都道府県	施設名	郵便番号	住所	電話番号
佐賀県	佐賀大学医学部附属病院リハビリテーション部	〒849-8501	佐賀市鍋島5丁目1-1	0952-34-3285
長崎県	健康保険諫早総合病院　神経内科	〒854-8501	諫早市永昌東町24-1	0957-22-1380
	長崎大学医学部　小児科	〒852-8501	長崎市坂本1-7-1	095-819-7298
宮崎県	宮崎大学医学部　神経内科	〒889-1692	宮崎郡清武町大字木原5200	0985-85-2965
	宮崎大学医学部　小児科	〒889-1692	宮崎郡清武町大字木原5200	0985-85-1510
鹿児島県	医療法人あおぞら会岩尾病院　神経内科	〒892-0387	鹿児島市甲突町17-18	099-225-3838
	鹿児島大学医学部　神経内科	〒890-8520	鹿児島市桜ヶ丘8-35-1	099-275-5332
	鹿児島県立大島病院　神経内科	〒894-0015	奄美市名瀬真名津町18-1	0997-52-3611
	鹿児島市医師会病院　神経内科	〒890-0064	鹿児島市鴨池新町7-1	099-254-1125
	鹿児島共済会 南風病院	〒892-8512	鹿児島市長田町14-3	099-226-9111
	鹿児島市立病院 小児科	〒892-8580	鹿児島市加治屋町20-17	099-224-2101
沖縄県	琉球大学医学部　神経内科	〒903-0215	中頭郡西原町字上原207	098-895-3331
	琉球大学医学部　小児科	〒903-0215	中頭郡西原町字上原207	098-895-3331

（滋賀健介）

CHAPTER 7

Spinocerebellar Ataxia with Axonal Neuropathy

Cheryl Walton, Heidrun Interthal, Ryuki Hirano, Mustafa A.M. Salih, Hiroshi Takashima and Cornelius F. Boerkoel*

Abstract

Spinocerebellar ataxia with axonal neuropathy (SCAN1) is an autosomal recessive disorder caused by a specific point mutation (c.1478A>G, p.H493R) in the tyrosyl-DNA phosphodiesterase (*TDP1*) gene. Functional and genetic studies suggest that this mutation, which disrupts the active site of the Tdp1 enzyme, causes disease by a combination of decreased catalytic activity and stabilization of the normally transient covalent Tdp1-DNA intermediate. This covalent reaction intermediate can form during the repair of stalled topoisomerase I-DNA adducts or oxidatively damaged bases at the 3′ end of the DNA at a strand break. However, our current understanding of the biology of Tdp1 function in humans is limited and does not allow us to fully elucidate the disease mechanism.

Introduction

Disorders of DNA repair can result in multiple pathological phenotypes, depending on the nature of the defect.[1,2] One of the most common features is neurological disease,[3] which can manifest as a developmental malformation or more commonly as a degenerative disorder during later life (Table 1).

Predisposing to these neurological manifestations are the poor renewal of neural tissues and the requirement that the tissue function for decades of life. As a consequence of their high oxygen requirement, neurons must cope with the DNA damage from oxidative and metabolic stress[5-7] and consequently require efficient DNA strand-break surveillance and repair mechanisms. Consistent with these observations, many studies link aging with a decline in DNA repair activity.[8-12] Also, individuals who incur genetic mutations inactivating these repair pathways show accelerated neuronal death.[4,13]

Spinocerebellar ataxia with axonal neuropathy (SCAN1) is an autosomal recessive disorder of DNA repair that clinically only affects the nervous system. Its neurodegenerative features include cerebellar atrophy with ataxia and axonal loss with peripheral neuropathy. The absence of affects on other tissues suggests that it is a good model for understanding the role of DNA repair in the nervous system.[14] SCAN1 is very rare and has only been reported for one extended family in Saudi Arabia.[14]

*Corresponding Author: Cornelius F. Boerkoel—Provincial Medical Genetics Program, Department of Medical Genetics, Children's and Women's Health Centre of BC, 4500 Oak St., Rm. C234, Vancouver, British Columbia, V6H 3N1 Canada. Email: boerkoel@interchange.ubc.ca

Diseases of DNA Repair, edited by Shamim I. Ahmad.

Table 1. DNA processing disorders with prominent neurological features

Disorder	Disease Gene	Neurological Signs	Other Symptoms
Nucleotide excision repair			
Xeroderma pigmentosum	*XPA, ERCC3, ERCC2, ERCC4, ERCC5, POLH, XPC, DDB2*	Microcephaly Neurodegeneration Peripheral neuropathy	Photosensitivity; skin cancer; poikiloderma; hearing loss; cognitive impairment
Cockayne syndrome	*ERCC3, ERCC2, ERCC5, ERCC8, ERCC6*	Microcephaly Neurodegeneration Peripheral neuropathy	Photosensitivity; growth retardation; hearing loss; cognitive impairment; progeria
Trichothiodystrophy	*ERCC3, ERCC2, GTF2H5, TTDN1*	Microcephaly	Photosensitivity; short brittle hair; cognitive impairment; ichthyosis; decreased fertility; short stature
Cerebro-oculo-facio-skeletal syndrome	*ERCC6, ERCC2, ERCC5, ERCC1*	Microcephaly Microphthalmia	Spasticity; neurological impairment; growth retardation
DSB repair			
Ataxia telangiectasia	*ATM*	Ataxia Neurodegeneration Oculomotor apraxia Choreoathetosis Peripheral neuropathy	Infections; immune defects; malignancy
Ataxia telangiectasia-like disorder	*MRE11A*	Ataxia Neurodegeneration Oculomotor apraxia Choreoathetosis Peripheral neuropathy	

continued on next page

Table 1. Continued

Disorder	Disease Gene	Neurological Signs	Other Symptoms
Nijmegen breakage syndrome	*NBS1*	Neurodegeneration Microcephaly	Short stature; cognitive impairment; premature ovarian failure; infections; immune defects; malignancy; short stature
ATR-Seckel syndrome	*ATR*	Microcephaly	Dwarfism
Primary microcephaly	*MCPH1*	Microcephaly Neural migration defect	Short stature
LIG4 syndrome	*LIG4*	Microcephaly	Infections; immune defects; malignancy; cognitive impairment
Immunodeficiency with microcephaly	*NHEJ1*	Microcephaly	Immune defects; malignancy; growth retardation
SSB repair			
Spinocerebellar ataxia with axonal neuropathy Type 1 (scan 1)	*TDP1*	Ataxia Peripheral neuropathy	
Ataxia and oculomotor apraxia	*APTX*	Ataxia Neurodegeneration Oculomotor apraxia	Hypoalbuminemia Hypercholesterolemia

Symptoms of SCAN1

SCAN1 is a progressive neurodegenerative disorder that begins in late childhood with the gradual onset of ataxic gait and loss of touch, pain and vibration sensation in the extremities.[14] As the disease progresses patients develop areflexia, gaze nystagmus, cerebellar dysarthria and *pes cavus*. This leads to decreased foot dorsiflexion, a steppage gait and eventually loss of independent walking. Additional features identified by Takashima et al included mild hypercholesterolemia, borderline hypoalbuminemia and seizures.[14] Unlike many diseases caused by deficiencies in DNA repair, patients with SCAN1 do not develop neoplasia, immunodeficiency, or photosensitivity and have normal intellect, fertility and longevity.

