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hypoperfusion in the cerebellum. At the age of 52, he was admitted to a hospital, and this demonstrated dysarthria and a broad-based gait. Brain MRI and peripheral nerve conduction study revealed no apparent abnormalities. At the age of 54, he became irascible and began to experience difficulty in swallowing.

At the age of 55, the patient was transferred to our hospital for medical care and recuperation. He was unable to walk without assistance. Tremor was observed in the upper extremities. Vertical ocular movement was limited. Tongue tremor, scanning and slurred speech, and postural and intension tremor were also evident. There were no paroxysmal discharges on the EEG. Brain MRI showed suspicious frontotemporal cortical atrophy with mild enlargement of the lateral ventricles; atrophy of the brainstem and cerebellum was not evident. At the age of 56, dementia became evident: his score on the revised version of the Hasegawa dementia scale (a mental examination method commonly used in Japan that utilizes a scale of 0 to 30, where <21 represents dementia; [10]) was 19. Dysphagia progressed gradually, and action myoclonus appeared. Thereafter, he became bedridden with repeated episodes of aspiration pneumonia, necessitating tube feeding. At the age of 59, the patient died of pneumonia.

During the course of his illness, the patient was treated with an anticonvulsant clonazepam (2.5 mg/day); this was effective against myoclonus in the early stage, but became much less effective in the late stage (≤6.5 mg/day). A general autopsy was performed about 10 h after death, at which time the brain weighed 1,400 g. The kidneys (left, 220 g; right, 165 g) were grossly unremarkable. The cause of death was aspiration pneumonia in both lungs.

Case 2

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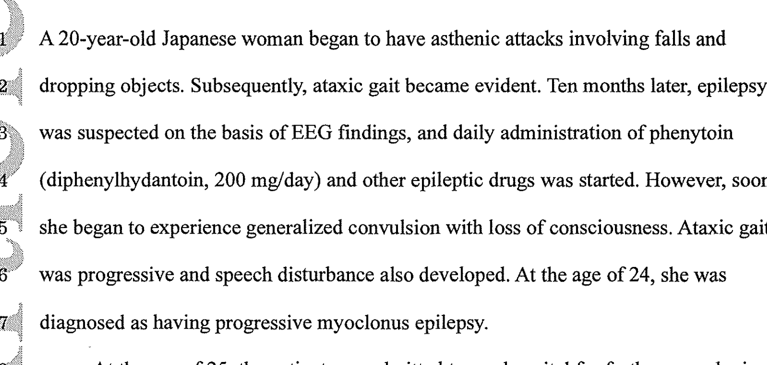

A 20-year-old Japanese woman began to have asthenic attacks involving falls and dropping objects. Subsequently, ataxic gait became evident. Ten months later, epilepsy was suspected on the basis of EEG findings, and daily administration of phenytoin (diphenylhydantoin, 200 mg/day) and other epileptic drugs was started. However, soon she began to experience generalized convulsion with loss of consciousness. Ataxic gait was progressive and speech disturbance also developed. At the age of 24, she was diagnosed as having progressive myoclonus epilepsy.

At the age of 25, the patient was admitted to our hospital for further neurological evaluation. Tongue tremor, truncal ataxia, dysphagia, and action myoclonus in the upper and lower extremities were observed. She was unable to walk without assistance. Cognitive decline (intellectual deterioration) was also evident. Liver biopsy revealed no Lafora bodies. Thereafter, her general condition gradually worsened. She suffered from repeated urinary infection and pneumonia, as well as frequent convulsions. EEG showed multifocal spike and wave complexes. At the age of 28, she died of bronchopneumonia.

During the course of her illness, chorea was not observed. No clinical or laboratory findings suggestive of renal dysfunction were evident. The serum concentration of phenytoin was well controlled (<20 µg/ml). The entire clinical picture was considered to be indistinguishable from that seen in DRPLA (juvenile-onset) [11]. A general autopsy was performed about 2 h after death, at which time the brain weighed 1,370 g. The kidneys (left, 70 g; right, 95 g) were apparently small in size. The cause of death was bronchopneumonia in both lungs.

Mutational analysis of the *SCARB2* gene

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Direct sequencing of exon 11 of *SCARB2* in case 1 and the controls revealed a homozygous frame-shift mutation, c.1385_1390del6insATGCATGCACC in case 1 (Figure 2A), resulting in a production of aberrant SCARB2 protein, in which the C-terminal trans-membrane domain was lost (Figure 2**C**). This frame-shift mutation resulted in loss of the BamHI cleavage site, and created a cleavage site for SfaNI. Thereby, restriction analyses showed that the PCR fragments of exon 11 of *SCARB2* in case 1 were digested by SfaNI, and not by BamHI (Figure 2**B**), supporting the data obtained from direct sequencing.

On the other hand, direct sequencing of exon3 in case 2 and the controls revealed a homozygous nonsense mutation, c.361C>T, p.R121X in case 2 (Fig. 7A), resulting in a production of an extremely short SCARB2 protein defective in the majority of its luminal and C-terminal regions (Figure 2**C**).

Neuropathological findings

In both cases, the cerebellum appeared somewhat small. In sections, although mild, folial atrophy was noted in the superior parts of the vermis and hemispheres. In addition, shrinkage of the pontine tegmentum was evident. In case 2, the globus pallidus (both internal and external segments) appeared somewhat atrophic.

Presence of extraneuronal brown pigment

In both cases, the most striking features was the presence of granules of brown pigment of various sizes (major axis ~10 μm) scattered widely in the brain, including the cerebellar cortex (Purkinje cell layer), cerebral cortex (layer I and II, rarely extending to layer III), basal ganglia (globus pallidus and neostriatum), substantia nigra (pars

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reticulata) and choroid plexus. These granules were abundant in the cerebellar and cerebral cortices and were apparently located extraneuronally (Figure 3**A**, **E**). They were clearly recognizable by Masson-Fontana silver staining (Fig. 3**B**, **F**) and showed autofluorescence (Figure 3**C**). Only in the cerebellar cortex, the granules were often PAS-positive (Figure 3**D**), but never digested by α–amylase or diastase. In both cases, such granules were not evident in the hippocampal formation (hippocampus, dentate gyrus and subiculum).

Immunohistochemically, these pigment granules were completely unreactive with any of the antibodies used in the present study, including those against polyglutamine stretches, ubiquitin and p62.

Ultrastructurally, in both cases, the pigment granules appeared as membrane-bound, round to oval solitary dense bodies, or compound dense bodies formed by conglomeration of solitary dense bodies; individual dense bodies lacked the characteristic lipid droplets seen in lipofuscin granules (Figure 4**A**, **B**). These features were consistent with those of lysosomes [12]. Importantly, the granules were localized exclusively in the astrocytic cytoplasm (Figure 4**B**).

Presence of neurodegeneration

In both cases, moderate to severe loss of Purkinje cells with proliferation of Bergmann's glia was observed in the cerebellar cortex (Figure 5**A**, **B**). Although mild, loss of granule cells was also noted. In sagittal sections of the vermis and cerebellar hemispheres, the loss of Purkinje cells was rather diffuse without particular predilection sites. In the dentate nucleus, mild neuronal loss and gliosis were noted; however, no grumose degeneration [13] was observed. In the white matter, myelin pallor was not

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conspicuous and gliosis was only mild with accentuation in the amiculum of the dentate nucleus.

In the cerebral cortex, neuronal loss and gliosis were not evident On the other hand, neuronal loss and gliosis were present in some regions of the subcortical gray and brainstem. In the globus pallidus, mild to moderate neuronal loss with gliosis was observed in the external segment, being more marked in case 2 (Figure 5**C**, **D**). In the subthalamic nucleus, only gliosis was noted in case 1, and mild neuronal loss was also observed in case 2. The pontine tegmentum was atrophic, and showed diffuse mild myelin pallor and gliosis without evident neuronal loss (Figure 5**E**). Neuronal loss and gliosis were also observed in the vestibular and inferior olivary nuclei. In the inferior olivary nucleus, neuronal loss was diffuse (Figure 5**F**) and appeared to well correspond to the diffuse loss of Purkinje cells in the cerebellar cortex [14]. These changes observed in the brainstem appeared to be more severe in case 2, except for the inferior olivary nuclei, which were almost equally affected.

In the spinal cord, myelin pallor and axon loss were evident in the anterolateral column and the central part of the posterior column, being more evident in case 2 (Figure 6**A**). Paucity of motoneurons, with a number of dystrophic swollen neurites, was also observed in the anterior horns (lumbar > cervical) (Figure 6**B**). Of interest was that the motoneurons were occasionally seen to have coarse granular eosinophic inclusions in their cytoplasm (Figure 6**C**); these eosinophilic inclusions were much larger than normal lipofuscin granules and PAS-negative, and immunopositive for ubiquitin (Figure 6**D**) and p62 (Figure 6**E**). However, ultrastructurally, they were electron-dense, irregularly shaped structures resembling lipofuscin granules (Figure 6**F**). In the dorsal root ganglia, scattered residual nodules (Nageotte nodules) were evident.

