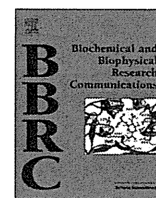
Biochemical and Biophysical Research Communications 453 (2014) 368–374

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



A novel evaluation method of survival motor neuron protein as a biomarker of spinal muscular atrophy by imaging flow cytometry

Masayuki Arakawa [a,*,1], Reiko Arakawa [b,1], Shinichi Tatsumi [c], Ryoko Aoki [b], Kayoko Saito [b], Akio Nomoto [a]

[a] Institute of Microbial Chemistry (BIKAKEN), Tokyo, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan
[b] Institute of Medical Genetics, Tokyo Women's Medical University, 10-22 Kawadacho, Shinjyuku-ku, Tokyo 162-0054, Japan
[c] Merck Millipore Merck Ltd., Arco Tower 5F, 1-8-1 Shimomeguro, Megro-ku, Tokyo 153-8927, Japan

ARTICLE INFO

Article history:
Received 6 September 2014
Available online 27 September 2014

Keywords:
Spinal muscular atrophy
Survival motor protein
Biomarker
Imaging flow cytometry
Valproic acid

ABSTRACT

Spinal muscular atrophy (SMA) is caused by mutations within the survival motor neuron 1 (*SMN1*) gene. These mutations result in the reduction of survival motor neuron (SMN) protein expression and SMN complex in spinal motor neurons and other tissues. SMN protein has been used as a therapeutic biomarker in recent SMA clinical studies using enzyme-linked immunosorbent assay (ELISA). Here, we investigated whether imaging flow cytometry can be a viable source of quantitative information on the SMN protein. Using a FlowSight imaging flow cytometer (Merck-Millipore, Germany), we demonstrated that imaging flow cytometry could successfully identify different expression patterns and subcellular localization of SMN protein in healthy human fibroblasts and SMA patient-derived fibroblasts. In addition, we could also evaluate the therapeutic effects of SMN protein expression by valproic acid treatment of SMA patient-derived cells *in vitro*. Therefore, we suggest that imaging flow cytometry technology has the potential for identifying SMN protein expression level and pattern as an evaluation tool of clinical studies.

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1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by mutations of the survival motor neuron 1(*SMN1*) gene, leading to progressive limb and trunk muscle weakness associated with muscle atrophy [1,2]. SMN protein is ubiquitously expressed in mammalian tissues; it plays a critical role in RNA metabolism, participating in small ribonucleoproteins (sRNPs) biogenesis and in pre-mRNA splicing [2]. Two *SMN* genes translate SMN proteins: a telomeric copy (*SMN1*) and an inverted centromeric copy (*SMN2*). The *SMN2* gene is present in all patients, but is not able to compensate for *SMN1* gene defects completely, resulting in low levels of the full-length SMN protein in order to have a single point mutation in exon7 of the *SMN2* gene [3,4]. In SMA clinical specimens, reduction of SMN has been assessed by several methods. Typically, immunocytochemistry and Western blotting have been used on primary dermal fibroblasts and leukocyte cell lines, leading to a correlation between healthy human controls and SMA patients in preclinical studies [5,6]. However, this method is not sufficiently reliable for clinical and diagnostic use. Recently, enzyme-linked immunosorbent assay (ELISA) has also been used in preclinical and clinical studies of SMA [7–10]; nevertheless, SMN protein levels in human peripheral-blood mononuclear cells (PBMC) were not correlated between healthy controls, carriers, and SMA phenotypic severity in clinical trials [9,10]. These methods should be optimized to allow detection of SMN protein in human cells for SMA clinical studies.

In this study, we focus on the imaging flow cytometry technique as a new assay method of SMN protein evaluation. Usually, standard flow cytometry cannot be used to assess the localization of molecules within specific cellular compartments. However, imaging flow cytometry can evaluate intact proteins, using a digital microscope system, and immunological technologies [11,12]. SMN proteins are localized intracellularly throughout the cytoplasm and nucleus, as a multi-protein complex. Specifically, SMN proteins form SMN complexes in the nucleus, where they accumulate in structures called Gemini of Cajal bodies (Gems) that play an essential role in the assembly of spliceosomal snRNPs and biogenesis during mRNA processing [13,14]. The predicted outcome of decreased snRNPs assembly is an alteration in gene splicing, containing minor introns due to reduced snRNPs levels [2,15]. In SMA-derived cells, gems formation is clearly decreased compared to that of healthy controls [16].

* Corresponding author at: Institute of Microbial Chemistry (BIKAKEN), Tokyo, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan. Fax: +81 3 3441 7589.
E-mail address: arakawam@bikaken.or.jp (M. Arakawa).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.09.087
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At present, there is no effective treatment for SMA. Some therapeutic approaches are recently under investigation; therapies aim at increasing the amount of full-length SMN protein levels produced by *SMN2* promoter activation, while reducing *SMN2* exon7 alternative splicing, using small molecules, and antisense oligonucleotides [6,17–20]. Therefore, a new method to accurately measure SMN protein levels is needed, to assess disease severity and response to treatment.

The aim of this study was to evaluate SMN protein expression and to qualitatively assess its cellular localization using imaging flow cytometry. We therefore explored the applicability of this new technology for evaluating SMN protein as a biomarker in SMA clinical trials.

2. Materials and methods

2.1. Materials

Human fetal dermal fibroblasts (from healthy controls) were obtained from Cell Applications, Inc., SMA patient-derived dermal fibroblasts were obtained from skin biopsies of SMA patients. The patient having SMA type I was a 7-month-old female who had not acquired head control with *SMN1* deletion and two copies of *SMN2*, as assessed by molecular diagnoses. Ethical approval for tissue collection was granted by the Institutional Review Board of Tokyo Women's Medical University, Japan. For immunocytochemical analyses, we used a mouse monoclonal FITC-conjugated anti-SMN, (clone 2B1, Merck Millipore, Germany), and a mouse monoclonal anti-SMN antibody (BD Transduction Laboratories, San Diego, USA).