Genetic Basis of SCAN1

SCAN1 was associated with a mutation in the *TDP1* gene by linkage analysis and positional cloning.[14] The *TDP1* mutation identified in SCAN1 patients is the missense change H493R that disrupts the active site of Tdp1.[14-16] As explained below, the unusual properties of this mutation likely account for the rarity of SCAN1 and the absence of detectable *TDP1* mutations among other diseases associated with ataxia and peripheral neuropathy.[14,17]

Tdp1 Function

TDP1 encodes the enzyme tyrosyl-DNA phosphodiesterase 1 (Tdp1) that participates in the resolution of DNA damage caused by stalling of topoisomerase I (Topo I).[16-18] It can also process protruding 3′-phosphoglycolate termini that form in response to oxidative stress, ionizing radiation and specific chemotherapeutic agents such as bleomycin.[19-23]

Topo I is an essential enzyme that cleaves supercoiled DNA in order to relieve the torsional stress generated by key nuclear processes such as replication and transcription.[24] The active site of Topo I contains a tyrosine residue which cleaves one strand of the DNA by a nucleophilic attack upon a phosphodiester bond in the DNA backbone. Normally the result is a transient DNA single strand break with the Topo I covalently bound to the 3′ phosphate terminus of the break via its nucleophilic tyrosine.[24-26] After DNA relaxation has occurred a nucleophilic attack by the 5′ hydroxyl group on the phosphotyrosyl linkage between Topo I and the 3′ end of the DNA at the nick usually religates the DNA and the topoisomerase dissociates. The anticancer drug camptothecin (CPT) or endogenous DNA damage, such as abasic sites, nicks and mismatched base pairs, can prevent removal of Topo I from the DNA, often by causing a misalignment of the 5′ hydroxyl end of the DNA and preventing it from acting as a nucleophile.[25,27,28]

Importantly, collision of the DNA replication machinery or RNA polymerase with the Topo I-DNA covalent intermediate can cause irreversible DNA breaks. In the former case, the collision results in replication fork arrest and formation of a double-strand DNA break,[29,30] whereas in the latter case, collision of RNA polymerase with a Topo I-DNA complex on the template strand results in transcription arrest at single-strand DNA breaks.[31]

Processing of stalled Topo I-DNA complexes likely requires proteolytic degradation of the stalled Topo I, removal of the peptide remnant from the DNA by Tdp1 and then repair of the break by the DNA single strand break repair complex.[32-35] Processing of dead-end Topo I-DNA complexes at double strand breaks is less well understood. Tdp1 has two modified conserved HxKx4Dx6G(G/S) motifs, known as the HKD motifs[15,36,37] and these two HKD motifs together form a single active site.[38] In the human enzyme, amino acids 263 and 493 are the conserved histidines of the HKD motifs.[15,39] In removal of the Topo I peptide from DNA, H263 acts as a nucleophile attacking the phosphotyrosyl bond between the topoisomerase and the 3′ end of the DNA. H493 acts as a general acid to protonate the leaving group tyrosine. In this reaction intermediate, Tdp1 is covalently bound to the DNA via H263; H493 then acts as a general base and activates a water molecule to hydrolyse the bond between H263 and the DNA 3′ phosphate releasing Tdp1 from the DNA.[15,35,39,40]

Molecular Basis of SCAN1

Since the SCAN1-associated mutation of Tdp1 (c.1478A>G) changed histidine 493 to arginine (H493R) disrupting the active site,[14] the hypothesis was that loss of functional Tdp1 gave rise to the neurodegenerative disease. To test this, three groups analyzed mouse models.[17,41,42] None of these Tdp1 null mice recapitulated the SCAN1 phenotype.[17,41,42] These results suggested that mice may have redundant pathways for stalled Topo I or other Tdp1 substrates similar to yeast,[43] or that SCAN1 does not arise simply from loss of Tdp1 enzymatic activity as suggested earlier by Interthal et al.[16] This hypothesis was based on biochemical analysis of the mutant Tdp1 ($Tdp1^{H493R}$) associated with SCAN1. Although $Tdp1^{H493R}$ showed an approximately 25-fold reduction in catalytic activity for its phosphotyrosyl substrate, it became trapped on the DNA as a covalent reaction intermediate and had an extended half-life of approximately 13 min.[16,17,41] Thus, $Tdp1^{H493R}$ essentially just replaced the stalled topoisomerase. Consistent with the autosomal recessive inheritance of SCAN1, the only identified enzyme capable of resolving this covalent $Tdp1^{H493R}$-DNA intermediate was wild type Tdp1.[16,35] This suggested that SCAN1 might arise, at least in part, from stabilization of the Tdp1-DNA reaction intermediate (Fig. 1).[16,17,41]

An alternative and less considered possibility is that Tdp1 has a function in humans that is not conserved in mice. In the comparative profiling of Tdp1 expression in mice and humans, Tdp1 always exhibited nuclear expression in the mice, whereas it had cytoplasmic expression in some human cell types.[17] Interestingly, the cell types with the most cytoplasmic expression are those most likely affected in SCAN1, namely spinal anterior horn motor neurons, cerebellar Purkinje cells and dentate nucleus neurons.[17] As precedent for a cytoplasmic function, *glaikit*, the *Drosophila* homolog of Tdp1, has only been detected in the cytoplasm. During *Drosophila* embryogenesis, *glaikit* was essential for epithelial polarity and for neuronal development; it localized proteins to the apical lateral membrane of epithelial cells and its deficiency led to a severe disruption of central nervous system architecture.[44] In contrast to *Drosophila*, however, Tdp1 does not have a major role in human neurodevelopment because SCAN1 patients have normal neurodevelopment.[14,17] Therefore, if human Tdp1 has a cytoplasmic function in human neurons analogous to that of *glaikit*, it is likely a maintenance function that leads to neurodegeneration when disrupted.

Current and Future Research

Current and future research on Tdp1 focuses on four areas: (1) the DNA repair processes interacting with and dependent on Tdp1, (2) Tdp1 as a cancer therapeutic target, (3) the role of Tdp1 in biological processes other than DNA repair and (4) further delineation of the mechanism underlying SCAN1. Each of these areas is rapidly advancing and should open new understanding of neurodegenerative diseases, aging, cancer and fundamental human biology.

Emerging data in mammalian systems and earlier studies in yeast suggest that Tdp1 interacts with many DNA repair processes.[43,45-47] Understanding of the distribution and function of these redundant pathways in the human brain may shed light on the peculiar sensitivity of the human nervous system to expression of $Tdp1^{H493R}$.[17] Additionally, if transgenic mice expressing human $Tdp1^{H493R}$ recapitulate the SCAN1 phenotype, this will suggest that it is the specific H493R mutation that is responsible for disease and not solely the loss of functional Tdp1. In this situation, SCAN1 is potentially treatable by directed inhibition of $Tdp1^{H493R}$.

Besides its role in neural maintenance, Tdp1 has been regarded as a promising therapeutic target for cancer. Tdp1 is a promising cotarget of Topo I in cancer therapy as it counteracts the effects of Topo I inhibitors, such as camptothecin (CPT) and its clinically used derivatives.[35] Also, resistance to CPT is frequently encountered in nonsmall cell lung cancer and has been attributed to overexpression of Tdp1.[48] It is hypothesized therefore that Tdp1 inhibitors can augment the anticancer activity of Topo I inhibitors by reducing the repair of Topo I-DNA lesions.[17,49,50] The DNA double strand breaks caused by replication forks that encounter CPT-trapped Topo I are considered to be the major cytotoxic lesion caused by CPT based cancer therapy.[29] Additionally, since Tdp1 deficiency increases sensitivity to radiation, bleomycin, oxidative DNA damage and radiation, Tdp1 is also a promising cotarget for several other cancer therapies.