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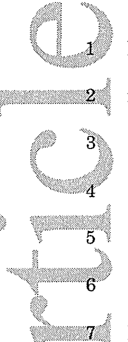

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In case 1, mild neurogenic atrophy was noted in the examined scalenus and iliopsoas muscles, and diaphragm.

In both patients, there was no immunohistochemical evidence suggestive of the involvement of tau, α-synuclein, TDP-43 or CAG repeat expansion (polyglutamine streches).

Immunohistochemical expression of SCARB2 (LIMP II)

With regard to pathology in the kidney, there were no apparent histological abnormalities except that individual glomeruli were generally smaller in cases 1 and 2 than in controls; the glomeruli in case 2 were much smaller than in case 1 (Figure 7**A**, **B**, **C**). SCARB2-immunoreactive cytoplamic granular or vesicular staining was ubiquitously present in the kidney and brain cells of the controls (Figure 7**A**, **D**, **G**, **J**). In the kidney, such staining was seen mainly in the tubular epithelium and vascular endothelial cells (Figure 7**A**). Strong staining was detected in neurons and white matter oligodendrocytes in the brain regions examined (Figure 7**D**, **G**, **J**). In addition, dot-like or coarse granular staining was also seen in the neuropil of the Purkinje layer and white matter (Figure 7**D**, **G**); some of this staining was also seen in astrocytes. In case1, the staining intensity was markedly decreased in nearly all cells (Figure 7**B**, **E**, **H**, **K**), except for neurons of the cerebellar dentate nucleus (Figure 7**E**, inset). In case 2, SCARB2 was undetectable in all of the sections examined (Figure 7**C**, **F**, **I**, **L**).

Discussion

We have described two autopsied patients with PME without renal failure (cases 1 and 2), carrying novel homozygous frame-shift and nonsense mutations in the *SCABR2* gene,

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respectively. The present study is noteworthy from several aspects. The frame-shift mutation is a hitherto undescribed, unique type of *SCABR2* gene mutation. It is the first to have described patients with PME-SCARB2, with details of their neuropathological features and to have demonstrated the presence of system neurodegeneration in the affected brains. It is also the first to have demonstrated immunohistochamically decreased levels and no expression of SCARB2 protein in both the brain and kidney tissues, respectively

AMRF is a progressive, autosomal recessive, neuronal-renal disease in adolescents and young adults: the age at onset ranges from14 to 26 years (mean 20.0 years) [1, 2, 15] and the ages at death ranges from 25 to 35 years (mean 30.1 years) [1, 2]. The neurological features include tremor, action myoclonus, generalized seizures and cerebellar dysfunction; tremor is often the initial symptom [1, 2, 15]. Recently, reported cases of PME-SCABR2 have been accumulating [5-7, 16-18], confirming the clinical aspect of the disease, including the ages at onset (14-26 years; man 18.8 years).

With regard to the *SCABR2* gene mutation, the frame-shift mutation detected in case 1 ought to result in production of SCARB2 with an almost normal length, including the coiled-coil motif in the luminal domain (Figure 2). Immunostaining analyses of brain and kidney samples with an antibody specific for the C-terminus of SCARB2 (LIMP II) showed that the protein was not severely decreased in case 1 (Figure 7). On the other hand, the R121X mutation detected in case 2 (Figure 2) was a type similar to those in cases reported previously [5, 17]. Such nonsense mutations generate premature translation termination codons (PTCs) and generally facilitate degradation of SCARB2 mRNA by nonsense-mediated mRNA decay (NMD). Immunostaining revealed no expression of the protein in case 2 (Figure 7).

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With regard to the pathology, we have been able to find only two autopsy cases of AMRF [1, 2] through a PubMed-MEDLINE search, one of which had homozygous mutations (W146SfsX16) in *SCARB2* that were identified later [4]. According to the published descriptions, in both cases, extraneuronal, abnormal brown pigmentation in the cerebral and cerebellar cortices as well as the basal ganglia (globus pappidus and putamen) and substantia nigra, was a feature. However, neither showed any features of neurodegeneration, i.e. neuronal loss or associated gliosis, in the affected regions.

However, it is interesting to note one autopsy case report of a "pigment variant of neuronal ceroid lipofuscinosis (Kufs' disease)" in a 24-year-old woman, which had been published before AMRF was first described [19]; at present, this patient is not considered to have had Kufs' disease [20, 21]. The patient's clinical history and neuropathology appeared to be typical of AMRF [1, 2]. Importantly, in this patient, moderate loss of Purkinje cells and occurrence of neuronal cytoplasmic inclusions were also observed in the cerebellar cortex and lumbosacral enlargement, respectively [19].

Only a small number of clinical and genetic features of PME-SCARB2 have so far been described; it still appears difficult to discuss the genotype-phenotype correlations of SCARB2. In the two Japanese cases we examined here, extrapyramidal signs (tremor and action myoclonus) and cerebellar features (ataxia, dysarthria and intension tremor) were evident. In addition, generalized seizures were observed in case 2, and dementia in both cases. Neither patient had any evident renal disease. It was also noteworthy that in case 2, the clinical features, including dementia, were very similar to those of dentatorubral-pallidoluysian atrophy (DRPLA), although the family history was non-contributory [11]. DRPLA, which is most prevalent in Japan, is an autosomal dominant neurological disease caused by an expansion of the CAG repeat in the *DRPLA*

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gene [13]. At present, one clinical feature “progressive myoclonus epilepsy” links the two hereditary neurological diseases, AMRF and DRPLA, together [20, 21]. Surprisingly, the age at onset in case 1 (45 years) was much older than that in case 2 (20 years); in this connection, the oldest age at disease onset reported previously was 26 years, the patient being an Italian with compound heterozygous mutations (c.424-2A>C and H363N) in *SCARB2* [5, 7]. The much later disease onset in case 1 may have been attributable to the type of *SCARB2* mutation resulting in production of mutant SCARB2 with an almost normal length (Figure 2). This might also have explained the comparatively slow progression of the disease, without generalized seizures.

In the present two cases, the neuropathological study revealed not only widespread deposition of abnormal brown pigment in the brain but also neurodegeneration, i.e. neuronal loss and associated gliosis, in some brain and spinal cord regions. The distribution pattern and morphological profiles of the brown pigment were actually the same as those demonstrated previously in cases of AMRF [1, 2]. The neurodegeneration was rather widely spread in the nervous system, including the pallido-luysian and cerebello-olivery systems.

The present study is the first to have demonstrated neurodegeneration in patients with PME-SCARB2. The degree of neuronal loss and gliosis, as well as myelin pallor, were in general less severe in the brain and spinal cord lesions in case 1 than in case 2. Again, the less severe degenerative changes may have been attributable to the above-mentioned molecular genetic background detected in case 1. It was also of interest that the spinal motoneurons occasionally possessed ubiquitin- and p62-positive coarse eosinophilic inclusions. It was difficult to distinguish the present neuronal cytoplamic eosinophlic inclusions (Fig. 6C) from the lumbosacral neuronal cytoplamic

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inclusions (Lafora bodies of the protein type) reported by Horoupian and Ross [19: see Fig. 5].

In the present cases, neuronal loss and gliosis in the pallidoluysian and cerebello-olivary systems must have been associated with the patients’ involuntary movements and cerebellar dysfunction, respectively. Moreover, degenerative changes observed in the upper and lower motor neuron systems as well as in the dorsal root ganglia strongly suggested that both the motor and sensory neuron systems were also involved in the disease process in PME-SCARB2. It is interesting to note that peripheral neuropathy, cardiomyopathy and hearing loss can occur in some patients with PME-SCARB2 [7, 16-18].

Finally, dementia (or cognitive decline), which has never been described in patients with AMRF [1, 2, 15] or PME-SCAB2 [5-7, 16-18], was evident in the present two patients. It is important to note that marked dementia was evident in the final year of life in the 24-year-old woman who was diagnosed postmortem as having a “pigment variant of neuronal ceroid lipofuscinosis (Kufs’ disease)” [19]. We presumed that in the present two patients, this clinical aspect was “cortical-subcortical” in nature; in this context, widespread deposition of brown pigments in the cerebral cortex as well as neurodegeneration in the basal ganglia (globus pallidus) and pontine tegmentum (reticular formation) appeared to be of great importance. However, one could argue that the pigment itself is innocuous, since there was no evident neuronal loss or gliosis in the cortex.

In conclusion, we have described two Japanese cases of PME with novel *SCARB2* mutations. The frame-shift mutation detected in case 1 was a hitherto undescribed, unique type, resulting in production of mutant SCARB2 with an additional 16 amino

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acids at the end of the C-terminus. Pathological investigation convincingly demonstrated the co-occurrence of extraneuronal (astrocytic cytoplasmic) brown pigment deposition and system neurodegeneration in the affected brains. It was considered that dementia (cognitive decline) could be one of the important clinical features of the disease. Of great interest was why the present two patients had neurodegeneration (neuronal loss and gliosis) as well as dementia. In one patient (case 1), it is tempting to suggest that the relatively long survival was responsible for these features. However, such an explanation would not have been applicable to the other patient (case 2). Further studies are needed to explore the role of SCARB2 in brain and kidney function, and the molecular pathogenesis of PME-SCARB2.