2.2. Cell culture and valproic acid treatment

Human dermal fibroblasts (from healthy controls) and type I SMA patient-derived dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) including 1.0 g/L glucose and supplemented with 20% fetal bovine serum (FBS) without antibiotics. These fibroblasts were cultured in six-well plates for 24 h and then treated with the histone deacetylase inhibitor, valproic acid (VPA: 0, 0.1, 1, 10 mM) diluted in PBS for 24 h at 37 °C with 5% CO_2.

2.3. Immunocytochemical staining

After cells were cultured for 48 h, 1.5×10^6 cells were rinsed twice with cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and then rinsed three times for 5 min with PBS. The cells were then treated with 0.2% TritonX-100 in PBS for 10 min at room temperature. The cells were incubated in blocking buffer (10% normal goat serum in PBS) for 60 min at room temperature. Following blocking, the cells were incubated with a mouse monoclonal anti-SMN antibody (1:100, BD) at room temperature for 60 min and then cells were then visualized using an Alexa Fluor 488-conjugated goat anti-mouse (1:400, Molecular Probes) for 60 min at room temperature. The cells were treated with Hoechst 33342 (0.5 μg/mL) to stain the cell nuclei for 5 min at room temperature. Image photographs were taken using a Leica fluorescent microscope system.

2.4. Quantitative RT-PCR analysis

Cells were cultured for 24 h after VPA treatment, and total RNA was isolated using the RNeasy kit (QIAGEN Sciences, USA) according to the manufacturer's instructions. For reverse transcription reactions, 500 ng of total RNA was used with PrimeScripts RT Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Aliquots of cDNA were mixed with SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) each containing 400 nM primers. Quantitative PCR was performed on Thermal Cycler Dice Real Time Systems (Takara Bio Inc., Shiga, Japan). Primers used in this paper were as follows: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 5′-GCACCGTCAAGGCTGAGAAC-3′ for forward and 5′-TGGTGAAGACGCCAGTGGA-3′ for reverse; *SMN2*, 5′-AACCTG TGTTGTGGTTTACACTGGA-3′ for forward and 5′-CAGATTTGGGCTT GATGTTATCTGA-3′ for reverse. All samples were assayed in duplicate.

2.5. Western blotting

Cells were cultured for 24 h VPA untreatment or treatment and then washed twice with PBS. Cells were homogenized on ice in protein lysis buffer (ER4, Enzo Life Sciences, Farmingdale, NY). After incubation on ice for 15 min, the samples were centrifuged at 10,000 rpm for 10 min at 4 °C. Sample protein concentrations were determined by the BCA method (Pierce, Rockford, IL). The amount of total protein was adjusted to equal levels between samples with SDS sample buffer, and the samples (10 μg of protein) were subjected to electrophoresis on 10% SDS polyacrylamide gels. Proteins were transferred to a PVDF membrane (Millipore Corp., Billerica, MA) and treated with blocking buffer (5% skim milk in 0.1% Tween20 in TBS) for 1 h at room temperature. After blocking, the membrane was treated with monoclonal anti-SMN antibody (1:2000, BD) diluted in blocking buffer for 1 h at room temperature and then incubated with an HRP-conjugated anti-mouse IgG antibody (1:2000, DAKO) for 1 h at room temperature. The membrane was treated with a substrate (ECL plus substrate kit). To test for equal amounts of loaded protein, membranes were stripped and incubated with monoclonal anti-α-tubulin antibody (1:5000, Sigma) as described above. Proteins were visualized using a luminescent image analyzer ImageQuant LAS-1000 (Fuji Photo Film, Tokyo, Japan). All samples were assayed in duplicate.

2.6. ELISA

Cells were cultured for 24 h after VPA treatment, and then washed twice with PBS. Cells were homogenized in protein lysis buffer on ice as described above. Aliquots of protein extracts were diluted with lysis buffer. SMN ELISA kit (Enzo Life Sciences, Farmingdale, NY) was carried out according to the manufacturer's instructions. All samples were assayed in duplicate.

2.7. Immunostaining for the imaging flow cytometry

SMA patient-derived fibroblasts were rinsed twice with PBS and trypsinized. Cells were then washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min on ice. After they were washed three times with PBS, we permeabilized the cells using chilled BD Phosflow Parm buffer II for 30 min on ice. Cells were washed with Stain buffer (BD) and counted; 10 μl FITC-conjugated human SMN antibody (Millipore) or normal mouse Ig (Sigma) was added in 1×10^6 cells/90 μl and incubated at room temperature for 45–60 min. After incubation, and a single wash with PBS, the cells were treated with Hoechst 33342 (5 μg/mL) in PBS for 5 min at room temperature.

2.8. Imaging flow cytometry analysis

Samples were analyzed on a FlowSight imaging flow cytometer (Merck-Millipore, German). Data from a minimum of 10,000 cells (counts) were acquired, utilizing the 405, 488, and 785-nm lasers to calculate cell granularity, at a 20× magnification, using INSPIRE

software. Compensation was performed using single-color staining for all channels. Single cells were first identified based on a scatter plot of bright field area versus the aspect ratio. A gate was drawn around the population containing putative single cells based on the criteria of the area being large enough to exclude debris, and the aspect ratio being greater than ~0.6, which eliminates debris and clusters. Focused-Single cells were plotted on the SMN-FITC and Hoechst dye intensity. Both double positive population was gated and confirmed by image gallery to determine correct gate. Cellular localization of SMN protein was analyzed using the Bright Detail Intensity (BDI) feature algorithm. Acquired data were analyzed using the IDEAS analysis software.