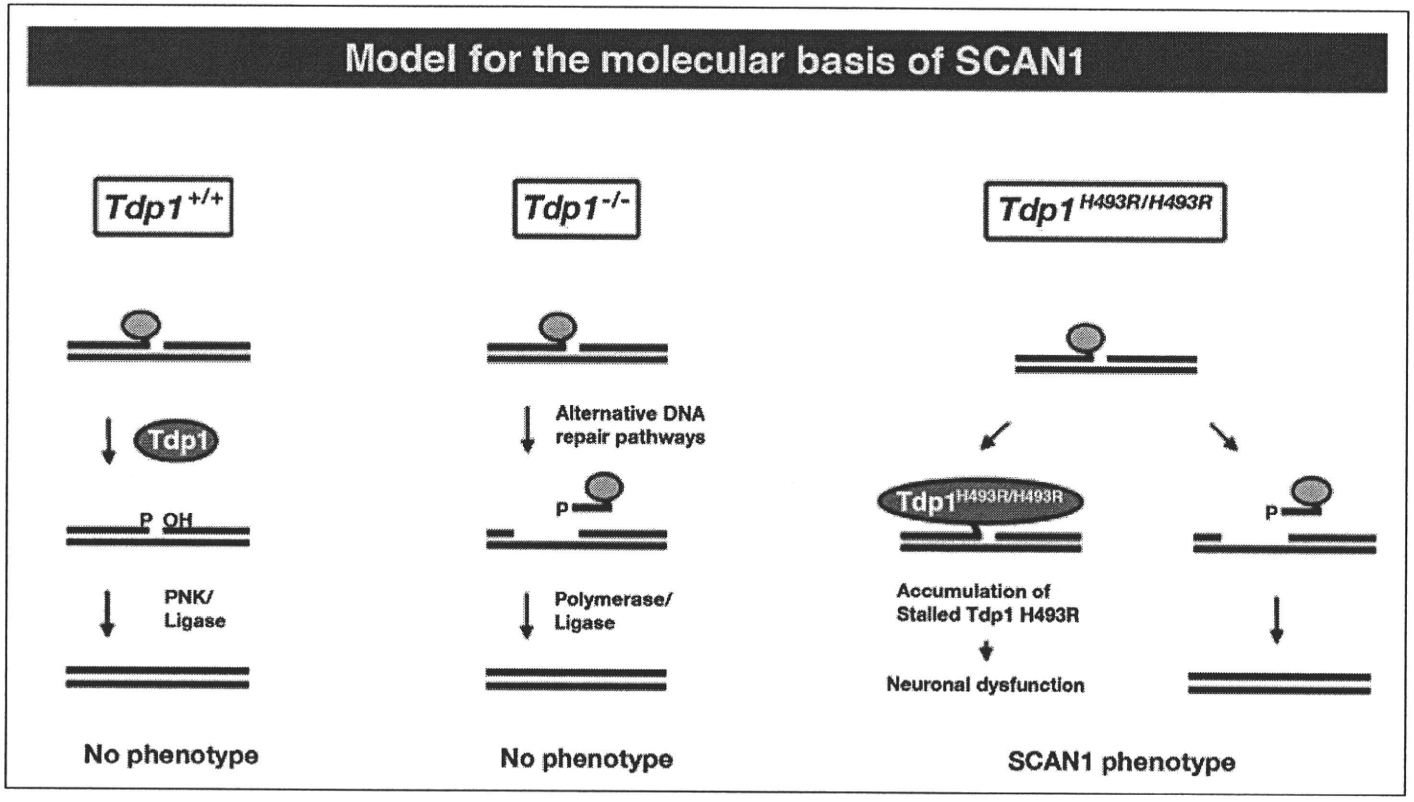

Figure 1. A model for the molecular basis of SCAN1. DNA breaks with blocked 3′ ends (e.g., Topo I or phosphoglycolate) undergo Tdp1-facilitated DNA repair via both DNA single-strand break repair and double-strand break repair mechanisms. With loss of functional Tdp1 ($Tdp1^{-/-}$), there is sufficient redundant activity for adequate DNA repair by alternative pathways (e.g., endonuclease-dependent pathways) unless the system is further stressed as by DNA damaging agents such as radiation, oxygen free radicals, or chemotherapy. In contrast, when Tdp1 carries the H493R mutation, it not only has a quantitative reduction in overall activity, but also a qualitative change resulting in accumulation of Tdp1-DNA complexes. These complexes are efficiently removed from the DNA by wild-type Tdp1 in all tissues of heterozygotes, whereas they are only removed in replicating cells of homozygotes by alternative DNA strand break repair mechanisms. According to this model, the transcriptional interference and/or apoptosis resulting from the Tdp1-DNA complexes in nondividing neurons causes SCAN1 via neurodegeneration.

Based on these precedents as well as the association of differences in Tdp1 expression with outcome in breast cancer,[51] identification of Tdp1 inhibitors is being actively pursued in order to treat cancers resistant to Topo I inhibitors and bleomycin as well as to predict outcome. A number of Tdp1 inhibitors have been described, including vanadate,[52,53] tungstate, the aminoglycoside neomycin,[54] NSC 305831, NSC 118695, NSC 88915[50] and furamidine.[55]

Conclusion

In summary, the discovery of the association of SCAN1 with a specific mutation of Tdp1 has spurred understanding of DNA repair in human biology and suggested a novel mechanism of human disease. SCAN1 may be the first example of a human genetic disease that results from a failure to repair DNA-protein covalent complexes. SCAN1 likely arises not only from a quantitative change in Tdp1 activity but also from a qualitative change that renders the enzyme different from wild type Tdp1 causing it to become covalently trapped on the DNA.[16,17,41] Additionally, the absence of detectable acute effects of Topo I inhibitors and bleomycin treatment on the nervous system of mice deficient for Tdp1 suggests that nonproliferating cells of the nervous system are sufficiently insensitive to Topo I-DNA complexes and 3′ phosphoglycolate-DNA damage that short-term administration of these chemotherapeutic agents is unlikely to induce neurological disease even in the absence of functional Tdp1.[17]

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CASE REPORTS

A new phenotype of mitochondrial disease characterized by familial late-onset predominant axial myopathy and encephalopathy

Yusuke Sakiyama · Yuji Okamoto · Itsuro Higuchi · Yukie Inamori · Yoko Sangatsuda · Kumiko Michizono · Osamu Watanabe · Hideyuki Hatakeyama · Yu-ichi Goto · Kimiyoshi Arimura · Hiroshi Takashima

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Abstract Axial myopathy is a rare neuromuscular disease that is characterized by paraspinal muscle atrophy and abnormal posture, most notably camptocormia (also known as bent spine). The genetic cause of familial axial myopathy is unknown. Described here are the clinical features and cause of late-onset predominant axial myopathy and encephalopathy. A 73-year-old woman presented with a 10-year history of severe paraspinal muscle atrophy and cerebellar ataxia. Her 84-year-old sister also developed late-onset paraspinal muscle atrophy and generalized seizures with encephalopathy. Computed tomography showed severe atrophy and fatty degeneration of their paraspinal muscles. Their mother and maternal aunt also developed bent spines. The existence of many ragged-red fibers and cytochrome *c* oxidase-negative fibers in the biceps brachii muscle of the proband indicated a mitochondrial abnormality. No significant abnormalities were observed in the respiratory chain enzyme activities; however, the activities of complexes I and IV were relatively low compared with the activities of other complexes. Sequence analysis of the mitochondrial DNA from the muscle revealed a novel heteroplasmic mutation (m.602C>T) in the mitochondrial $tRNA^{Phe}$ gene. This familial case of late-onset predominant axial myopathy and encephalopathy may represent a new clinical phenotype of a mitochondrial disease.

