She had neither deafness nor a history of episodic headache and nausea. Neurological findings revealed a slight cerebellar ataxia of the trunk and limbs, transient myoclonus of the upper extremities, and an episode of generalized convulsion. She appeared childish–during conversation, but intellectual ability was normal. Her visual acuity and field were intact. External ophthalmoplegia was not observed. Serum creatine kinase, transaminases, lactate dehydrogenase, amylase, hemoglobin A1c, and creatinine were within normal limits, but lactate was elevated (16.4 mg/dL; normal <16.0 mg/dL). Transthoracic ultrasonic cardiography indicated normal wall motion of the heart. A brain MRI showed no definite abnormalities. She showed left dominant spikes and waves on an EEG and was diagnosed with myoclonic epilepsy at age 19. For about one and a half years, myoclonic

epilepsy was well controlled with carbamazepine; however, at age 21 the frequency of the epileptic seizures increased. Despite changes to, or additions of, other antiepileptic drugs (phenytoin, zonisamide, topiramate, sodium valproate), control of seizures was difficult, and she required intensive care with mechanical ventilation twice during status epilepticus (SE). Moreover, in the course of treatment for the second SE, abdominal pain and elevated serum amylase and lipase occurred. A CT scan and an ultrasonogram detected no abnormal changes in the pancreas. Following treatment with camostat mesilate, ursodeoxycholic acid, and ulinastatin, the symptoms were relieved and serum pancreatic enzymes were normalized in one month.

II-1; Mother of III-1

The proband's mother, at age 51, was asymptomatic.

mtDNA mutation analysis

The examination of mtDNA mutations was first conducted in II-2 at another institution. She appeared to express the features of both MERRF and MELAS. Therefore, two established pathogenic mutations in mtDNA found in these phenotypes, m.8356T>C and m.3243G>A, were selected and screened for using commercial-based analyses of blood (SRL Inc.); both

point mutations were found. Therefore, we also screened for these mtDNA mutations in blood and muscle of III-1, and in the preserved muscle specimen of II-2. The m.8356T>C and m.3243A>G mutations were detected by PCR-restriction fragment length polymorphism analysis. Genomic DNA was extracted from blood and muscle specimens and amplified by PCR. PCR primers for m.3243A>G were adopted from Goto et al. [8], while mismatch primers to detect m.8356T>C substitution were adopted from Silvesti et al [4]. PCR products were digested with *Apa*I and *Dra*I at positions 3243 and 8356, respectively. Plasmids containing only wild type or mutant mtDNA were constructed and used as controls. PCR products were also sequenced to confirm the base substitutions. Blood samples of two other family members whom we had chances to examine (II-1 and III-6) were also analyzed with the same procedure. Moreover, whole mtDNA genome analyses in blood and muscle of III-1, and in blood of II-1 were conducted（mitoSEQr resequencing system, for resequencing the entire mitochondrial genome with 46 RSAs. Applied Biosystems, USA).

RESULTS

Symptoms and phenotypes

The phenotypes of the proband (III-1) and cousin (III-6) were MERRF, the aunt (II-2) was MERRF/MELAS overlap syndrome, and the mother (II-1) was an asymptomatic carrier.

Three other members (I-1, III-5, and III-7) also had some symptoms that could not be categorized in distinct entities. Of the three patients with MERRF (III-1 and III-6) and MERRF/MELAS overlap syndrome (II-2), two (II-2 and III-1) showed short stature and low body weight with bilateral sensorineural deafness from childhood. All three patients suffered from myoclonic epilepsy from the late teens to the mid twenties. Patient II-2 subsequently exhibited episodic headache in her mid thirties, with a stroke-like episode that repeatedly occurred afterwards. The other two patients (III-1 and III-6) had not reached their thirties when this study was conducted.

Blood and cerebrospinal fluid analysis

Of the three patients examined (II-2, III-1 and III-6), two showed elevated lactate (II-2 and III-6) in serum and one (II-2) had impaired glucose tolerance; at the same time, other items in serum chemistry, involving creatine kinase, were within normal limits in all three patients. No hormonal abnormalities except short stature were found in blood of III-1. Patient III-1 showed high levels of lactate in cerebrospinal fluid (CSF was not examined in the other patients).

Radiological examinations and EEG findings

Of the three patients examined, brain MRIs in two of them (II-2 and III-1) showed diffuse

brain atrophy, with stroke-like lesions in II-2, while patient III-6 exhibited a normal brain MRI. Transthoracic ultrasonic cardiographies demonstrated normal cardiac wall motion in the two patients examined (III-1 and III-6). Thoracic and abdominal CT scans detected no abnormalities in III-1. Two patients (III-1 and III-6) underwent EEG. III-1 showed generalized slow waves, and III-6 exhibited focal spike-and-waves.