Acknowledgments

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Author Contributions

HT was project leader who conceived and managed the research and was principally responsible for writing. IA*, TN, HN and MN examined the patients and carried out the clinical analysis, YJF*, MT, YT, ST, AK and HT performed the neuropathological observation and evaluation, and MT* and OO performed the sequencing and restriction analysis. These authors* contributed equally to this work.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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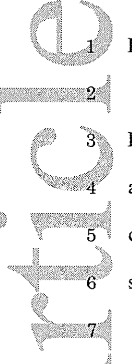

Figure legends

Fig. 1 An abbreviated pedigree of case 1. Filled symbols and slash marks indicate affected and deceased individuals, respectively. An arrow indicates the proband (III-1, case 1). Grandparents (I-1 and I-2) and parents (II-4) of the proband were first and second cousins, respectively.

Fig. 2 Sequence and restriction analyses of the *SCARB2* gene. (A) Direct sequencing of exon 11 of case 1 and controls showing homozygosity for c.1385_1390del6insATGCATGCACC (deletion/insertion) mutation (left panel). Direct sequencing of exon 3 of case 2 and controls showing homozygosity for c.361C>T (non-sense) mutation (right panel). (**B**) Restriction analyses showing that the PCR fragments of exon 11 of *SCARB2* in case 1 were digested by SfaNI, but not by BamHI. (**C**) Exonic structure of *SCARB2* cDNA and domain structure of the SCARB2 protein. TM, transmembrane domain.

Fig. 3 Deposition of brown pigment in the brain. Sections from case 1 (cerebellar vermis; **A-D**) and case 2 (middle frontal cortex layer II; **E, F**). (**A**) Many coarse brown pigment granules are recognizable in the Purkinje cell layer of the cerebellar cortex in the haematoxylin-eosin (HE)-stained section. (**B, C**) The granules are stained black by the Masson-Fontana silver method and show autofluorescence. (**D**) In the cerebellar cortex, most of the granules are also stained pink by the PAS method. (**E, F**) Such pigment granules are also recognizable in the cerebral cortex in the HE-stained section and stained black by the Masson-Fontana silver method. Scale bars = 20 µm.

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Fig. 4 Ultrastructure of the brown pigment granules observed in the Purkinje cell layer (case 1). (**A**) Isolated and conglomerated pigment granules, composed of fine granular and filamentous materials varying in electron density are evident. (**B**) In this pigment-bearing cell, longitudinally and transversely cut bundles of intermediate filaments (glial filaments) are evident in the cytoplasm (nucleus: lower left). (**A**). Scale bars = 0.5 μm.

Fig. 5 Degenerative changes in the brain. Sections from case 1 (**A**, **B**, **F**) and case 2 (**C**, **D**, **E**), stained with HE (**A**, **D**), and Holzer (**C**) and Klüver-Barrera (KB) methods (**E**, **F**), and immunostained with antibodies against calbindin D-28k (**B**) and phosphorylated neurofilament protein (**G**). (**A**) Loss of Purkinje cells is evident in the cerebellar vermis. An increased number of small, round nuclei of Bergmann's glia are also evident in the Purkinje cell layer. (**B**) A few scattered Purkinje cells are seen in the cerebellar hemisphere. Also note the marked reduction of Purkinje cell dendrites in the molecular layer. (**C**) Fibrillary gliosis is evident in the external segment of the globus pallidus. (**D**) In the central area of the external segment shown here, no neurons can be seen. (**E**) In the pons, the proportion of the tegmentum to the base is markedly reduced (tegmental shrinkage). (**F**) Diffuse neuronal loss is evident in the inferior olivary nuclus (the right lateral part is shown). Scale bars = 50 μm for (**A**, **B**, **D**), 2 mm for (**C**), 5 mm for (**E**) and 100μm for (**F**).

Fig. 6 Degenerative changes in the spinal cord. Sections from case 2(**A**) and case 1 (**B-F**), stained with KB method (**A**, **B**) and HE (**C**), and immunostained with antibodies

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against ubiquitn (**D**) and p62 (**G**). (**A**) The cervical cord (C7), showing myelin pallor in the bilateral anterolateral columns, especially in the lateral and anterior corticospinal tracts, where sudanophic droplets were also evident. The change in the white matter is reminiscent of that seen in amyotrophic lateral sclerosis. (**B**) A small population of motoneurons is evident in the lumbar anterior horn (L4). (**C**) A cytoplasmic area containing coarse granular eosinophilic inclusions is evident in a lumbar motoneuron (L3) (**D**) Such inclusions observed in the spinal motoneurons show positivity for ubiquitin. (**E**) Serial section of (**D**), showing that these inclusions are also positive for p62. (**F**) Recycled electron microscopy specimen of the eosinophilic inclusions seen in (**C**), showing irregularly shaped structures consisting of electron-dense materials and associated empty vacuoles. Scale bars = 2 mm for (**A**), 500 μm for (**B**), 20 μm for (**C-E**) and 1 μm for (**F**).

Fig. 7 SCARB2 immunostaining performed on kidney and brain sections. SCARB2-immunoreactive granular staining is evident in the cytoplasm of cells in the renal cortex (**A**), cerebellar cortex (**D**), cerebellar white matter (**G**), and temporal cortex layer II (**J**) in a control (a 64-year-old female). The degrees of staining intensity shown in the control are clearly decreased in the corresponding areas in case 1 (**B**, **E**, **H**, **K**); note that the cerebellar dentate nucleus neurons are an exception, showing well preserved staining intensity (**E**, **inset**). No immunoreactivity is evident in any areas in case 2 (**C**, **F**, **I**, **L**). Note that although the glomerulus in case 1 (**B**) and that in case 2 (**C**) are apparently small compared with that in the control (**A**), no obvious abnormalities are evident in these glomeruli. Arrows indicate SCARB2-immunonegative brown pigment granules (**E**, **F**, **K**, **L**). Scale bars = 50 μm

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1 for (**A-C**) and 20 μm for (**D-L**).

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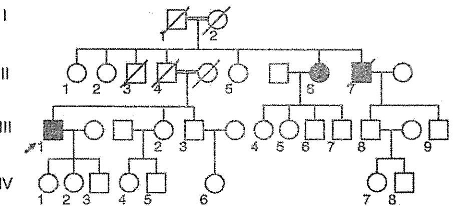
Accepted Article



Fu et al.

Figure 1

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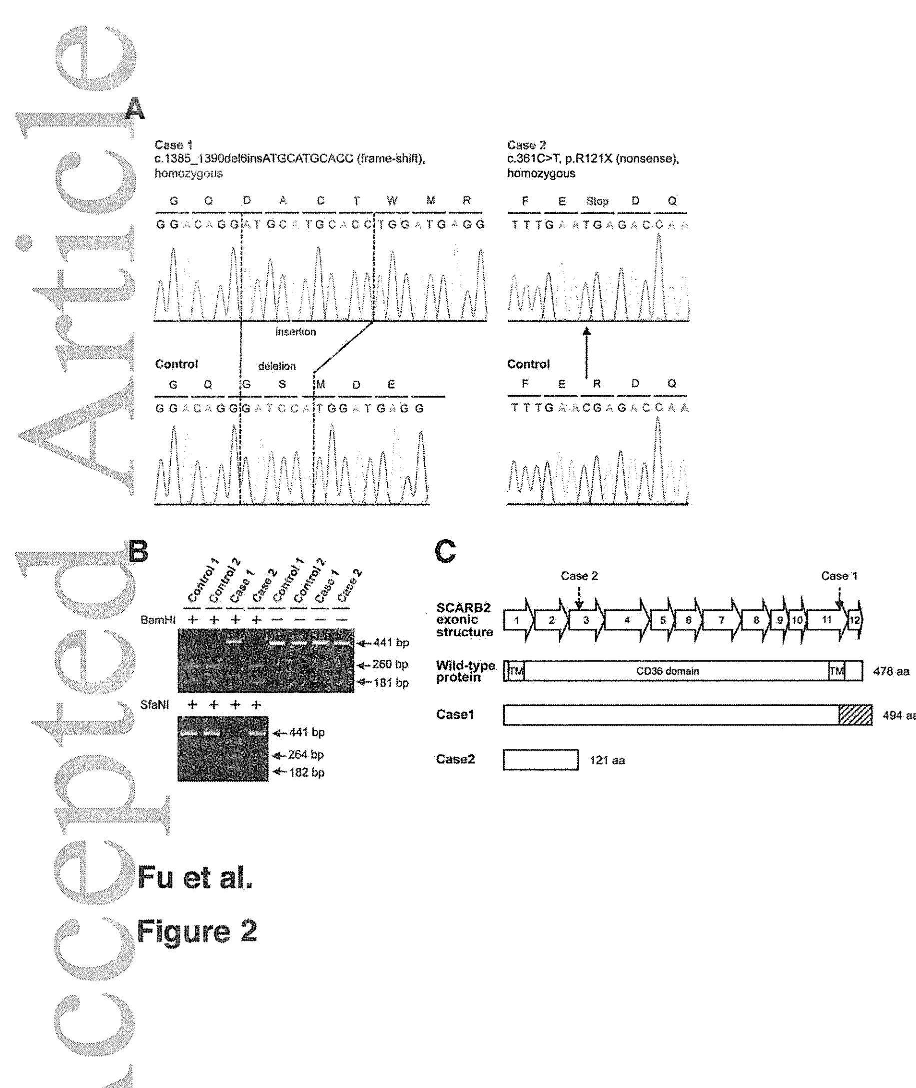