Keywords Mitochondrial disease · Predominant axial myopathy · Encephalopathy · Late-onset · Familial case

Introduction

Camptocormia, a term coined by Souques and Rosanoff-Saloff from two Greek words (*kamptos* meaning bent and *kormos* meaning trunk), is characterized by involuntary trunk flexion in the erect position that disappears in the supine position. Camptocormia was initially described as a hysterical phenomenon that occurred in male soldiers during World Wars I and II [1, 16]. However, in the last 20 years camptocormia has been reported to be present with various organic diseases, including muscular dystrophies, inflammatory myopathies, dystonia, amyotrophic lateral sclerosis, myasthenia gravis, paraneoplastic syndrome, Parkinson's disease, multiple system atrophy, and spinal deformities, as well as in an idiopathic form. Camptocormia is also referred to as "bent spine syndrome" [1, 32].

Axial myopathy has been described as the selective involvement of the paraspinal muscles in camptocormia or dropped head. Axial myopathy has heterogeneous etiologies, including primary and various other neuromuscular disorders. Primary axial myopathy is characterized by the

Y. Sakiyama · Y. Okamoto · I. Higuchi · Y. Inamori · K. Michizono · O. Watanabe · H. Takashima (✉)
Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences,
8-35-1 Sakuragaoka, Kagoshima City,
Kagoshima 890-8520, Japan
e-mail: thiroshi@m3.kufm.kagoshima-u.ac.jp

Y. Sangatsuda
Department of Psychiatry, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

H. Hatakeyama · Y. Goto
Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience,
National Center of Neurology and Psychiatry, Tokyo, Japan

K. Arimura
Division of Neurology, Okatsu Hospital, Kagoshima, Japan

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insidious and progressive weakness of the extensor muscles of the spine, normal or slightly elevated serum creatine kinase (CK) levels, and a myogenic pattern on electromyography in the elderly. Muscle biopsies show nonspecific myopathic changes with fibrosis, fatty replacement, and variations in fiber size. In addition, some ragged-red fibers and complex I and III deficiencies have been observed; these findings are considered to be the age-related accumulation of various mitochondrial abnormalities [21, 31].

Some cases of autosomal dominant inheritance patterns of familial primary axial myopathy were reported several years ago; however, the genetic analyses that were used have not been described [31]. Recently, a novel heterozygous dominant mutation in the skeletal muscle ryanodine receptor gene was identified in the central cores of muscle biopsy specimens that were excised from sporadic cases of axial myopathy [15]. Furthermore, facioscapulohumeral muscular dystrophy with isolated axial myopathy has also been reported [19]. Five cases of axial myopathy that were associated with mitochondrial dysfunction have been previously reported; however, no familial cases of mitochondrial gene mutation have been reported [8, 11, 28, 30, 32].

In this paper, we have reported about a mitochondrial disease that is characterized by familial late-onset predominant axial myopathy and encephalopathy. In addition, the pathogenicity of a novel, familial, mitochondrial tRNA gene mutation is discussed.

Methods

Subjects

Patient 1

A 73-year-old woman (Fig. 1, III-8) presenting with abnormal posture and gait disturbance. Since the age of 63, the patient had a slight stooping posture and a pushed-out waist. At 68 years of age, she started using a walking stick because of her unstable gait. She was diagnosed with hypothyroidism by her family physician and administrated with 25 μg/day levothyroxine; however, her symptoms did not improve. At 70 years of age, it gradually became more difficult for her to climb the stairs. At 71 years of age, she was admitted to another hospital. Doctors suspected myopathy because of elevated serum CK levels. She visited our hospital presenting with prominent paraspinal muscle atrophy and mild proximal weakness of limbs. Hypothyroidism-related myopathy was suspected in her, and hence, the levothyroxine dose was increased to 50 μg/day; however, her symptoms did not improve. She had a family history of bent spine, i.e., in her elder sister (patient 2, Fig. 1, III-5), mother (Fig. 1, II-3), and maternal aunt (Fig. 1, II-4). Physical examination on arrival revealed a marked atrophy of the paraspinal muscles and abnormal posture (Fig. 2a, b). She also presented with right ptosis, dysarthria, bilateral cataracts, and hearing loss. Her eye movements were normal. But there was moderate weakness of the neck flexion and mild weakness of the proximal limb muscles. Tendon reflexes were symmetrical, and Babinski's sign was absent. She had poor balance with tandem gait without limb ataxia. Sensory systems were intact and Romberg's sign was negative. She scored poorly on the attention and calculation tests that are a part of the Mini-Mental State Examination (score: 25 points).

Laboratory data were as follows: serum CK level was 290 IU/l (normal range 45–163 IU/l), resting blood and cerebrospinal fluid (CSF) lactate levels were normal, thyroid-stimulating hormone levels were slightly low at 0.47 μIU/ml (normal range 0.5–5.0 μIU/ml). Under the administration of 50 μg/day levothyroxine; antithyroglobulin antibody levels were high at 7.0 U/ml (normal range <0.3 U/ml), antithyroid peroxidase antibody levels were high at 46.5 U/ml (normal range <0.3 U/ml), rheumatoid factor levels were high at 152.3 IU/ml (normal value <15.0 IU/ml), antinuclear antibody levels were mildly elevated (titer of 1:80). Autoimmune analyses, including anti-Jo-1, anti-RNP, anti-SS-A, and anti-SS-B, were negative. The oral glucose tolerance test (75 g) was within normal limits, but Holter monitoring revealed high-frequency premature contractions. Pure-tone audiometry indicated sensorineural and high-frequency hearing loss.

Needle electromyographic findings of the biceps brachii and rectus femoris muscles indicated mild myopathic features. Computed tomography (CT) of the thoracic spinal nerve 10 (T10) revealed severe atrophy and fatty degeneration of the paraspinal muscles (Fig. 2c). Brain magnetic

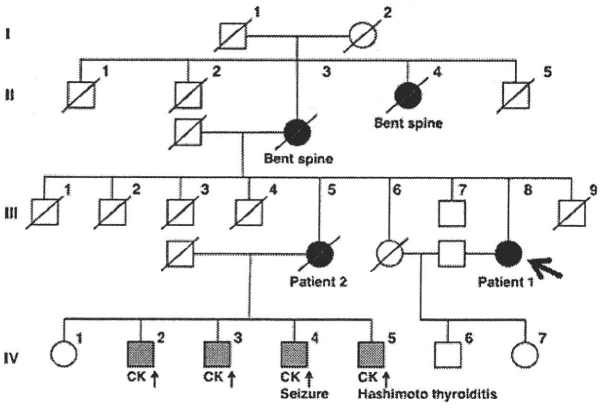

Fig. 1 Pedigree of the family. The *arrow* indicates the proband. The affected individuals are represented by the *solid black symbols*; *open symbols* represent healthy individuals. *Gray symbols* indicate individuals with elevated CK levels