Muscle pathology

Muscle biopsies were conducted in two cases (II-2 and III-1). II-2 had already expressed stroke-like episodes at the time this procedure was conducted. The two patients had similar morphological findings: fiber size variability, RRFs, focal COX deficiency, SSVs, and a mosaic distribution of COX activities in both RRFs and SSVs (Figure 2). The proportion of the SSVs with COX deficiencies was higher in III-1 than in II-2.

Genetic analysis

Results of genetic analyses are shown in Figure 4. Genetic analysis of mtDNA in III-1 revealed heteroplasmic m.8356T>C and m.3243A>G mutations in blood. This double mtDNA mutation was also detected and was heteroplasmic in the blood of II-1 and III-6, although the proportions of both mutations were clearly less in asymptomatic II-1. In muscle of III-1 the

proportion of m.3243A>G was higher than that in blood and m.8356T>C appeared to be homoplasmic. In muscle of II-2, m.3243A>G was heteroplasmic and m.8356T>C also appeared to be homoplasmic. Screening of the entire mtDNA genomes from blood and muscle of III-1, and from blood of II-1 revealed no mutations other than the m.8356T>C and m.3243G>A transitions.

Discussion

All four patients that we encountered carried the double point mutations m.8356T>C and m.3243A>G in mitochondrial tRNA genes. Both mutations were heteroplasmic in blood, while m.8356T>C appeared to be homoplasmic in muscle. The proportion of m.3243A>G transitions was higher in the muscle of III-1, and seemed to be associated with clinical severity because all symptomatic patients had significant proportions of the mutation but the asymptomatic patient II-1 carried the lowest mutational load. The m.8356T>C mutation appeared to be homoplasmic in muscle of III-1 and II-2, although a low level of heteroplasmy cannot be excluded due to the limitation of our current semi-quantitative PCR analysis. Nevertheless, it is fair to say that the m.8356T>C transition was higher in muscle than in blood, at least in III-1. The proportion of m.8356T>C transitions also appeared to be associated with clinical severity.

The four patients showed phenotypes of MERRF (III-1 and III-6), MERRF/MELAS overlap syndrome (II-2), and of an asymptomatic carrier (II-1). There were several other symptomatic family members (I-1, III-5, and III-7). We suggest that the typical course of disease in this family is as follows: the onset is characterized by short stature with emaciation and bilateral sensorineural deafness in childhood followed by cerebellar ataxia and myoclonic epilepsy; MERRF expression follows in young adulthood. By middle age the disease progression is characterized by the addition of migraine, vomiting, and stroke-like episodes, symptoms of MELAS expression, that indicates completion of the MERRF/MELAS overlap syndrome. Muscle pathology is compatible with MELAS before and after phenotypic expression of MELAS [9, 23]. However, a quantitative association between SSVs with COX deficiencies and expression of MELAS was not found in this study. This might mean that these family members gain the potential to express MELAS from the mutation loads in their primary phases. The characteristic brain MRI and EEG findings were not observed in this family. Neither external ophthalmoplegia nor cardiomyopathy were detected.

We presume that these mutations are pathogenic for the observed clinical phenotypes based on the following criteria; i) m.8356T>C and m.3243A>G transitions are pathogenic for MERRF [5, 6] and MELAS [8], respectively; ii) both mutations were heteroplasmic in blood of all four cases; iii) no mutations other than m.8356T>C and

m.3243A>G were detected in total sequences of mtDNA from blood of II-1 or from blood and muscle of III-1; iv) other symptomatic members of the pedigree had symptoms indicative of mitochondrial dysfunction [2].

Patients with the m.8356T>C transition frequently express both MERRF and MELAS [5, 6]. Moreover, some patients with the m.3243A>G transition exhibit MERRF [10, 11] or MERRF/MELAS overlap syndrome [12, 13]. As we did not quantitatively evaluate these mutations or clarify their thresholds, we were unable to determine whether both mutations simultaneously affected the observed clinical phenotypes.