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Figure 2

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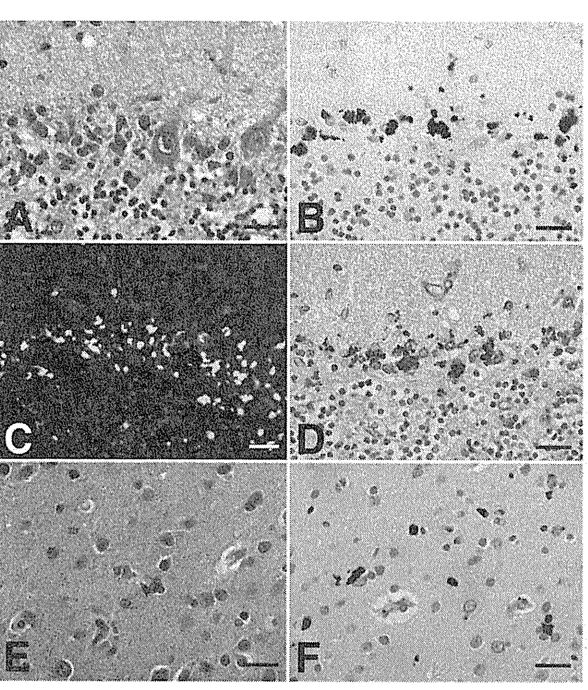

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Figure 3

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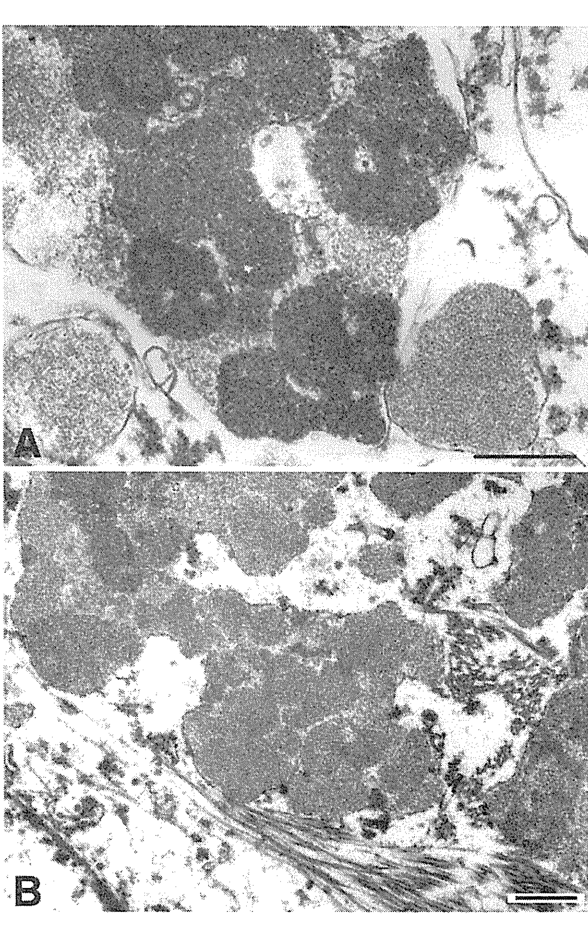

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Figure 4

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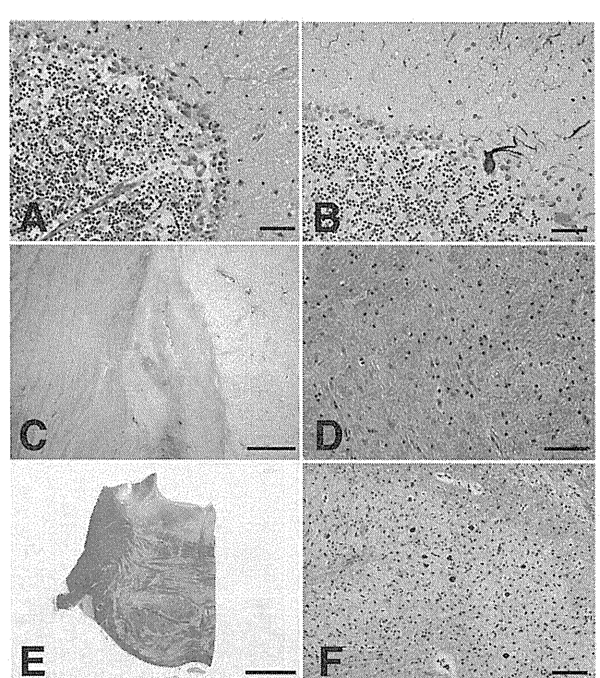

Fu et al.

Figure 5

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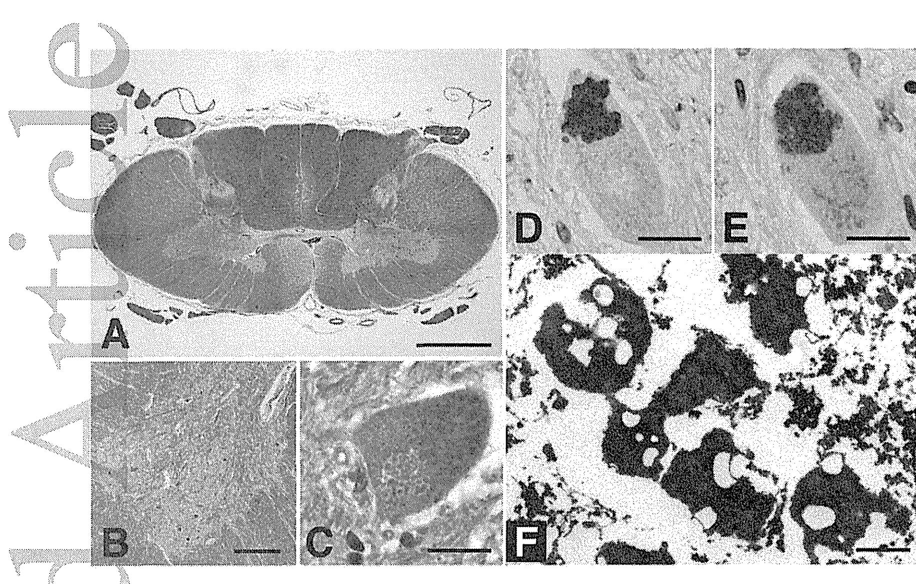

Fu et al.
Figure 6

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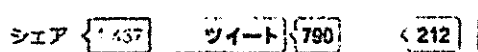
Fu et al.
Figure 7

2014.02.03 Mon

地球で生きる宇宙飛行士――『宇宙兄弟』はなぜALSを描いたのか？

川口有美子×佐渡島庸平

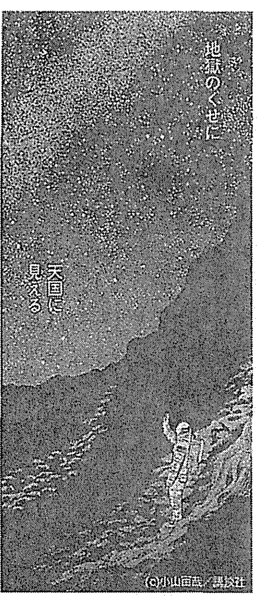


ALS患者は宇宙飛行士

川口　『宇宙兄弟』を読む前から、ALS患者と宇宙飛行士ってすごく似ているなってずっと思っていたんです。だから『宇宙兄弟』でALSがでてきたとき「やっぱり！」って。本当にぴったりな病気を選ばれたと思います（笑）。

ALSの人って宇宙空間に投げ出された状態と一緒なんですよね。呼吸器に24時間繋がれていないといけなくて外れたら死んじゃうでしょ。息する機械に対して120パーセントの信頼がもてないと生きていけない。。

日々人が月面を探索中にクレーターに落ちてしまって、真っ暗闇の中で絶望に陥りかけるシーンがありますよね。ALSの患者さんはみんなそれを体験しているんです。しかもね、日々人がそうだったように負けないんです。究極の孤独の中にいるのに明るい気持ちを保とうとしている。



クレーターに落ちた日々人

佐渡島　ALSになったシャロンは宇宙兄弟の気持ちを誰よりもわかるということかもしれませんね。

川口　そう思いますよ。

私は人間と機械の友好的な関係が好きなんです。医療専門職の中には患者さんの人間性を医療機械が奪ってしまう場面をいっぱい見ているから機械に対して批判的な人が多いんです。だから呼吸器をつけないと生きていけないようになってしまったときに、呼吸器をつけさせたがらないことがあるの。でも私はALS患者さんと20年間も一緒に活動してきたから、死ぬくらいならむしろ機械をつけても生きるほうが自然に思えてる。

佐渡島　宇宙飛行士にとって宇宙服が仲間なのと一緒。

川口　そうそう！　人類の科学の粋を集めた機械に対する一体感をALSの患者さんは持っている。それにね、いま自分がどんなに辛くても、頑張って生きていれば、いつか科学が追いついてくれる。自分は治らなくても、人類がALSに勝つ日がやってくると信じている。一人ひとりが主人公なんですよ。身体が動かなくても呼吸器をつけて、ただ息をしているだけの状況で、20年、30年生き続けてALSに負けないでいるの。