2.9. Statistical methods

Analysis of statistical significant between SMN levels between the VPA-treated groups was done by Student's *t*-test. Values are presented as mean ± standard deviation value (SD). Statistically significant differences were defined as $p < 0.05$.

3. Results

3.1. Detection of SMN protein levels in healthy human fibroblasts, and type I SMA patient-derived fibroblasts by immunocytochemistry and Western blotting

In control fibroblasts from healthy individuals, endogenous SMN protein is expressed in the cytosol and nucleus, and accumulates in discrete nuclear foci known as gems. In this study, we checked SMN protein expression in human controls by immunocytochemistry and Western blotting using a specific antibody (Fig. 1A and B). On the other hand, type I SMA patient-derived fibroblasts showed decreased SMN protein levels resulting from *SMN1* gene loss, compared to healthy controls (Fig. 1A and B).

3.2. Effects of valproic acid treatment on type I SMA patient-derived fibroblasts

In our preclinical studies, we investigated the effects of VPA (concentrations used were 0, 1, and 10 mM) on cell morphology, full-length *SMN2* mRNA transcription levels and changes in SMN protein levels in SMA patient-derived fibroblasts (VPA; 0, 0.1, 1, 10 mM) at 24 h after treatment.

Our results showed that 24 h VPA treatment did not cause any changes in cell morphology or any toxicity (Fig. 2A). Moreover, these treatments were dose-dependent. Thus, a 10 mM dosage of VPA significantly increased full-length *SMN2* mRNA transcription levels, detected by quantitative RT-PCR method, compared to non-treated SMA cells ($p < 0.05$, Fig. 2B). Quantification of full-length *SMN2* mRNA transcription levels was determined with respect to a standard curve constructed using serial dilutions of cDNA. We used mRNA transcription levels of a housekeeping gene, *GAPDH* as internal control. On the other hand, SMN protein levels were also increased when examined by two independent methods, Western blotting (Fig. 2C), and ELISA ($p < 0.05$, Fig. 2D), after VPA treatments.

3.3. Detection of SMN protein expression and cellular localization by imaging flow cytometry

In this study, we report for the first time the use of imaging flow cytometry to assess the intracellular expression and localization of SMN protein in fibroblasts from healthy controls and SMA patients. Cells were labeled with the same mouse monoclonal FITC-conjugated anti-SMN antibody (2B1), and Hoechst 33342 nucleic staining, as described in materials and methods. There were significant differences in fluorescence intensity in the number of FITC-labeled SMN-positive cells between SMA cells and healthy controls (Fig. 3A). Moreover, the expression of SMN protein in SMA cells was clearly lower, compared to healthy controls. Nonetheless, at least 20–30% of cells were strongly positive for SMN as identified by a plotted histogram of FITC-SMN-positive cells (Fig. 3A). Furthermore, in order to investigate the accumulation area of SMN protein in healthy control and SMA cells, we used the BDI modulation to measure the distribution of SMN protein. The BDI features compute the intensity of localized bright spots within a masked area in the image where the background has been removed around the spots. SMN proteins are generally known to localize both within the cytoplasm and within nucleus, especially in nuclei foci called gems, where the SMN complex is composed. Our results from immunocytochemical analysis showed that the SMN protein accumulated in the nucleus and cytoplasm, and appeared as cellular dots in healthy controls (Fig. 1A). However, we could not detect SMN complex like gems or accumulation in our SMA cells under the fluorescent microscopic observation (Fig. 1A). On the other hand, using imaging flow cytometry, we also

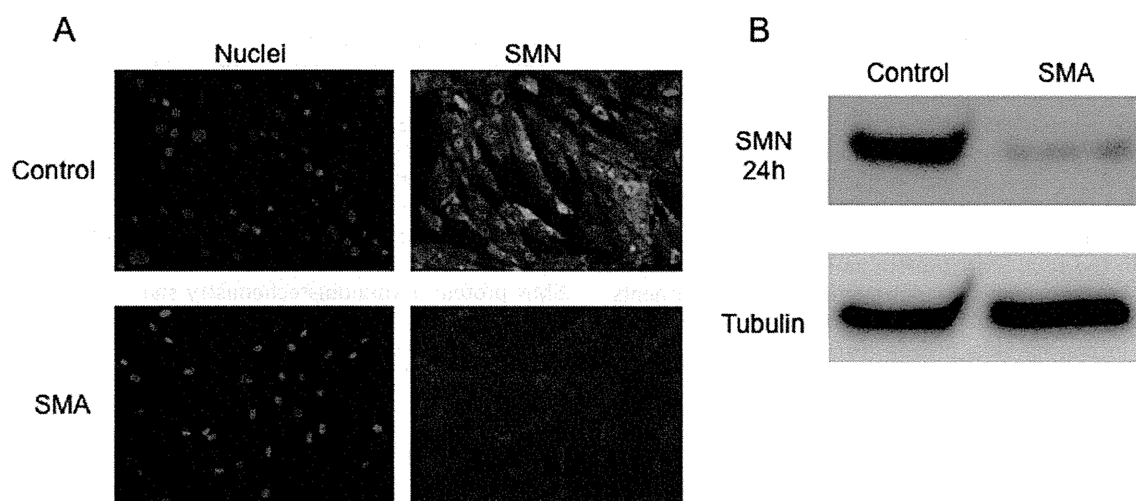

Fig. 1. Detection of SMN protein in human fibroblasts from healthy controls and type I SMA patient-derived fibroblasts. (A) Micrographs showing SMN protein immunocytochemistry. Cells were cultured for 24 h and then stained for SMN using a monoclonal anti-SMN antibody. SMN expression is reduced in SMA patient-derived fibroblasts when compared to healthy controls. (B) Detection of SMN protein by Western blotting analysis, using the same antibody as described above, shows reduced SMN expression in SMA patients.