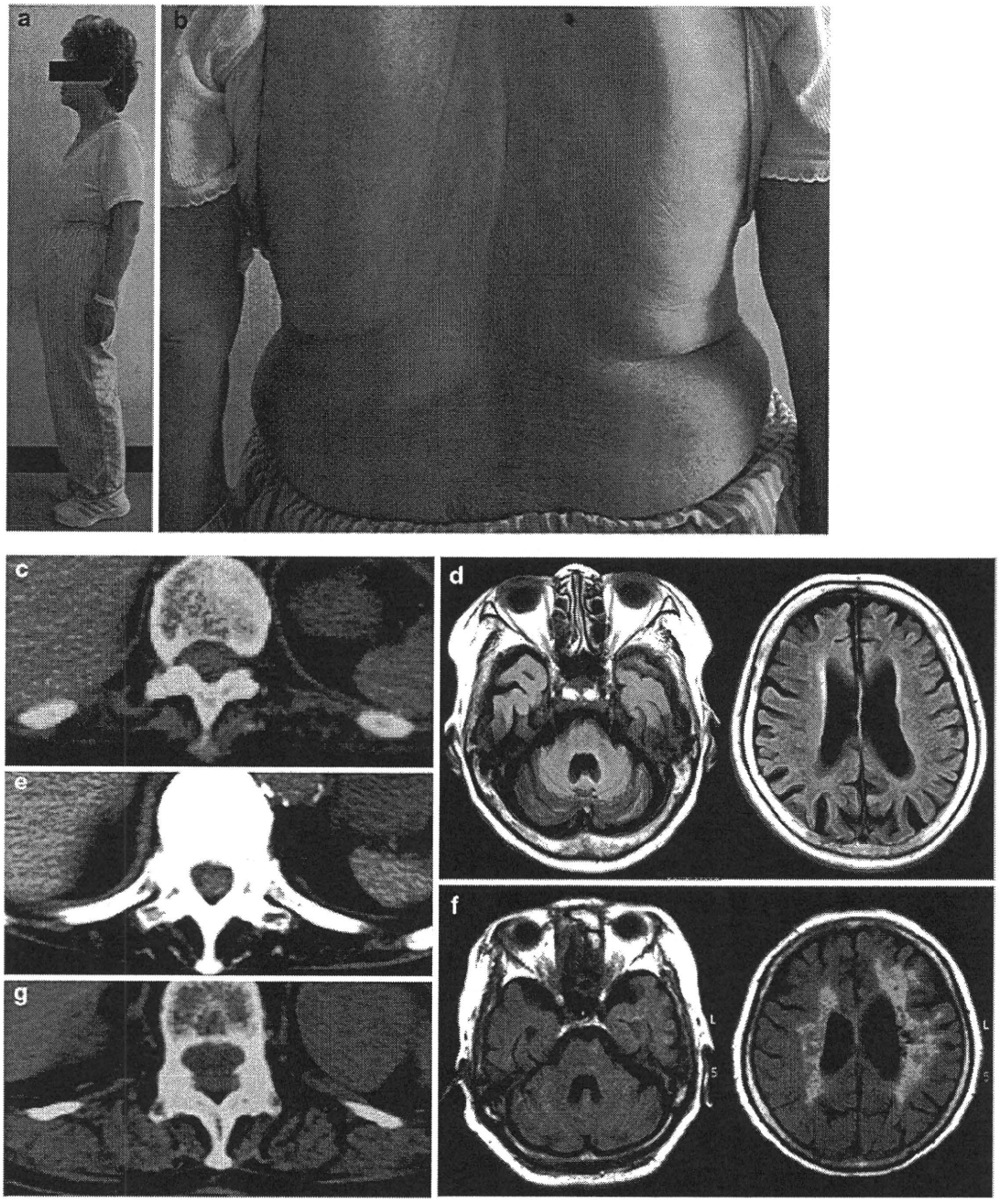

Fig. 2 **a** The full-length figure indicates the posture of patient 1 showing her pushed-out waist. **b** The dorsal view shows the marked atrophy of the paraspinal muscles in patient 1. CT of T10 of **c** patient 1 (age 71), **e** patient 2 (age 82), and **g** a healthy female (age 74) reveals the profound atrophy of the paraspinal muscles in **c** patient 1 and **e** patient 2, but not in **g** the healthy female. Brain MRI studies revealed several differences between the patients 1 and 2. **d** Axial FLAIR images of patient 1 show moderate cerebellar atrophy and some cerebral cortical atrophy. **f** The same images of patient 2 revealing hyperintense lesions around the white matter

resonance imaging (MRI) with fluid-attenuated inversion recovery imaging showed moderate cerebellar and temporo-parieto-occipital lobe atrophy (Fig. 2d). MR spectroscopy revealed the absence of increased lactate peaks. 123I-IMP single photon emission CT revealed hypoperfusion that was indicative of atrophic brain lesions.

Patient 2

The elder sister of patient 1 was an 84-year-old woman with a stooping posture presenting with tremors since the age of 60. In her 70s she started walking with the aid of a walking stick. At 82 years of age, she was hospitalized for

generalized seizures and disturbed consciousness. CT of T10 revealed severe atrophy and fatty degeneration of the paraspinal muscles (Fig. 2e). Brain MRI revealed hyperintense lesions around the white matter (Fig. 2f); elevated serum and CSF lactate levels were also noted at this time. The mitochondrial DNA analysis of the lymphocytes did not indicate MELAS (m.3243A>G) or MERRF (m.8344A>G) mutations. The patient's condition remained undiagnosed and she died at the age of 84. CK levels in all her four sons were found to be elevated and her third son was diagnosed with epilepsy. She and her fourth son had also been previously diagnosed with Hashimoto thyroiditis (Fig. 1).

Patient 1 was examined using pathological, biochemical, and genetic analyses. The Institutional Review Board of Kagoshima University approved this study. Patient 1 gave the written and informed consent for her participation in this study.

Histochemical and immunohistochemical studies

Frozen biopsies of the biceps brachii muscle specimens were obtained from patient 1. The specimens were sliced into 8 μm sections and placed on aminosilane-coated slides. Histochemical and immunohistochemical procedures were performed as previously described [13].

Biochemical studies

Enzyme activity levels, blue native polyacrylamide gel electrophoresis (BN-PAGE), and other biochemical measurements of the frozen muscle specimens from patient 1 were performed as previously described [6, 33, 36].

Mitochondrial DNA analysis

In case of patient 1, the total DNA was extracted from the peripheral blood leukocytes and the frozen muscle specimens using the DNeasy Blood & Tissue kit (Qiagen). MitoChip v2.0 (The GeneChip® Human Mitochondrial Resequencing Array 2.0), which provides a standard assay for the complete sequence analysis of human mitochondrial DNA, was obtained from Affymetrix. The patient's entire mitochondrial DNA was sequenced using MitoChip v2.0 as previously described [37]. Analysis of the microarray data obtained with MitoChip v2.0 was performed using GeneChip Sequence Analysis Software v4.0 (Affymetrix) [24].

In order to reveal the mutations that were confirmed by MitoChip v2.0, a 465-base pair PCR product that spanned all of the mutation sites was screened by DNA sequencing. In brief, 50 ng of the patient's genomic DNA was amplified using the hot-start PCR method and a forward (5′-CACCATTCTCCGTGAAATCA-3′) and reverse primer (5′-AGGCTAAGCGTTTTGAGCTG-3′) [5, 29]. Each PCR product was generated under the following conditions: 15 min at 95°C, 42 cycles of amplification (95°C for 30 s, 61°C for 30 s, and 72°C for 1 min), and 30 min at 72°C. Using a presequencing kit (USB, Cleveland, OH, USA), the patient's PCR products with abnormal elution profiles were purified, and the appropriate PCR products from relatives and control chromosomes were obtained and sequenced by dye-terminator chemistry using an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA). The resulting sequences were then aligned and any mutations were evaluated using the Sequencher sequence alignment program (Gene Codes, Ann Arbor, MI, USA).