みんなに守られている

佐渡島　ALSの患者さんって、呼吸器をつけるという選択をした後に、瞼を開いたままにするか、閉じたまま真っ暗闇の中で生きるかって選択肢がありますよね。『逝かない身体』を読むと、川口さんのお母さんは目を閉じることを選ばれていました。

川口　私の母は「開けておくと目が乾いて痛くなるから閉じておいて」って言ったんです。悲しかったけど、「わかった、閉じておくね。でもときどきこっちで開けるからね」って。

佐渡島　急に強い光をみても目が痛くなりますよね。

川口　だからカーテンを締めて、部屋を薄暗くして。

朝が来るとホッとするみたいです。夜が怖いんですよね。動かないから体力を使っていなくて3時くらいには目が覚めちゃう。だいたい2時間半くらいしか寝てないんじゃないかなって。不安だから寝てもすぐに起きちゃうんだけど、家族を起こすのもかわいそうだから、身体が痛くても朝まで我慢している。

佐渡島　家族を起こすときは、ナースコールを押すんですか？

川口　ちょっとでも動くと反応するナースコールを身体の動くところに張り付けておくんです。うちの母も朝まで我慢していました。父が5時に起きて、枕元にあるラジオのスイッチをいれて「おはよう」って。私も「おはよう、朝が来たよ、今日も生き延びたね」って瞼を開いてあげて、テレビのスイッチをいれたり、朝ごはんを胃ろうに流し込んであげたり。

家族が起きてがちゃがちゃした生活音の中に入った途端に安心して眠りだすんですよね。それってわかる気がしません？　耳慣れた生活音に囲まれていると生きているって安心できるらしいんですよ。身体を動かすことはほとんどできないけれど、自宅で生活している。親しいみんながいることで自分の存在が確認できる。

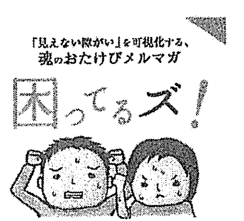
だから絶対に病室に一人で隔離するようなことはしちゃいけない。よっぽど心の強い人じゃないと不動と孤独になんて耐えられません。いつも誰かがそばにいて守られているって思えるから、生きていけるんです。

佐渡島　宇宙だって、実際はひとりでも、みんなに守られているって感じたときに安心できるような気がします。

川口　でしょう。ALSの患者さんって医療関係者、看護師さん、ヘルパーの方、家族、地域の人たち、みんな足したら最低でも100人くらいの人に支えられて生きているんです。

佐渡島　いまシャロンは入院していますが、もしかしたら家に帰って、昔のムッタみたいな大学生が集まる賑やかな中で暮らして行くかもしれませんね。

川口　そういう患者さんもいますよ。健康だった時は自宅で塾を開かれていた女性患者さんは、若い子の信頼を集めていたので学生がボランティアに入って介護していました。シャロンも家に帰って、地域の人に支えられて生き続けるのがいいんじゃないかな。



川口氏

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大人気漫画『宇宙兄弟』（現在22巻まで刊行。3月21日23巻発売予定）では、ふたりの登場人物がALS（筋萎縮性側索硬化症）として描かれている。筋肉が委縮し、呼吸器なしでは生きられなくなり、いずれは身体が動かなくなるALS。そんな難病をなぜ『宇宙兄弟』は取り上げたのか。『宇宙兄弟』（講談社）の編集・・・

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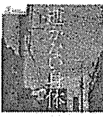
2012.08.09 Thu
いま、わたしたちに「死ぬ権利」は必要なのか？
川口有美子 / さくら会



2012年7月31日付けの「東京新聞」朝刊 http://www.tokyo-np.co.jp/article/politics/news/CK2012073102000091.html によると、超党派の国会議員がつくる「尊厳死法制化を考える議員連盟」（以下、議連）が、今国会への法案2案の上程・・・

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復興アリーナ　困ってるズ！　ABOUT　COMPANY　STAFF　CONTACT

2014.02.03 Mon

地球で生きる宇宙飛行士――『宇宙兄弟』はなぜALSを描いたのか？

川口有美子×佐渡島庸平

シェア ツイート 780 212

瞼の裏で幸せな夢を見る

佐渡島　ずっと気になっていたんですけど、病気が進行してどんどん身体が動かせなくなっていくと得られる情報も減ってきますよね。患者さんがみる夢って変わるんですか？

川口　患者さんに夢インタビューをしたことがあるんですけど、ある人は「SMAPと六本木で飲み歩いている夢を見た」って言っていました。あと釣り好きの患者さんは釣りの夢を見たって。

佐渡島　ぼくたちはよく不安な夢を見るじゃないですか。仕事に失敗する夢とか（笑）。患者さんも不安な夢を見るのか気になって。

川口　呼吸器が外れちゃう夢も見るみたいですね。でも話を聞くとね、だいたいがいい夢の話。まだ身体が動いていたときの。ALSの患者さんは、昔の思い出を宝物のように、何度も反芻しているみたい。私の母は、父と一緒に山登りにいって見慣れた小道を歩いて、一緒にお団子食べる夢をみたって言っていました。そういう話を聞いていると目が潤んじゃうんだけど。

『潜水服は蝶の夢を見る』って左目のまぶたしか動かせなくなった人の映画がありますよね。その映画でも蝶のように自由に羽ばたいている夢とか昔の彼女の夢を見ていましたね。

佐渡島　うまく眠れたら幸せなのかもしれませんね。

川口　そうですね。いい夢みたあとに母が「ずっと寝ていたい」って言うので、「いい夢ばかりとは限らないよー？」って言っていたんですど（笑）

佐渡島　子どものときに発症する筋ジストロフィーの患者さんはどうなんでしょう？

川口　筋ジストロフィーは、学校に通えなかったりして、社会経験が少ないって聞くから、またちょっと違うのかも。

佐渡島　実はALSについて調べているときに、筋ジストロフィーにも出会っているんです。

宇宙兄弟で出しているムックに掲載するために、筋ジストロフィーの研究をされている磯出良博さんに取材に行ったところ、いま筋ジストロフィーの薬が宇宙で開発されて、犬に与えたところ、走れるようになるまで回復したって結果がでているんです。

川口　そういえばせりかも宇宙で新薬を開発しようとしていますね。

佐渡島　宇宙空間はたんぱく質の結晶が綺麗に見えるそうです。それでワクチンの研究が一気に進んで。あとは誰かがお金を出してくれれば、筋ジストロフィーの患者さんは助かるかもしれないところまで来ているらしいです。でも筋ジストロフィーの患者さんって20歳以下でなくなってしまう方が多いんですよね。人口の割合も少ないから製薬会社も儲からない。だからなかなか難しいらしくて。

川口　でも最近はケアがよくなってきて、20歳をこえても生きている子も大勢いるんですよね。未来ではちゃんとお薬が開発されていると思う。

力強く生きるALS患者たち

佐渡島　最近話題になっている徳田虎雄さんもALSですよね。ぼく、徳田虎雄が好きなんですよ。彼の生き方を尊敬しているんです。青木理さんが書いた『トラオ　徳田虎雄　不随の病院王』（小学館）もすごくいいノンフィクションですよね。

川口　あの本は表紙もいいですね、徳田さんの目が。

徳田先生とも交流があるんですけど、先生は自分の正義に素直な人なんですよね。私の周りにいるALSの人は、みんなそんな感じ（笑）。

佐渡島　徳田さんはALSになってから仕事量が減ってない気がして。

川口　前に「先生、ALSは不便でしょ？」って聞いたら、「飲み会とかゴルフとか付き合いしないでずっと仕事していられるから効率が良くなった」って（笑）。

佐渡島　それはすごいなあ（笑）。徳田さんがもっと元気だったらいまの騒動もちょっと違ったものになっていたかもしれませんね。

川口　重度・重症の人って、弱々しくて、なんだかいい人に見えるイメージがあると思うんですけど、徳田先生は見事にそれを打ち壊してくれましたよね。もうほとんど動かないのに、あんな風に生きられるんだって（笑）。

佐渡島　『こんな夜更けにバナナかよ』（北海道新聞社）の鹿野靖明さんも力強い印象を受けますよね。

川口　そうそう。それにさくら会の理事長である橋本操さんも力強い方ですよ。発症したのが30歳くらい、昨年還暦を迎えたので、30年以上ALS患者として生きています。

物語を書き変えて現実を越えていく

川口　橋本さんは特殊な喋り方をするんです。唇はほとんど動かないんだけど、本当に微妙な唇の形をヘルパーさんが読み取って会話するの。

佐渡島　それはヘルパーもすごいですね。

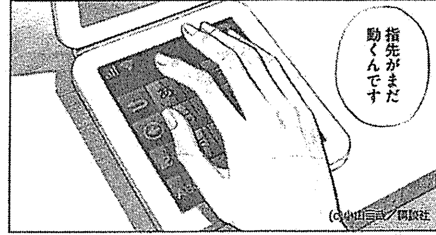
川口　大学生がバイトでやってます。

佐渡島　口の読み取りができるようになるんですか？

川口　私はできませんよ。でも学生は半年くらい読み取る訓練をしてできるようになる。

佐渡島　すごいなあ。『宇宙兄弟』ではALSで身体がほとんど動かせなくなってしまったシャロンが、スマートフォンのようなデバイスを使ってムッタと会話をする描写があるんです