In II-2, with MERRF/MELAS overlap syndrome, MERRF preceded MELAS. In the next generation, the proband (III-1) expressed MERRF without MELAS. However, her muscle pathology showed RRFs and SSVs with COX activities, which are more characteristic of MELAS than MERRF [23], and SSVs might be a predictor of stroke-like episodes [24] and additional MELAS expression. This finding leads us to speculate that the m.3243A>G transition might be pathogenic for expressing MELAS. Because the proportion of another mtDNA point mutation in muscle (m.12320A>G) was shown to increase over time [25], we hypothesize that the possibly increasing m.8356T>C transitions in our family might first overcome the threshold for the biochemical and symptomatic expressions of MERRF, and the increasing m.3243A>G transitions might then finally reach the threshold of symptomatic

MELAS expression. It is presumed that neither mutation had reached its threshold in the asymptomatic family members. However, to demonstrate the pathogenicity of the m.3243A>G transition in the expression of MELAS, single muscle fiber studies and more precise mtDNA species analyses are required. Without these data, it remains unknown whether a stroke-like episode of patient II-2 may be ascribed to m.3243A>G, because the m.8356T>C mutation itself can lead to the expression of MELAS/MERRF overlap syndrome [5, 6]. Among further studies that we are planning, the screening for m.3243A>G transitions in urinary sediment samples will also be informative for determining clinical severity. Data have shown that assessment of mtDNA mutational load in urinary cells correlates closely with that observed in skeletal muscle [26, 27], and is also a reliable measure of clinical severity [28].

Most of the reported cases of patients with more than one mtDNA mutation carried double point mutations [15-22]; however, cases with two heteroplasmic mtDNA point mutations were observed by Bidooki et al. in only one family with m.10010T>C and m.5656A>G transitions in the tRNA^{Gly} gene and in the non-coding region between the tRNA^{Asn} and tRNA^{Aln} genes, respectively [16]. The former mutation was pathogenic, while the latter was silent, and the associated phenotypes have not been generally established even in the former [16]. By contrast, both of the mutations in our family are known to express

established phenotypes. The difference between this work and the 1997 publication by Bidooki et al [16]. is that in the current study, there are potentially two pathogenic mtDNA mutations, rather than a pathogenic mutation and a SNP. However, the causal effects must be investigated experimentally.

There are, as yet, no reports of double mtDNA mutations in which no evolutionary associations with one another were shown [15-22]. Thus, we presume that some of the double mutations in our family were in the same mitochondria and some occurred as single mutations in distinct mitochondria. If further studies demonstrate this condition (i.e., the recombination of mtDNA [16] and one mutation's effect arising along with another in mtDNA [29]), single muscle fiber analysis and quantitative evaluations of the heteroplasmic rate of both mutations in each tissue might provide more profound insights into mtDNA mutations.

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Figure Legends

Figure 1

Proband's family pedigree. ○= female; □ = male; / = deceased. Solid symbols are symptomatic members. The arrow shows the proband. * Patients we encountered.

Figure 2

Pathology observed in *rectus femoris* muscle biopsies in III-1 (A and B) and II-2 (C, D, E and F). A & B, C & D and E & F are pairs of adjacent sections stained as indicated. A; Scattered ragged-red fibers (RRFs). Modified Gomori trichrome stain, ×400. B; Occasional RRFs with (arrow heads) or without (arrow) cytochrome c oxidase (COX) activities. COX stain, ×400. C; Strongly succinate dehydrogenase-reactive blood vessels (SSVs) (arrow). Succinate dehydrogenase (SDH) stain, ×400. D; SSVs with COX activities (arrow). COX stain, ×400. E; SSVs (arrow heads). SDH stain, ×100. F; SSVs without COX activities (arrow heads) and with mild COX activities (arrow). COX stain, ×100.