The polymorphic and pathogenic natures of the confirmed mutations were checked against two databases: the MITOMAP (http://www.mitomap.org/) and GiiB-JST mtSNP database (http://mtsnp.tmig.or.jp/mtsnp/index.shtml).

Results

Histological and immunohistochemical characterizations

The muscle fibers ranged from 10 to 80 μm in diameter. Sixty-nine of the 609 Gomori trichrome stained muscle fibers (11.3%) were ragged-red fibers (Fig. 3a). Cytochrome *c* oxidase (COX) activity was deficient in many of the ragged-blue fibers that were stained with succinate dehydrogenase (SDH) and COX (233 of 881 muscle fibers, 26.4%) (Fig. 3b, c), and no blood vessels showing strong SDH reactivity were observed. In NADH dehydrogenase-reactive sections, focal decreases and increases in oxidative enzyme activities were observed. Adenosine monophosphate (AMP) deaminase activity was normal. The random checkerboard distribution of the histochemical fiber types was preserved as shown in the ATPase-reactive sections. Acid phosphatase activity was slightly high in some fibers. Muscle fiber glycogen contents appeared normal and the lipid contents were slightly high in some fibers. Electron microscopy showed abnormal proliferation of mitochondria with paracrystalline inclusions (Fig. 4).

Biochemical studies

All respiratory chain enzyme activities, which are expressed as a percentage of the normal control values relative to the citrate synthase activity, were greater than 20% (Table 1). BN-PAGE revealed no abnormalities in either the respiratory chain complexes or their molecular assembly structures.

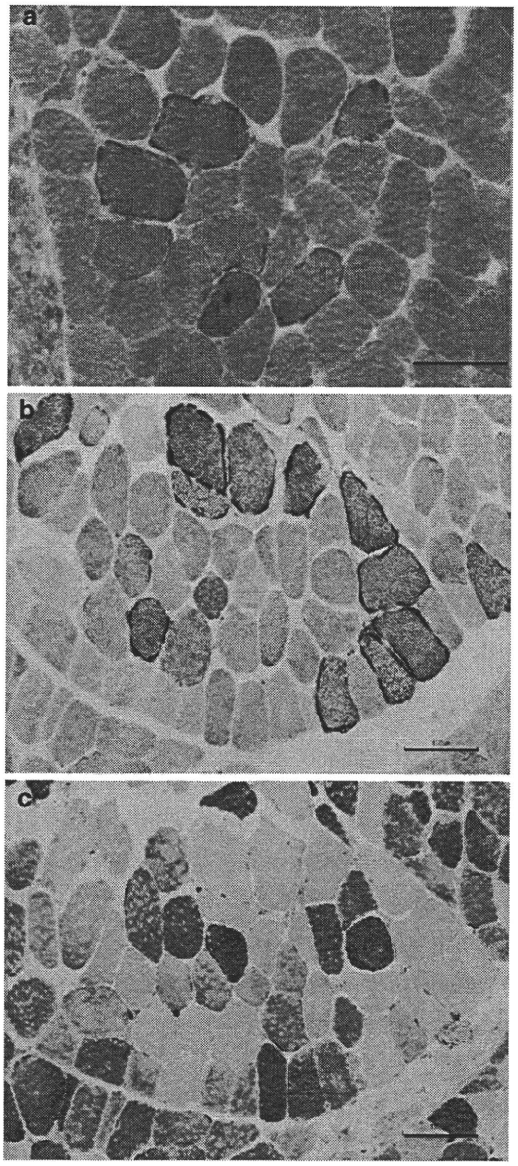

Fig. 3 Histochemical analysis of the right biceps brachii muscle. **a** Gomori trichrome staining reveals typical ragged-red fibers. Histochemical analysis of serial sections of samples stained with **b** SDH or **c** COX shows a number of ragged-blue fibers with COX deficiency. **a**–**c** *Bar* 100 μm

Mitochondrial DNA analysis

Using MitoChip v2.0, 37 missense variants were detected in the mitochondrial DNA of the peripheral blood lymphocytes. All of these variants show polymorphisms and are listed in the MITOMAP and GiiB-JST mtSNP databases. Two additional missense variants were detected in the mitochondrial DNA of the muscle homogenate; the variants were m.602C>T in the $tRNA^{Phe}$ gene and m.16111C>G in the D-loop. The variant m.16111C>G is listed as a polymorphism, but the variant m.602C>T is not

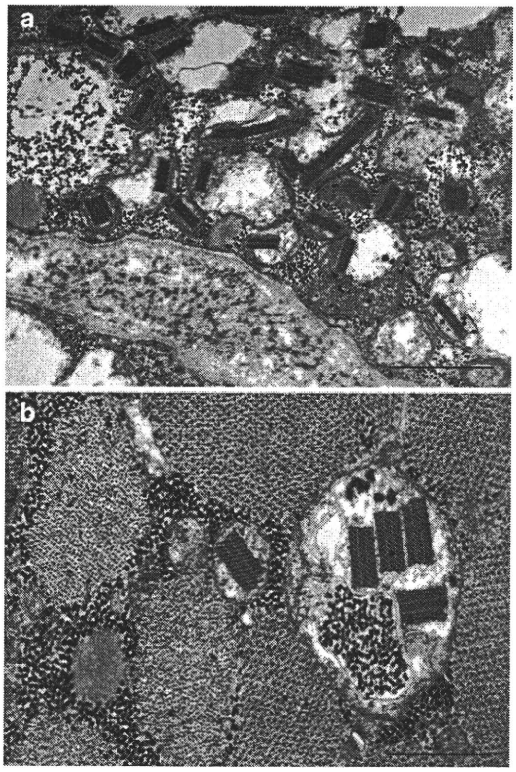

Fig. 4 Electron micrograph of abnormal mitochondria in the right biceps brachii muscle. Abnormal mitochondria with paracrystalline inclusions that are suggestive of mitochondrial myopathy are shown. **a** *bar* 1 μm, **b** *bar* 500 nm

reported in either database. The m.602C>T variant was also confirmed by direct sequencing. The sequence chromatogram showed a heteroplasmic m.602C>T transition in the muscle homogenate mitochondrial $tRNA^{Phe}$ gene (Fig. 5a). The proportion of mutant mitochondrial DNA in the muscle was 64.7 ± 1.2% (mean ± SD; the operation was performed thrice). Mutant mitochondrial DNA was not detected in the blood lymphocytes when measured using real-time amplification refractory mutation system quantitative PCR analysis (RT-ARMS qPCR), as previously described [2, 10]. Healthy Japanese controls ($n = 100$) did not show these mutations in their blood lymphocytes, at least not within the limits of Sanger's method for DNA sequencing.