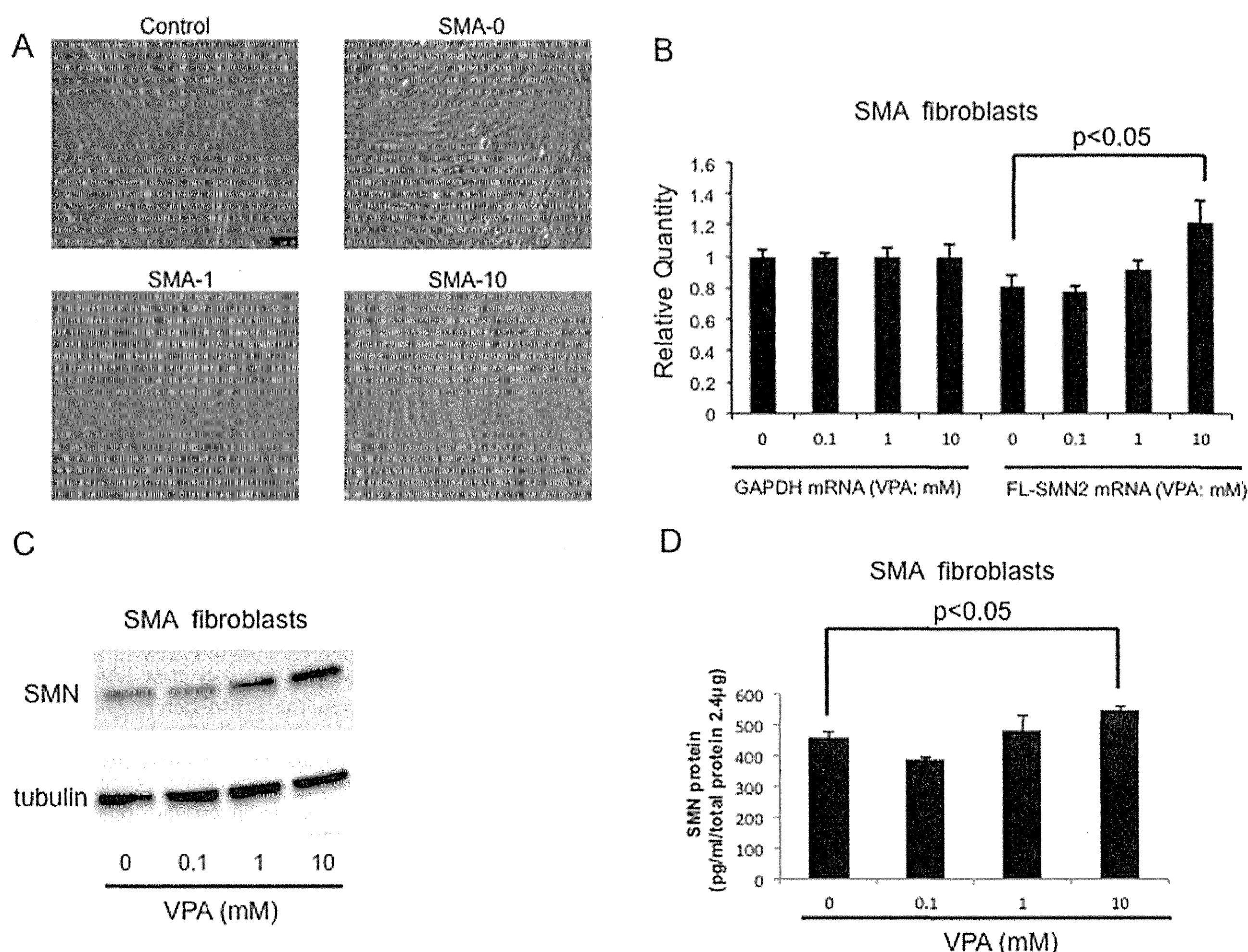

Fig. 2. Effects of VPA treatment on SMA patient-derived fibroblasts. (A) Effects on cellular morphology. Cells were treated with 0, 1, 10 mM VPA for 24 h. All treatments did not cause any morphological changes and any toxicity. (B) Real-time PCR analysis of *SMN2* mRNA expression after VPA treatment in SMA-derived fibroblasts. Cells were treated with 0, 0.1, 1, 10 mM VPA for 24 h, and then total RNA was extracted and subjected to real-time PCR. *GAPDH* was used as internal control gene. (C) Western blotting analysis of SMN protein expression after VPA treatment in SMA-derived fibroblasts. Cells were treated with 0, 0.1, 1, 10 mM VPA for 24 h and then total protein levels were analyzed by Western blotting. (D) ELISA data showing SMN protein expression in SMA-derived fibroblasts treated with VPA. Cells were treated with 0, 0.1, 1, 10 mM VPA for 24 h and then total protein was analyzed using an ELISA kit (Enzo Life Sciences). Error bars represent the mean ± S.D. obtained from three independent samples.

observed that the accumulation of SMN protein was significantly decreased in SMA cells (Fig. 4A and B).

3.4. Evaluation of SMN protein expression and cellular localization in VPA-treated SMA patient fibroblasts using imaging flow cytometry

To investigate the change of SMN protein expression and cellular localization in SMA patient-derived fibroblasts treated with VPA, we used imaging flow cytometry technology to detect SMN protein-positive cells by a specific anti-SMN antibody as described above. In this technology, data from SMA cells exhibited a significant increase in the total SMN protein amount, translated from each *SMN2* locus, under dose-dependent VPA-treatments (Fig. 3B–D). In addition, BDI analysis showed that VPA treated-cells were not only increasing SMN protein levels, but also SMN was accumulating in the nucleus and cytoplasm (Fig. 4C–E). Using imaging flow cytometry, we could first detect significant increases in SMN protein accumulation in discrete nuclear foci after VPA treatment (Fig. 4C–E).