けど、いまは皆さんどんな方法を使っているんですか？



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川口　意思伝達の方法っていろいろあるんですよ。いまは世界的にiPadを使っていますね。力入れなくてもシュシュッて指で軽く画面を動かせるでしょ。

もっと進行すると橋本さんみたいに口の筋肉のかすかな動きを読み取っていったり、透明な文字盤を使ったり。意思伝達装置などのハイテクもすごいけど、ローテクもすごい。人間同士の生のコミュニケーションそのものも本当にすごいって思います。

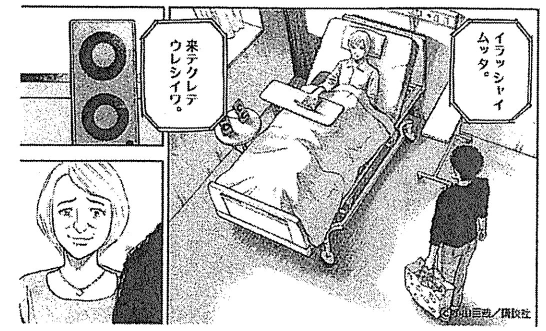
佐渡島　六太がそんなシャロンをみたら、テンション高くなるかもしれないなあ。

川口　宇宙兄弟って、「ナラティブの書き変え」をしてくれる漫画だと思うんですね。ムッタはその天才。どんな窮地に陥っても発想を変えて、前向きに進んでいくでしょ。

ALSの患者さんは、最悪を生きていくためのヒントをいっぱい持っているんです。呼吸器は眼鏡みたいなもので、生きていくために必要不可欠。だから呼吸器をつけている、とかね。「命の選択の問題」ではない、とかね。そうやって、世間で思われているような悲惨なイメージとは違う物語に書き変えていくんです。そうやってテンションを高めて生きている。

ちなみに『宇宙兄弟』ってモデルはいるんですか？

佐渡島　いや、いませんよ。実は、小山さんは一人っ子なんです。僕には、兄弟がいるんですけど、小山さんは、僕や兄弟のいる友人から、兄弟のエピソードをたくさん聞いて、発想を膨らませていったのです。



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2012.08.09 Thu

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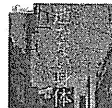

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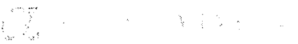

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Review

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Spinal Muscular Atrophy: From Gene Discovery to Clinical Trials

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Summary

Spinal muscular atrophy (SMA) is a common neuromuscular disorder with autosomal recessive inheritance, resulting in the degeneration of motor neurons. The incidence of the disease has been estimated at 1 in 6000–10,000 newborns with a carrier frequency of 1 in 40–60. SMA is caused by mutations of the *SMN1* gene, located on chromosome 5q13. The gene product, survival motor neuron (SMN) plays critical roles in a variety of cellular activities. *SMN2*, a homologue of *SMN1*, is retained in all SMA patients and generates low levels of SMN, but does not compensate for the mutated *SMN1*. Genetic analysis demonstrates the presence of homozygous deletion of *SMN1* in most patients, and allows screening of heterozygous carriers in affected families. Considering high incidence of carrier frequency in SMA, population-wide newborn and carrier screening has been proposed. Although no effective treatment is currently available, some treatment strategies have already been developed based on the molecular pathophysiology of this disease. Current treatment strategies can be classified into three major groups: *SMN2*-targeting, *SMN1*-introduction, and non-*SMN* targeting. Here, we provide a comprehensive and up-to-date review integrating advances in molecular pathophysiology and diagnostic testing with therapeutic developments for this disease including promising candidates from recent clinical trials.

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Keywords: Spinal muscular atrophy (SMA), survival motor neuron (SMN), diagnosis, clinical trials

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Introduction

Spinal muscular atrophy (SMA; OMIM 253300) is an autosomal recessive neuromuscular disorder characterized by the degeneration of motor neurons in the spinal cord. The incidence of the disease has been estimated at 1 in 6000–10,000 newborns, with an expected carrier frequency of 1 in 40–60 (Prior et al., 2010). SMA is clinically heterogeneous and can be classified into three subtypes depending on the age of onset and achievement of motor milestones: SMA type 1 (severe type with the onset before the age of 6 months, unable to sit without support), SMA type 2 (intermediate type with the

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onset before the age of 18 months, unable to stand or walk without support), and SMA type 3 (mild type with the onset after the age of 18 months, able to stand and walk independently until the disease progresses) (Zerres & Davies, 1999). Additionally, two other forms of the disease with the most severe phenotype with prenatal onset and the mildest phenotype manifesting after 20 years of age, have been reported as SMA type 0 and SMA type 4, respectively (Kolb & Kissel, 2011).

Genetic linkage studies have mapped all disease subtypes to chromosome 5q13 and the survival motor neuron genes (*SMN*) were identified as the disease-causing genes in SMA (Brzustowicz et al., 1990; Gilliam et al., 1990; Melki et al., 1990a, b; Lefebvre et al., 1995). The cloning and characterization of *SMN1* (OMIM 600354) and its homologue *SMN2* (OMIM 6001627) have led to an improved understanding of the molecular basis of SMA and have facilitated the development of techniques for molecular diagnosis of this disease.

Although the pathogenesis of SMA remains to be fully understood, there have been active investigations into pharmacological agents and other novel therapeutic strategies for the treatment of SMA. An in-depth understanding of disease pathophysiology is necessary to direct design of therapeutic strategies. Elucidation of mechanisms and efficacies of the therapeutic approaches is also essential to guide clinical application. Here, we discuss advances in diagnostic procedures, molecular pathophysiology, and therapeutic strategies in SMA. In this review, information representing significant findings in SMA was collected from scientific articles published between 1990 and 2013 retrieved from PubMed and MEDLINE databases.

The *SMN* Genes

Discovery of the SMA Causative Gene

The SMA locus contains multiple repetitive and inverted sequences resulting in two highly homologous copies of *SMN*, namely *SMN1* (telomeric *SMN*) and *SMN2* (centromeric *SMN*) (Lefebvre et al., 1995). Both genes differ by only five nucleotides. *SMN 1* is an SMA-causing gene, due to its homozygous deletion in ~95% of SMA patients (Hahnen et al., 1995). Among the remaining patients, some may retain both *SMN1* alleles carrying intragenic mutations or they may be compound heterozygotes for a deletion and an intragenic mutation in one allele of *SMN1* (Rochette et al., 1997). On the contrary, *SMN2* is a modifier for SMA phenotype with an inverse relationship between *SMN2* copy number and disease severity. High copy number of *SMN2* ameliorates the clinical severity in some patients (McAndrew et al., 1997). However, complete loss of *SMN2* has not been observed in any SMA patients with homozygous *SMN1* deletion (Lefebvre et al., 1995), suggesting that its complete loss may show embryonic lethality (Schrank et al., 1997; Hsieh-Li et al., 2000).

Splicing Regulation of the SMN Genes

Of the five nucleotide differences between the two *SMN* genes (Lefebvre et al., 1995), only one is present in the coding region at position +6 of exon 7 in *SMN1* (c.840C) and *SMN2* (c.840T). Although this mutation is translationally silent, the C-to-T transition alters the splicing pattern in *SMN2* exon 7 (Lorson et al., 1999). *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% of exon 7-lacking (Δ7) *SMN2* transcripts and ~10% of FL-*SMN2* transcripts (Jodelka et al., 2010). SMN protein produced by *SMN1* transcript including exon 7 (FL-SMN) oligomerizes by means of self-association via a domain encoded by exon 7 (Lorson et al., 1998) and interacts with other proteins to form a multimeric complex (Burnett et al., 2009). However, SMN protein produced by *SMN2* transcript lacking exon 7 (Δ7-SMN) is unable to oligomerize because of the absence of the domain encoded by exon 7. The instability of Δ7-SMN may be explained by protein conformation and/or incompetency of oligomerization and complex formation (Burnett et al., 2009). Cho and Dreyfuss also showed that the splicing defect of exon 7 creates a potent degradation signal (degron) at Δ7-SMN's C-terminal 15 amino acids which target Δ7-SMN to the proteasomal degradation pathway, making it unstable and vulnerable (Cho & Dreyfuss, 2010).

SMN exon 7 has weak 3′- and 5′-splice sites (Lim & Hertel, 2001; Singh et al., 2004b). Thus, to be correctly spliced, additional splicing elements are required: *cis*-elements and *trans*-acting splicing proteins. The *cis*-elements include exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs). These *cis*-elements are recognized by trans-acting splicing proteins. In the central region of *SMN* exon 7, there is an ESE which binds a positive splicing protein, Htra2-β1 (Hofmann et al., 2000). Together with other proteins such as SRp30c, hnRNP-G, and RBM, Htra2-β1 facilitates the inclusion of exon 7 (Hofmann et al., 2000; Hofmann & Wirth, 2002). However, these *cis*-elements are not sufficient to explain the differential splicing of exon 7 in *SMN1* and *SMN2*.