Discussion

A novel mitochondrial $tRNA^{Phe}$ gene mutation was identified in a patient with late-onset predominant axial myopathy and cerebellar ataxia (patient 1). She presented with a maternal history of bent spine, and her elder sister presented with elevated lactate levels, severe paraspinal muscle atrophy, and epilepsy. Furthermore, the sister's four

Table 1 Enzymatic activities for mitochondrial respiratory complexes in patient 1

	CI activity (CI/CS)	CII activity (CII/CS)	CIII activity (CIII/CS)	CIV activity (CIV/CS)	CS activity
Patient 1	0.1938 (0.7027)	0.2723 (0.9874)	1.2737 (4.6192)	0.0579 (0.21)	0.2757
Control	0.3194 (1.6183)	0.2751 (1.3444)	1.3132 (6.5512)	0.0826 (0.3840)	0.2151
Patient 1/control ratio	60.7% (43.4%)	98.9% (73.4%)	97.0% (70.5%)	70.1% (54.7%)	

Enzymatic activities for individual mitochondrial respiratory complexes are given in nmol/min protein, and represent percentage of normal control ($n = 10$) mean relative to a reference enzyme of citrate synthase (CS)

The activities are relatively low in complex I and complex IV compared with other complexes

CI complex I, *CII* complex II, *CIII* complex III, *CIV* complex IV

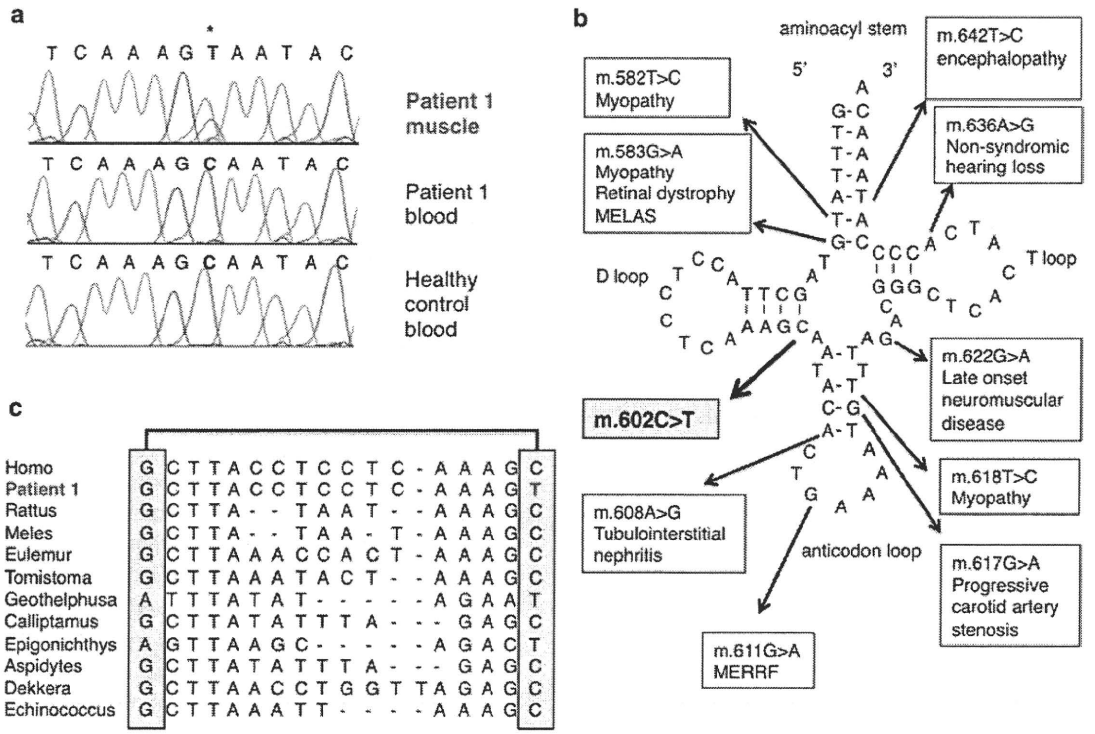

Fig. 5 **a** Sequence chromatogram of the mitochondrial DNA region that encompasses the m.602C>T alteration (*asterisk*) that was obtained from the skeletal muscle of patient 1 (reverse complement). **b** Schematic diagram of the mitochondrial $tRNA^{Phe}$ cloverleaf structure showing previously reported mutations and the m.602C>T alteration in the D-stem. **c** Comparison of mitochondrial $tRNA^{Phe}$ from different species. Base pairs, including the 602 nucleotides, are shown in *boxes*

sons presented with elevated CK levels, among which one had epilepsy. Patient 1 also presented with other symptoms associated with mitochondrial disease, including mild blepharoptosis, cataracts, hearing loss, and arrhythmia. Morphological examination revealed many ragged-red fibers and a partial deficiency in COX activity. One of the major diagnostic criteria for respiratory chain disorders in adults is less than 20% activity in any of the tissue complexes, but the data of the present study did not fulfill this condition [4]. However, the activities of complexes I (43.4%) and IV (54.7%) were lower than those of the other complexes. The decreased activities of complexes I and IV are probably due to the deficiency in COX activity that was measured in the muscle fibers. These clinical, morphological, and biochemical manifestations indicate that the patient most likely had a mitochondrial disease.

The marked atrophy of the paraspinal muscles was the most interesting feature found in patients 1 and 2. Axial myopathy has been defined as muscle weakness that is limited to the spinal and neck muscles [21]. Therefore, the symptoms of patient 1 are incompatible with pure axial myopathy because of the muscle weakness and mitochondrial abnormalities that were observed in the biceps brachii muscle. The most characteristic feature of axial myopathy is the remarkable atrophy of the paraspinal muscles rather than the atrophy of the muscles of the limbs, which is

different from the clinical symptoms of conventional mitochondrial myopathy. Thus, based on the available evidence, we believe that patients 1 and 2 can be diagnosed with mitochondrial predominant axial myopathy.

Axial myopathy may occur secondary to various diseases. However, only five cases of mitochondrial axial myopathy associated with the prominent involvement of the extensor muscles of the spine have been previously reported (Table 2) [8, 11, 28, 30, 32]. All these cases presented with abnormal trunk flexion that developed during walking and disappeared when the patient was in a supine position. In the cases described here, only patient 2 presented with camptocormia. These common symptoms, including late-onset, mildly elevated serum CK levels, ragged-red fibers, and the partial deficiency in COX activities, were observed in patient 1 and also in the above mentioned cases. However, biochemical analysis was performed in only one case that showed deficiencies in complexes I and III [32]. No case has been previously reported that describes a family history of similar symptoms. In addition, no genetic cause of any mitochondrial axial myopathy has been previously reported.

This study is unable to conclusively prove or disprove the pathogenicity of the m.602C>T mutation. However, three reasons that support the pathogenicity of this mutation are apparent. First, the heteroplasmic m.602C>T point mutation disrupts a conserved Watson–Crick cytosine–guanine (C–G) base pairing within the D-stem of the mitochondrial $tRNA^{Phe}$ gene, which would most likely affect the stability of the secondary structure of mitochondrial tRNA (Fig. 5b). Almost 94% of mitochondrial tRNA pathogenic mutations occur in this stem structure, and the disruption of Watson–Crick C–G base pairing is a significantly more common feature of pathogenic mutations than neutral variants [23]. Second, after performing a sequence homology search using CLUSTALW (http://clustalw.ddbj.nig.ac.jp/top-j.html), it was determined that this base pairing is largely conserved in other species as C–G or adenine–thymine base pairings (Fig. 5c). Third, the mutation is heteroplasmic and present in the affected skeletal muscles but not in the peripheral blood lymphocytes. Almost all pathogenic mitochondrial tRNA mutations in clinically affected tissues have a high proportion of heteroplasmy compared with unaffected tissues [23].

However, the decreased activities of complexes I and IV that were observed during the biochemical examination cannot be completely explained by the disruption in mitochondrial protein synthesis that could have been caused by the mitochondrial tRNA mutation. In addition, data obtained from the single muscle fiber analyses were limited due to the small sample size, and therefore, are not sufficient to prove the pathogenicity of the m.602C>T mutation.

Any additional evidence of the pathogenicity of the cybrid cells was not obtained. Therefore, 10 points (out of a maximum score of 20 points) was applied to the scoring criteria of the mitochondrial tRNA mutations listed in MITOMAP, which indicated that the m.602C>T mutation is possibly pathogenic [23].