4. Discussion

In this study, we developed a new method of SMN protein evaluation using imaging flow cytometry. This method can be easily and clearly detect SMN protein levels in healthy human fibroblasts and type I SMA patient-derived fibroblasts. In our preclinical studies 24 h after VPA treatment, the endogenous full-length *SMN2* mRNA and SMN proteins derived from full-length *SMN2* mRNA were significant increased in type I SMA patient-derived fibroblasts. VPA treatment may stimulate the transcriptional system of *SMN2*. The SMN protein expression, therefore, increased in VPA-treated SMA cells. These results indicate that VPA may serve as a promising therapeutic candidate for SMA.

Using imaging flow cytometry analysis, we found that SMA patient fibroblasts clearly expressed SMN protein at least at 20–30% of normal levels. In mammalian neural cells and tissues, SMN protein immunohistochemistry shows heterogeneous staining [21]. Therefore, the imaging flow cytometry analysis can be used to examine the population of SMN protein-positive cells acquired from SMA patients. Moreover, we first demonstrated that VPA-dependent SMN protein expression was also significantly increased, resulting in the accumulation of SMN to the cell nucleus as shown by BDI analysis. Our results suggest that the imaging flow cytometry system can play a role as a novel evaluation tool of SMN protein analysis for clinical studies in SMA.

The SMN protein is considered as the most suitable and sensitive molecular biomarker for SMA by many researchers. So far, several techniques have been used for SMN protein quantification.

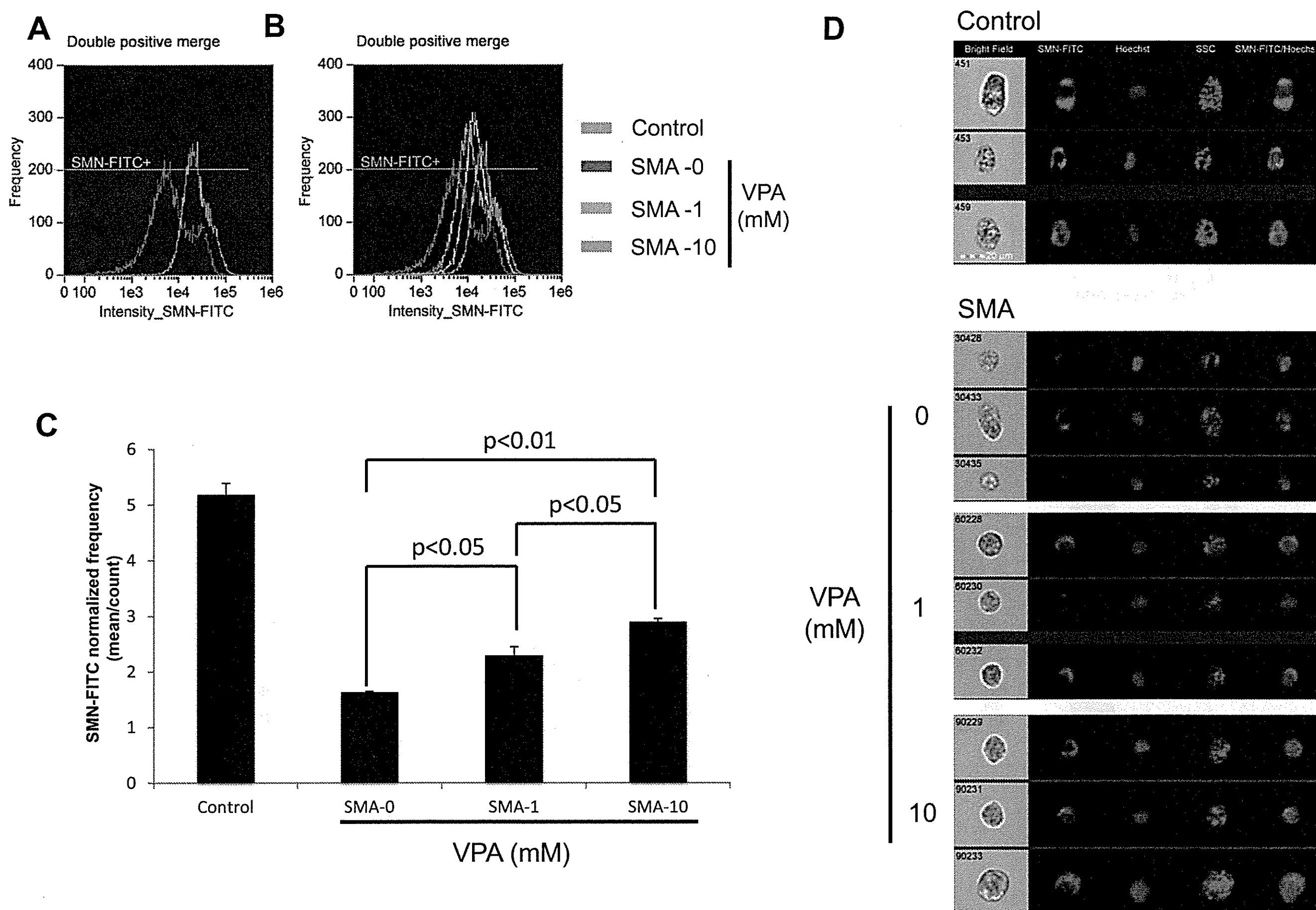

Fig. 3. SMN protein analysis by imaging flow cytometry. (A) Cells were cultured for 48 h, and then trypsinized and stained for SMN protein using a monoclonal FITC-conjugated anti-SMN antibody. Histogram represents the mean FITC intensity versus frequency (count). (B) Detection of SMN protein expression by imaging flow cytometry analysis after treatment with VPA (0, 1, 10 mM) for 24 h. The SMN protein was increasing in a dose-dependent manner. (C) Evaluation of SMN protein expression after VPA treatment. Values are represented as FITC-SMN normalized frequency (mean/count). (D) Cellular localization of SMN protein by imaging flow cytometry. FITC-SMN (green), Hoechst 33342 (blue), side scatter (red) and bright-field digital images are shown for human healthy controls and SMA-derived fibroblasts untreated or treated with VPA. SMN staining is clearly visible in the cytoplasm and nucleus. Error bars represent mean ± S.D. obtained from three independent samples.