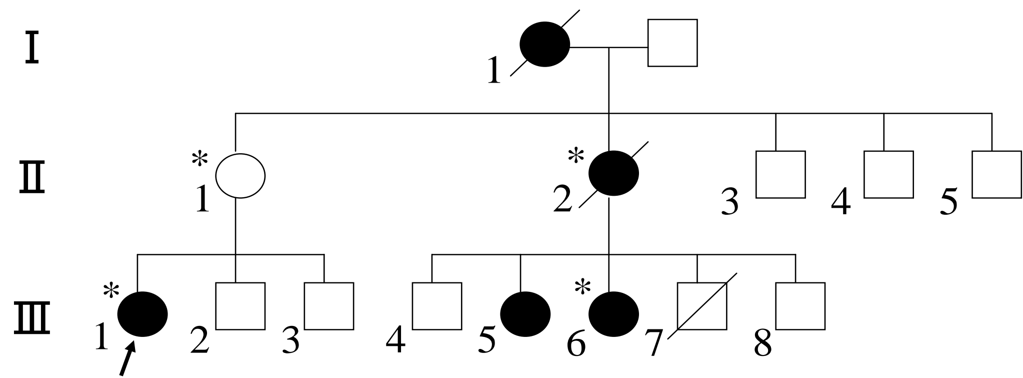
Figure 3

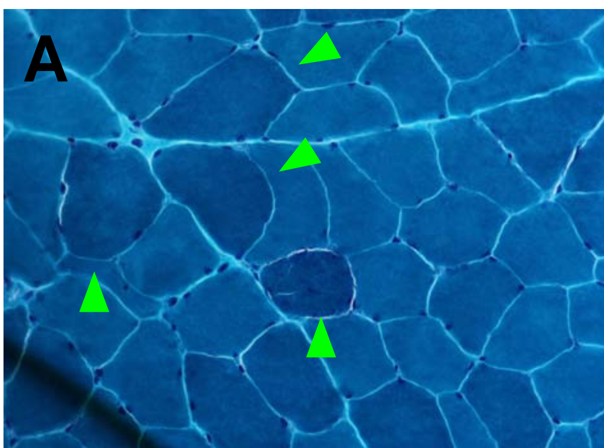
Brain MRI of II-2 during acute phase. FLAIR images detected a hyperintense lesion extending from the left occipito-parieto-temporal lobe at 38 years. R: right.

Figure 4

Restriction fragment length polymorphism analysis for the m.3243A>G and m.8356T>C mutations. Upper panel demonstrates variable heteroplasmy level of the m.3243A>G mutation in the pedigree. Uncut 294 bp band represents the wild type (3243A) while the 162 bp and 132 bp bands represent the mutant (3243G). Ratio of the mutant in muscle is significantly higher than in blood of III-1. Lower panel also shows variable heteroplasmy level of the m.8356T>C mutation. Uncut 141 bp band represents the mutant (8356C) while the 119 bp and 22 bp bands (only the 119 bp band is shown) represent the wild-type (8356T). The mutation rate in muscle is higher than in blood and appears to be homoplasmic in III-1 and II-2. WT and Mutant represent cloned plasmid DNAs containing only wild type and mutation sequences, respectively.

Figure 1　Proband's family pedigree

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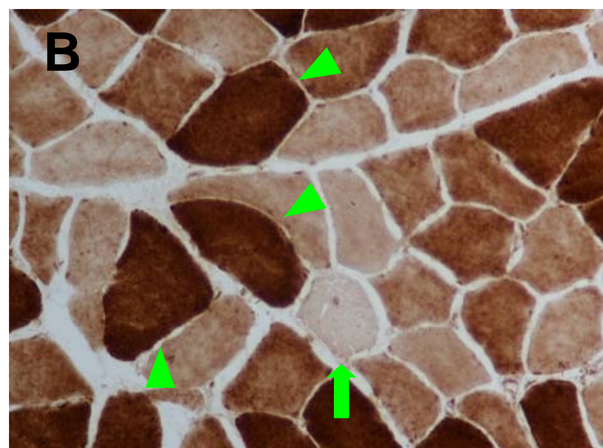
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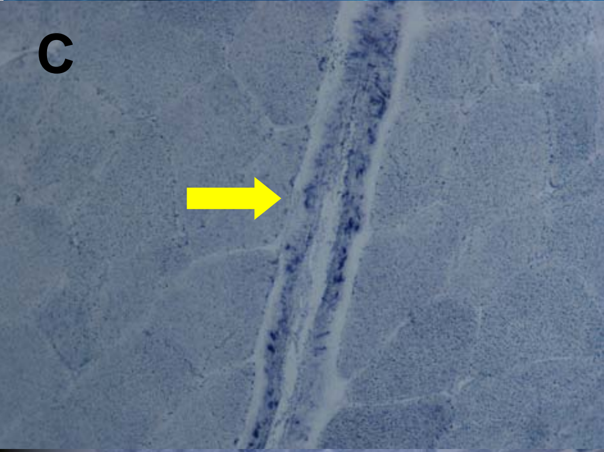
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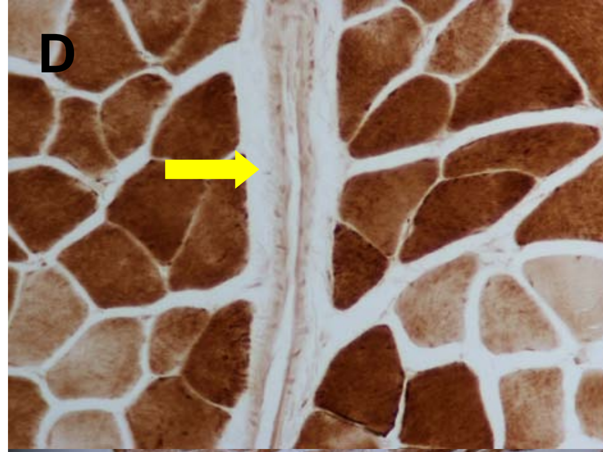
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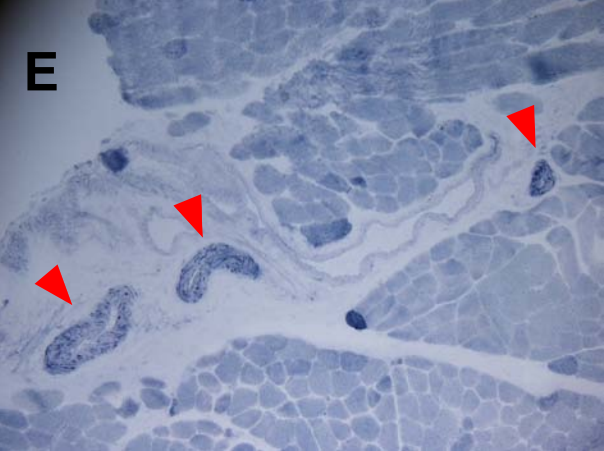
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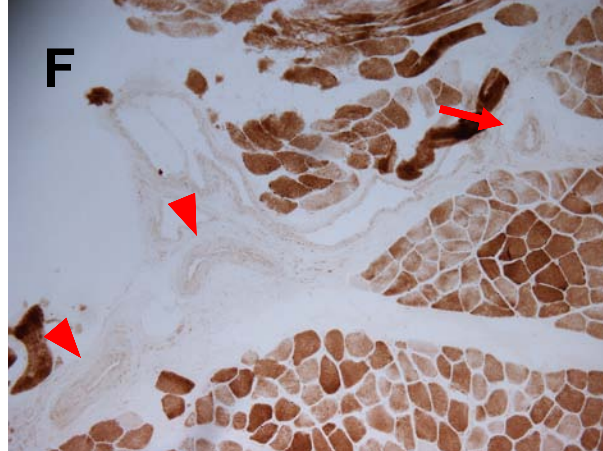
F