The mechanism of late-onset axial myopathy induced by mitochondrial dysfunction is unclear. Nine pathogenic mutations in the mitochondrial $tRNA^{Phe}$ gene have been previously described in various diseases (Fig. 5b), including a late-onset neuromuscular disease but not axial myopathy [7, 9, 12, 14, 17, 18, 22, 25, 34, 35]. A probable etiological mechanism for the presentation of such a myopathy in the elderly is the accumulation of mitochondrial tRNA pathogenic mutations that affect aging tissues [9]. If it is possible to get any information on the pathological status of the primarily affected muscles, this would perhaps be as informative as the differential involvement of the biceps and paraspinal muscles. Unfortunately, these data could not be obtained due to the remarkable fatty degeneration of the paraspinal muscles.

The patients described in this report are characterized by the combination of axial myopathy and CNS involvement. One report about a parkinsonian patient with mitochondrial axial myopathy suggested that mitochondrial dysfunction

Table 2 Clinical characteristics of patients with paraspinal muscle atrophy from mitochondrial myopathy

Age/sex [Ref.]	Onset age	Family history	CK (IU/l)	RRF	COX deficiency	mtDNA mutation	Neurological deficit
73/F [patient 1]	63	+	290	+	+	602C>T	Cerebellar ataxia
84/F [patient 2]	60	+	474	NE	NE	NE	Encephalopathy
65/M [32]	59	–	245	+	+	NR	–
65/M [30]	62	NR	NR	+	+	NR	Parkinsonism
78/M [11]	78	NR	501	+	+	NR	–
64/M [28]	NR	NR	Elevated	+	+	NR	–
55/M [8]	NR	NR	Normal	+	+	NR	–

M male, *F* female, *CK* creatine kinase, *RRF* ragged-red fiber, *NR* not reported, *NE* not evaluated, *COX* cytochrome *c* oxidase, *mtDNA* mitochondrial DNA, *Ref* reference

may lead to both axial myopathy and parkinsonism [30]. In the patients described here, CNS involvement was similar to that observed in myoclonus epilepsy with ragged-red fiber (MERRF) due to the accompanying cerebellar atrophy and epilepsy. In fact, MERRF has been previously reported to be associated with pathogenic mutations of the mitochondrial $tRNA^{Phe}$ gene [22].

Finally, mitochondrial dysfunction might be implicated in the development of Hashimoto thyroiditis in patients 1 and 2 and in the fourth son of patient 2; the relationship between mitochondrial diseases and Hashimoto thyroiditis has been previously described [3, 20, 26, 27].

In summary, this is the first report about familial mitochondrial disease with late-onset predominant axial myopathy and encephalopathy, which were confirmed by clinical and histological findings. This case expands the phenotypic spectrum of mitochondrial diseases. Future studies on the novel mitochondrial $tRNA^{Phe}$ 602C>T mutation may contribute to the understanding of late-onset predominant axial myopathy and encephalopathy.

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ORIGINAL ARTICLE

Morphological Progression of Myelin Abnormalities in IgM-Monoclonal Gammopathy of Undetermined Significance Anti–Myelin-Associated Glycoprotein Neuropathy

Yuichi Kawagashira, MD, Haruki Koike, MD, PhD, Minoru Tomita, MD, Saori Morozumi, MD, Masahiro Iijima, MD, PhD, Tomohiko Nakamura, MD, PhD, Masahisa Katsuno, MD, PhD, Fumiaki Tanaka, MD, PhD, and Gen Sobue, MD, PhD

Abstract

To characterize the morphological progression of neuropathy associated with immunoglobulin M–monoclonal gammopathy of undetermined significance with anti–myelin-associated glycoprotein antibody, we assessed histopathologic features of sural nerve specimens from 15 patients, emphasizing widely spaced myelin (WSM), demyelination, and tomaculous changes. The frequency of WSM correlated with that of demyelination and tomaculous appearance in teased-fiber preparations. In longitudinal sections at nodes of Ranvier and paranodal regions, the spaces between terminal myelin loops, particularly those adjacent to the node of Ranvier, were widened, indicating an early change before demyelination, and there was concomitant swelling of terminal myelin loops. Some conspicuously swollen terminal myelin loops were detached from the paranodal axolemma, thereby widening the nodes of Ranvier. Tomacula coexisted frequently with redundant myelin loops and WSM, particularly in the outermost layer of myelin sheaths, suggesting that loosening of the outer layers contributes to their formation. By immunofluorescence microscopy, immunoglobulin M and myelin-associated glycoprotein were colocalized in paranodal regions and Schmidt-Lanterman incisures. Confocal analysis revealed colocalization of immunoglobulin M and complement product C3d corresponding to the area of WSM. Thus, morphological changes in terminal myelin loops, formation of WSM at paranodes, and subsequent dissociation from paranodal axolemma (which may be associated with activation of the complement pathway) likely contribute to demyelination in this condition. Loosening of compact myelin seems to contribute to tomacula formation.

Key Words: Anti-MAG antibody, IgM monoclonal gammopathy of undetermined significance, Neuropathy, Widely spaced myelin.

INTRODUCTION

Polyneuropathy associated with immunoglobulin M (IgM) monoclonal gammopathy of undetermined significance (MGUS) with anti–myelin-associated glycoprotein (MAG) antibody is characterized by late age of onset, slow progression, and sensory-motor symptoms, particularly sensory ataxia caused by predominant impairment of large myelinated nerve fibers (1–6). In a recent study, anti-MAG antibody was found in approximately 70% of patients with neuropathy-associated IgM monoclonal gammopathy (7). Much of the MAG molecule is exposed on the membrane surface and would, therefore, be an accessible antigen. A pathogenetic role for anti-MAG antibody in this neuropathy has been indicated in several studies (8–14).

Pathological studies of IgM-MGUS anti-MAG neuropathy have demonstrated the presence of IgM antibody deposits at sites of MAG localization with loss of myelinated fibers, and teased-fiber preparations have demonstrated segmental demyelination, tomaculous appearance, and axonal degeneration (3, 8, 15–20). Ultrastructural studies have shown that widely spaced myelin (WSM), in which the distance between intraperiod lines is increased, is a nearly specific finding in this condition (11, 21–25). However, it is still unclear whether WSM is caused by the deposition of IgM antibody and how WSM relates to the onset of demyelination or tomacula. Furthermore, the morphological changes that occur during demyelination in this neuropathy, particularly where demyelination begins, what the earliest change is, and how demyelination may affect axonal structure are not known.

This study demonstrated morphological features of demyelination that are unique to IgM-MGUS anti-MAG neuropathy, focusing in particular on the correlation between WSM and the process of myelin alterations, including demyelination and tomaculous formation.

MATERIALS AND METHODS

Patients

Fifteen patients with anti-MAG neuropathy who were referred to the Department of Neurology of Nagoya University Hospital and who underwent sural nerve biopsy from 1996 to 2008 were investigated. We excluded patients who had evidence of a hematologic malignancy (multiple myeloma, Waldenstrom macroglobulinemia, lymphoma, or amyloidosis) or other diseases that may have contributed to their neuropathy. Determination of MAG autoantibody titer was performed by ELISA using purified human MAG antigen (Athena Diagnostics, Worcester, MA). Confirmation of all positive MAG

From the Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan.
Send correspondence and reprint requests to: Gen Sobue, MD, PhD, Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan; E-mail: sobueg@med.nagoya-u.ac.jp