Cytosine at position +6 of exon 7 may be essential for inclusion of the exon into mRNA, while thymine (or uracil in the pre-mRNA) at this position may cause exclusion of the exon (exon skipping). Cartegni and Krainer (2002) presented an enhancer model in which the C-to-T transition abrogates an essential ESE associated with positive splicing protein SF2/ASF. On the other hand, Kashima and Manley (2003) proposed a silencer model whereby the C-to-T transition creates a new ESS associated with a negative splicing protein, hnRNPA1. According to the extended inhibitory context (Exinct) model by Singh's group, the C-to-T transition strengthens an inhibitory context that covers a larger sequence than SF2/ASF and hnRNP-A1 binding sites

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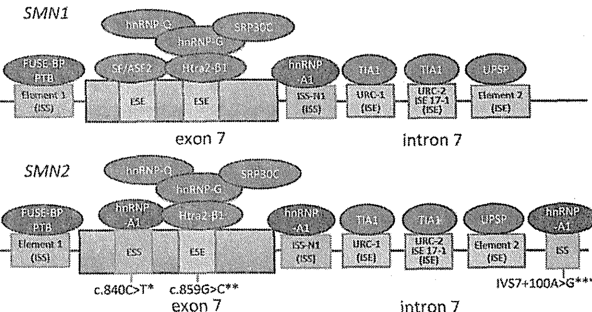

Figure 1 Splicing regulation of the *SMN* genes. Red boxes represent splicing enhancer motif sequences, and blue boxes represent splicing silencer motif sequences. UPSP denotes an unidentified positive splicing protein. *: The c.840C>T mutation (an *SMN2*-specific nucleotide) creates an hnRNP-A1 binding site (Kashima & Manley, 2003). **: The c.859G>C mutation disrupts an unforeseen hnRNP-A1 binding site, resulting in creation of a strong ESE (Vezain et al., 2010). ***: The IVS7+100A>G mutation (an *SMN2*-specific nucleotide) creates an hnRNP-A1 binding site (Kashima et al., 2007).

(Singh et al., 2004a). More recently, another new splicing regulator has been reported by Pedrotti et al. (2010): one of the multifactorial RNA-binding proteins, Sam68, binds to the C-to-T transition site in *SMN2* pre-mRNA exon 7 and triggers exclusion or skipping of the index exon. Collectively, the C-to-T transition at position +6 of exon 7 could create one or a combination of several situations including disruption of an enhancer, creation of a silencer, weakening of a stimulatory RNA structure, and strengthening of an inhibitory RNA structure (Singh et al., 2007).

However, a nucleotide change other than C-to-T transition at position +6 can also alter the splicing pattern of exon 7. It has been recently reported that a variant c.859G>C (at position +25 of exon 7), located in a composite splicing regulatory element in the center of *SMN2* exon 7, induces inclusion of exon 7 into *SMN2* transcript (Prior et al., 2009; Vezain et al., 2010). Besides exonic splicing motif sequences, intronic splicing motif sequences are involved in the regulation of alternative splicing in the *SMN* genes: one ISS has been found in intron 6 of *SMN*, three ISEs and two ISSs in intron 7 of *SMN* (Fig. 1). The ISS in intron 6 is known as element 1 (Miyajima et al., 2002). The ISEs identified in intron 7 are URC-1, URC-2 (or ISE I7–1), and element 2 (Miyajima et al., 2002; Miyaso et al., 2003). The ISSs in intron 7 are ISS-N1 (Singh et al., 2006) and *SMN2*-specific A-to-G transition at position +100 (Kashima et al., 2007). Thus, intron 7, especially the region in the vicinity of exon 7, may play a critical role in regulating *SMN* exon 7 splicing. Splicing proteins bound to the splicing motif sequences are shown in Figure 1. Splicing of *SMN* exon 7 with weak 3′- and 5′-splice sites is regulated in delicate balance among ESEs, ESSs, ISEs, ISSs, and their positive and negative splicing proteins.

Here, we mainly describe the alternative splicing behavior of *SMN2* exon 7. However, other alternative splicing patterns of *SMN1* and *SMN2* pre-mRNAs have been reported. Early studies showed that there are several isoforms generated by the *SMN* genes in muscle cells, indicating that exon 5 can be excluded in *SMN1* and *SMN2* pre-mRNAs (Gennarelli et al., 1995). Most recently, Singh et al. reported that the *SMN1* gene also generates surprising diversity of splice isoforms in some cell types, and that oxidative-stress can induce alternative splicing (Singh et al., 2012). An understanding of these alternative splicing mechanisms is important as strategies based on splicing correction of *SMN2* exon 7 may lead to novel treatment strategies for patients.

Molecular Diagnostics

Methods for Mutation Screening and Gene Dosages Analysis

To confirm the diagnosis of SMA, molecular genetic analysis to detect *SMN1* mutation is essential. Current methods for mutation screening in SMA are summarized in Table 1.

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Table 1 Molecular diagnostic methods for SMA.

Methods	Applications	References
Single strand conformation polymorphism (SSCP)	1	(Lefebvre et al., 1995)
Restriction fragment length polymorphism (RFLP)	1	(van der Steege et al., 1995)
Competitive PCR		
Radioisotope method	2	(McAndrew et al., 1997)
Nonradioisotope method	2, 3	(Chen et al., 1999, Wirth et al., 1999, Scheffer et al., 2000)
Real-time PCR		
Absolute quantifications		
Probe method	2, 2s	(Feldkötter et al., 2002)
Nonprobe method	2, 2s	(Feldkötter et al., 2002)
Relative quantifications		
Probe method	2, 2s	(Anhuf et al 2003, Gómez-Curet et al 2007)
Nonprobe method	2, 2s	(Cuscó et al., 2002, Tran et al., 2008, Abbaszadegan et al., 2011)
Denaturing high performance liquid chromatography (DHPLC)		
	1	(Sutomo et al., 2002)
	1s, 2, 2s	(Su et al., 2005)
	3	(Kotani et al., 2007)
High-resolution melting analysis (HRMA)		
Probe method	1, 1s	(Chen et al., 2009, Dobrowolski et al., 2012)
Nonprobe method	1, 1s, 2, 2s, 3	(Chen et al., 2009, Morikawa et al., 2011)
Multiplex ligation probe amplification (MLPA)	1, 1s, 2, 2s	(Arkblad et al., 2006, Scarciolla et al., 2006) (Passon et al., 2010, Su et al., 2011)
Liquid microbead array		
Tag-IT protocol	1, 1s	(Pyatt et al., 2007)
Multicode-PLx protocol	1, 1s	(Pyatt et al., 2007)

1: *SMN1* deletion screening, 1s: *SMN1* deletion test for newborn screening, 2: Quantification of *SMN* gene dosage, 2s: Quantification of *SMN* gene dosage for carrier screening, and 3: Detection of intragenic mutation in *SMN1* gene. It should be noted that to identify the intragenic mutation, nucleotide sequencing and assignment of the mutation to *SMN1* or *SMN2* are essential.

In order to detect *SMN1* deletion, single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) were initially used (Lefebvre et al., 1995; Van der Steege et al., 1995).

To detect *SMN* gene dosage or copy number analysis, a competitive PCR method was first described by McAndrew et al. (1997). This method used exogenous in vitro synthesized DNA as internal standards and radioisotope-labeled primers for autoradiograph analysis of the amplified products. This was later replaced with fluorescence-labeled primers and the amplified labeled products were analyzed on the auto-sequencer (Chen et al., 1999; Wirth et al., 1999; Scheffer et al., 2000; Harada et al., 2002).

Several quantitative real-time PCR approaches have been adopted for *SMN* gene dosage since then. These include absolute quantitative real-time PCR methods using *SMN1* or *SMN2* gene-specific primers (Cuscó et al., 2002; Feldkötter et al., 2002). However, a more convenient approach based on relative-quantification methods was later introduced utilizing an intrinsic gene existing in two copies as a reference (Anhuf et al., 2003; Gómez-Curet et al., 2007; Tran et al., 2008; Abbaszadegan et al., 2011; Chen et al., 2011). To ensure *SMN1*-specific and *SMN2*-specific detection, mismatched designed primer and/or probes (hybridization probes like FRET probes or hydrolysis probes like TaqMan probes) were used in the experimental procedures.

Several other technologies have also been introduced for *SMN* analysis: denaturating high-performance liquid chromatography (DHPLC) (Sutomo et al., 2002; Su et al., 2005; Chen et al., 2007), multiplex ligation probe amplification (MLPA) (Arkblad et al., 2006; Scarciolla et al., 2006) and high resolution melting analysis (HRMA) (Chen et al., 2009; Morikawa et al., 2011), and liquid microbead array (Pyatt et al., 2007). DHPLC, HRMA, and liquid microbead array can be applied for high throughput *SMN1* exon 7 deletion screening (Chen et al., 2007; Pyatt et al., 2007; Su et al., 2011; Dobrowolski et al., 2012) while DHPLC, MLPA, and HRMA techniques allow dosage analysis of *SMN* genes (Su et al., 2005; Scarciolla et al., 2006; Passon et al., 2010; Morikawa et al., 2011). Among these methods, only DHPLC and HRMA can facilitate both dosage and intragenic point mutation analysis (Kotani et al., 2007; Morikawa et al., 2011).