Western blot analysis, which was used in *in vitro* and *in vivo* studies, mainly aimed at evaluating possible variations of SMN protein levels related to pharmacological treatments [6]. However, this assay has several limitations, related to its semi-quantitative nature, thus requiring normalization versus housekeeping proteins, whose levels are subject to wide variations.

Kolb et al. developed an immunoassay suitable for total SMN protein quantification in PBMC, through which they could demonstrate a correlation with the number of *SMN2* copies [22]. However, they found a reduction in SMN levels only in PBMC of type I SMA patients, and they could not find any correlation between protein levels and phenotypic severity [22]. These findings clearly question the meaning of quantifying SMN protein levels in clinical trials. Generally, ELISA is considered more sensitive and adequate for protein quantification since it does not require normalization to other proteins, given that SMN levels are quantified with respect to a standard curve constructed with serial dilutions of purified protein. To date, these assays have been developed and validated [7–10]. These authors showed that their assay is sufficiently sensitive to measure SMN variations, related to a candidate drug treatment, and found that SMN protein levels in PBMC of SMA patients show a tendency to be reduced, compared to healthy controls [7–10]. Although these results are promising, the small number of samples analyzed, the absence of age-matched controls, of a placebo arm, and of clinical-molecular correlations, do not allow firm conclusions to be drawn on the validity of SMN protein dosage in clinical trials. SMA is a phenotypically heterogeneous disorder with variable disease onset and severity, which creates a series of issues in the design of clinical trials. Sensitive and accurate biomarkers are, therefore, needed that can be used as predictive, prognostic, and surrogate endpoint measures.

SMN protein, as a biomarker or surrogate outcome measure, presents some technical issues that need to be taken under consideration in the context of clinical trials. For example, the acquisition of SMA patient fibroblasts is an invasive procedure. On the other hand, obtaining peripheral whole blood cells and PBMC is a less invasive process, and more suitable samples for imaging flow cytometry, although peripheral blood draws are often hard to obtain from very young patients.

Moreover, with the imaging flow cytometry analysis, quantification of SMN accumulation could be considerably evaluated in intact cells, by using an algorithm of bright detail intensity. Generally, increases in gem numbers related to SMN complexes, were counted as gems per 100 cell nuclei [16]. In fact, our analysis may not only be reliable and beneficial for the evaluation of SMN protein expression, but also for the quantification of gems without counting cell nuclei. Therefore, our results suggest that imaging flow cytometry analysis can play a role as a novel tool for the evaluation of intact protein expression and localization of biologically active molecules, like the SMN protein.