Figure 3 Brain MRI of II-2 during acute phase

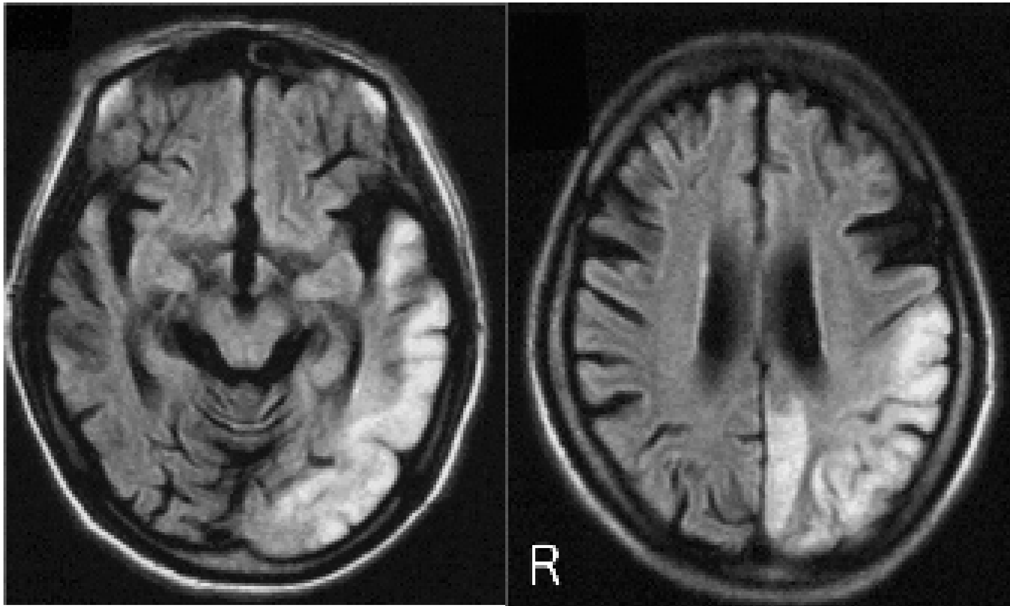

Figure 4 Restriction fragment length polymorphism analysis for the m.3243A>G and m.8356T>C mutations