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Homozygous deletion screening of *SMN1* represents the first-tier in diagnostic testing since around 95% of SMA patients carry homozygous gene deletion (Hahnen et al., 1995). The majority of the remaining 5% of SMA patients retaining *SMN1* are compound heterozygotes with one *SMN1* allele deletion and one intragenic mutation in the other *SMN1* allele. In addition, some patients may retain two *SMN1* alleles, each carrying a subtle sequence mutation (Bussaglia et al., 1995; Rochette et al., 1997). Thus, for the diagnosis of SMA patients retaining *SMN1*, it is necessary to determine *SMN1* copy number, screen for point mutations, and assign the mutation location to either *SMN1* or *SMN2*. For the latter, long-range PCR for genomic DNA (Clermont et al., 2004) or reverse-transcription PCR for mRNA is performed (Harada et al., 2002).

Based on our experience, the RFLP method (van der Steege et al., 1995) is well suited for those hospitals or laboratories that deal with a small population of subjects with SMA or with a high index of suspicion for SMA because this method does not require any specialized laboratory equipment. Based on this method, homozygous deletion of *SMN* exon 7 can be detected by a simple combination of a conventional PCR machine with a gel-electrophoresis detection apparatus.

Although the RFLP method for detecting homozygous deletion of *SMN1* is currently used in many laboratories, dosage analysis using MLPA is being increasingly adopted for first-tier diagnosis of SMA. The latter enables both *SMN1* deletion screening and *SMN2* copy number analysis to be carried out simultaneously. However, for population screening, the HRMA method may be better because of its low cost, rapid turn-around reporting time for results and ability to process high throughput samples.

SMN2 Gene Dosage and Disease Severity

Several studies have reported a phenotype–genotype relationship among the SMA patients suggesting that increased *SMN2* copy number is related to improved survival outcomes and maintenance of motor function (Velasco et al., 1996; Coovert et al., 1997; McAndrew et al., 1997; Taylor et al., 1998; Harada et al., 2002). Usually, type 1 SMA patients have one or two *SMN2* copies, type 2 patients have three *SMN2* copies, type 3 patients have three or four *SMN2* copies, and type 4 patients have four or more *SMN2* copies (Feldkötter et al., 2002; Wirth et al., 2006a). Individuals carrying 5 or more *SMN2* copies were observed to develop very mild SMA symptoms (Wirth et al., 2006b). Higher *SMN2* copy number in the patients with milder phenotype can be explained by the gene-conversion theory. Gene-conversion events in which *SMN1* is replaced by its centromeric counterpart, *SMN2*, results in higher *SMN2* copies in type 2 and type 3 patients as compared with type 1 patients (Bussaglia et al., 1995; Campbell et al., 1997).

Although the phenotype–genotype relationship may allow us to predict disease severity or prognosis by *SMN2* copy number to some extent in a research setting, specific correlation between disease severity and *SMN2* copy number on an individual level has not been proven. Our own experience also showed that a high *SMN2* copy number did not always guarantee complete protection against SMA (Harada et al., 2002). The correlation between SMA phenotype and *SMN2* copies is not absolute; other factors may also modify the SMA clinical phenotypes (Prior, 2007). Exceptional cases include SMA patients with zero copies of *SMN1* and two copies of *SMN2* who may show a milder phenotype than expected because of the presence of a single mutation in one of the *SMN2* alleles (Prior et al., 2009; Vezain et al., 2010). A single base substitution in *SMN2*, c.859G>C, was identified in exon 7 in these patients. This nucleotide change creates a new ESE element and increases the amount of full-length transcripts, thus resulting in less severe phenotypes. In addition, it may be impossible to predict clinical severity from gene dosage of *SMN2* alone in SMA patients retaining SMN1. Some SMA patients with one copy of mutated *SMN1* (with p.W92S mutation in the Tudor domain of SMN) and three copies of *SMN2* showed the severest phenotype (Kotani et al., 2007). The presence of a single mutation affecting the Tudor domain of SMN may hamper the formation of the SMN complex with other proteins. Recently, it has been reported that HuD binds to the Tudor domain of SMN (Fallini et al., 2012). HuD is a neuron-specific RNA-binding protein that interacts with mRNAs, which play a crucial role in axonal transport. Thus, Tudor domain mutations may deteriorate motor neuron growth and the residual functions of mutated *SMN1* may determine the prognosis of the patients.

Carrier Screening and Prenatal Diagnosis

Advances in methodologies for *SMN1* gene testing have allowed carrier testing and prenatal diagnosis to be offered to families with an affected child (Matthijs et al., 1998). However, prenatal diagnosis is more complicated in a family with an affected child heterozygous for a gene deletion and an intragenic subtle mutation, because it requires both the assessment of *SMN1* gene dosage and sequencing for subtle nucleotide mutations.

Prior to prenatal diagnosis, it is recommended that SMA carrier status be confirmed in both parents based on *SMN1* gene dosage (Wirth et al., 1999). Having one *SMN1* copy confirms carrier status (carrier with "1+0" genotype) (Ogino et al., 2002), whereas the presence of two *SMN1* copies generally excludes carrier status. However, false negative

Table 2 Population carrier frequencies of SMA.

Nation/Ethnic group	Carrier frequency	Subject number	Analytical method	Reference
Australia	1 in 49	146	Real-time PCR	(Smith et al., 2007)
China	1 in 63	569	Real-time PCR	(Chan et al., 2004)
China	1 in 42	1712	DHPLC	(Sheng-Yuan et al., 2010)
Germany	1 in 35	140	Real-time PCR	(Feldkotter et al., 2002)
Germany	1 in 25	100	Real-time PCR	(Anhuf et al., 2003)
Israel	1 in 62	9.037	MLPA	(Sukenik-Halevy et al., 2010)
Korea	1 in 47	326	Real-time PCR	(Lee et al., 2004)
Korea	1 in 50	100	MLPA	(Yoon et al., 2010)
Taiwan	1 in 48	107,611	DHPLC & MLPA	(Su et al., 2011)
USA				
Caucasian	1 in 35	1028	Real-time PCR	(Hendrickson et al., 2009)
Askhenawi Jewish	1 in 41	1002		
Asian	1 in 53	1027		
African America	1 in 66	1015		
Hispanic	1 in 117	1030		
USA				
Pan-ethnic	1 in 54	68,471	Real-time PCR	(Sugarman et al., 2012)
Caucasian	1 in 47	24,471		
Askhenawi Jewish	1 in 67	5806		
Asian	1 in 59	4647		
Hispanic	1 in 68	7655		
Asian Indian	1 in 52	4883		
African America	1 in 72	976		
Not provided	1 in 54	17,235		

exclusion can occur when a minority of carriers possess two *SMN1* copies on one chromosome and zero copies on the other chromosome (carrier with "2+0" genotype) (Ogino et al., 2002). Dosage analysis is also unreliable for carrier status prediction in germline mosaicism cases unless DNA samples from both gametes and peripheral blood are analyzed (Ogino & Wilson, 2002). Similarly, the rare occurrence of somatic mosaicism can also lead to ambiguous results in *SMN1* genotype analysis (Eggermann et al., 2005). In addition, individuals who carry an *SMN1* point mutation may be falsely identified as non-carriers based on deletion screening alone. Thus, genetic counseling for SMA families should always take these situations into consideration.

According to the Practice Guidelines of the American College of Medical Genetics (ACMG), routine SMA-carrier testing is recommended not only for SMA-affected families but also for population-based screening (Prior, 2008). This is due to the severity of the disease and the high carrier frequency in many countries (Table 2) (Feldkötter et al., 2002; Anhuf et al., 2003; Chan et al., 2004; Lee et al., 2004; Smith et al., 2007; Hendrickson et al., 2009; Sheng-Yuan et al., 2010; Sukenik-Halevy et al., 2010; Yoon et al., 2010; Su et al., 2011; Sugarman et al., 2012). However, the American College of Obstetricians and Gynecologist (ACOG) (ACOG, 2009) has expressed caution for preconception and prenatal screening of SMA for the general population due to logistics, education and counseling issues. Factors such as the wide phenotypic variation ranging from mild to severe disease forms in SMA, technical limitations of current routine screening methods which may not detect non-SMN deletion patients (Prior et al., 2010), limited cost-effectiveness of carrier screening (Little et al., 2010) and the absence of curative treatment for SMA (Gitlin et al., 2010), all contribute toward the lack of consensus in implementing a population screening program in many countries. Although such carrier testing would be voluntary and made available in conjunction with genetic counseling services, the implementation of such screening, whether offered only for couples-at-risk in affected families or for large-scale healthy populations, requires an understanding of the sensitivity and limitations of the tests so that individuals can make informed choices on the uptake of such screening. It should be noted that the purpose of carrier testing for couples is to identify risks for conceiving an affected child and that the carrier status, if undiagnosed, does not pose a threat to the health of the couples themselves or others in the community.