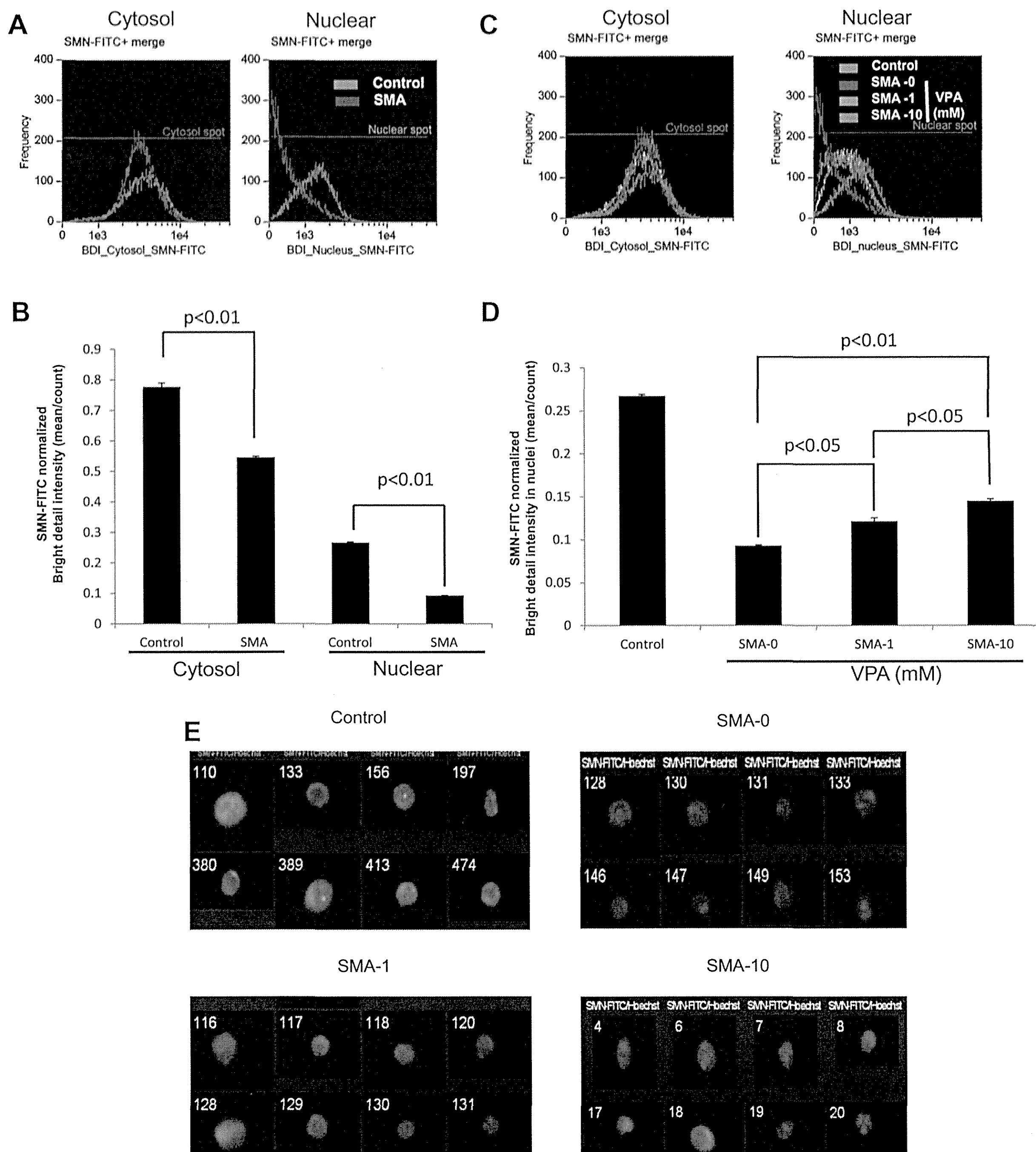

Fig. 4. Cytosolic and nuclear localization of SMN protein evaluated by imaging flow cytometry. (A) Comparison of bright detail intensity in the cytosol or nucleus of human healthy controls (green) and SMA patient-derived fibroblasts (red). Cells were cultured for 48 h and then subjected to imaging flow cytometry analysis. (B) Results show a significant decrease in SMN protein both in the cytosol and in nucleus in SMA patient-derived fibroblasts. (C) Effects of the SMN protein localization and accumulation on VPA-treated SMA patient-derived fibroblasts. Cells were treated with VPA (0, 1, 10 mM) for 24 h and then subjected to imaging flow cytometry analysis. Histogram represents cytosol spots or nuclear spots. (D) Accumulation of SMN protein in the nucleus of VPA-treated SMA patient-derived fibroblasts. Values are represented as FITC-SMN normalized bright detail intensity (mean/count). (E) Fluorescent micrographs showing localization of SMN protein by imaging flow cytometry. FITC-SMN (green) and Hoechst 33342 (blue) merged digital images are exhibited in human healthy control and SMA-derived fibroblasts treated with VPA (0, 1, 10 mM) respectively. Error bars represent mean ± S.D. obtained from three independent samples.

The imaging flow cytometry technique is a novel approach to qualitative and quantitative assessment of SMN protein expression in healthy human controls and SMA patient fibroblasts. The addition of digital images to standard quantitative and statistical measurements makes this the most sensitive flow cytometry method available for the assessment of cellular SMN accumulation and localization. We believe that imaging flow cytometry has a place as a first-line technique to assess the molecular genetic phenotype of cells acquired from SMA patients for clinical trials.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Nos. 23659530, 26461555. This work was also supported by Grants-

in-Aid from the Research Committee of spinal muscular atrophy (SMA), the Ministry of Health, Labor, and Welfare of Japan.

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Journal of Human Genetics (2015), 1–7
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ORIGINAL ARTICLE

A new method for *SMN1* and hybrid *SMN* gene analysis in spinal muscular atrophy using long-range PCR followed by sequencing

Yuji Kubo[1,2,3], Hisahide Nishio[4,5] and Kayoko Saito[1,2]

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by progressive loss of motor neurons in the spinal cord. Approximately 95% of SMA patients have a homozygous deletion of the survival motor neuron 1 (*SMN1*) gene, whereas 5% harbor compound heterozygous mutations such as an *SMN1* deletion allele and an intragenic mutation in the other *SMN1* allele. It is difficult to detect intragenic mutations in *SMN1* because of the high degree of homology shared between *SMN1* and *SMN2*. Current methods analyze a restricted region from exon 2a to exon 7 in *SMN1*. We propose a new, efficient long-range polymerase chain reaction (PCR) method for detecting intragenic mutations in *SMN1* (exon 1–8) and hybrid *SMN* genes. We analyzed 20 unrelated SMA patients using *SMN* copy number analysis, and the new long-range PCR method followed by sequencing. We thus confirmed a novel mutation in *SMN1* exon 1 (c.5C>T) in three patients with SMA type III who also had an *SMN1* deletion allele. Moreover, we confirmed three hybrid *SMN* gene types in eight patients. We report a novel *SMN1* mutation responsible for a relatively mild SMA phenotype and three hybrid *SMN* gene types in patients with SMA type III.

Journal of Human Genetics advance online publication, 26 February 2015; doi:10.1038/jhg.2015.16

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by degeneration of anterior horn cells in the spinal cord, leading to progressive proximal muscle weakness and atrophy.[1] Disease incidence has been estimated at 1 in 6000–10 000 live births, with a carrier frequency of 1 in 40–60.[2,3] SMA is a lower motor neuron disease and is clinically classified into four phenotypes: childhood-onset types I–III and adult-onset type IV.[4] SMA type I (also known as Werdnig–Hoffmann disease; OMIM 253300) is the most severe form, with onset before the age of 6 months. Unable to sit without support, patients must be ventilated to survive after the age of 2 years. SMA type II (OMIM 253550) is the intermediate form, with onset before the age of 18 months; patients with this form of SMA never gain the ability to stand and walk. SMA type III (also known as Kugelberg–Welander disease; OMIM 253400) is a mild form, with onset after the age of 18 months; patients are able to walk early in the disease course, but lose this ability as the disease progresses.[5] Adult-onset SMA is referred to as SMA type IV (OMIM 271150) and manifests after the age of 20.[4]

SMA is caused by deletion of the survival motor neuron (*SMN*) gene located on chromosome 5 (5q13). *SMN* is present in two homologous copies, a telomeric *SMN1* and a centromeric *SMN2*; the difference between these two genes is only five base pairs.[6] Both *SMN* genes encode the SMN protein, which has a role in pre-messenger RNA (mRNA) splicing in the anterior horn cells in the spinal cord.[7] Although transcription of *SMN1* produces full-length mRNA, transcription of *SMN2* yields only 15% full-length mRNA, whereas 85% of the mRNA is incomplete (lacking exon 7).[4]

SMN1 is the SMA-determining gene; ~95% of patients have homozygous disruptions of *SMN1* owing to deletion or conversion of *SMN1 to SMN2*.[8,9] Homozygous deletions of *SMN1* exon 7 are the result of a gene conversion of *SMN1 to SMN2*, yielding a hybrid *SMN* gene.[10,11] Approximately 5% of patients are compound heterozygotes with a deletion and an intragenic mutation in one *SMN1* allele.[12] *SMN2* copy numbers also vary among patients and are associated with disease severity.[13–15]

If no *SMN1* deletion is detected in a patient with suspected SMA, *SMN1* copy number analysis and intragenic mutation screening should be performed.[16] Real-time polymerase chain reaction (PCR) and multiplex ligation-dependent probe amplification are used to analyze *SMN1* copy number. Intragenic mutation screening of *SMN1* should be performed to determine whether *SMN1* or *SMN2* carries any intragenic mutations, because the sequences are largely homologous. Current methods include reverse-transcription PCR of mRNA

[1]Branch of Genetic Medicine, Advanced Biomedical Engineering and Science, Graduate School of Medicine and Global Center of Excellence (COE) program, Tokyo Women's Medical University, Tokyo, Japan; [2]Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan; [3]Technical Research Institute, Toppan Printing Co., Ltd, Saitama, Japan; [4]Department of Community Medicine and Social Health Care, Kobe University Graduate School of Medicine, Kobe, Japan and [5]Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan
Correspondence: Professor K Saito, Institute of Medical Genetics, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan.
E-mail: saito.kayoko@twmu.ac.jp
Received 2 December 2014; revised 15 January 2015; accepted 15 January 2015

or long-range PCR of genomic DNA, both of which have limitations.[6,14,17,18] It can be difficult to construct SMN1 complementary DNA because of the low expression level of SMN1 mRNA in peripheral blood leukocytes. Moreover, the current method does not detect intronic mutations. Although strategies have been developed to overcome some of the problems associated with this method, it remains limited to a restricted region (13.2 kb) from exon 2a to exon 7 in *SMN1* (20 kb). Therefore, the current method cannot be used to analyze upstream regions such as the 5′-untranslated region and exon 1 or regions associated with the hybrid *SMN* gene, such as exon 7, intron 7 and exon 8.

We have developed a more efficient and broadly applicable method using long-range PCR for specific amplification of *SMN1*. This new method was evaluated using controls and a sample from a previously reported patient with SMA type I, who is a confirmed compound heterozygote for *SMN1*, with one deleted *SMN1* allele and an intragenic mutation (c.275G>C, p.W92S) in the other allele.[19] We identified a novel missense mutation in *SMN1* exon 1 (c.5C>T), leading to an alanine-to-valine substitution at amino acid 2 (p.A2V) in three Japanese patients with SMA type III. We also identified three hybrid *SMN* gene types in eight Japanese patients with homozygous deletions of *SMN1* exon 7.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of Tokyo Women's Medical University and was performed with the written informed consent of all patients.

Patients

We analyzed 10 controls and 20 unrelated patients with SMA type I ($n=1$), type III ($n=18$) and type IV ($n=1$). All patients met the diagnostic criteria for proximal SMA established by the International Consortium for SMA.[5] Some patients did not clearly fit a single category; for those patients, we assigned SMA type by giving priority to each patient's highest function over age of onset. Our new method was evaluated in Patient 9 with SMA type I. Patient 9, as reported previously,[19] was known to be compound heterozygous for *SMN1*, with one deleted *SMN1* allele and the other allele containing an intragenic mutation (c.275G>C, p.W92S). The remaining 19 patients (patients 1–8 and 10–20) were analyzed to demonstrate and characterize the presence of homozygous or heterozygous deletions in *SMN1* exon 7, intragenic mutations and hybrid *SMN* genes. Family members 1–1 and 1–2 were analyzed as part of our evaluation of Patient 10.

DNA extraction and *SMN1* deletion test

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and adjusted to a final concentration of 100 ng μl^{-1}. The *SMN1* exon 7 deletion was detected by PCR-restriction fragment length polymorphism.[6,20]

SMN copy number analysis using the multiplex ligation-dependent probe amplification method

We used the SALSA multiplex ligation-dependent probe amplification KIT P021-A1 SMA (MRC-Holland, Amsterdam, Netherlands) to determine *SMN* copy numbers. This kit contains a mixture of probes specific to exon 7 of the *SMN1* (NM_000344) and *SMN2* genes (NM_017411); exon 8 of the *SMN1* and *SMN2* genes; exons 1, 4, 6 and 8 of the *SMN1* and *SMN2* genes; and probes for genes located near *SMN* (for example, the *NAIP* and *H4F5* (*SERF1*) genes); other chromosomes; and reference probes. After multiplex ligation-dependent probe amplification, DNA fragments were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with GeneMapper software v4.1 (Applied Biosystems).

Specific amplification of *SMN1* by long-range PCR

Conventional long-range PCR was performed using a specific *SMN1* exon 7 reverse primer to amplify a 13.2-kb region that includes exons 2a–7 of *SMN1*. Our new long-range PCR (nLR-PCR) method for specific amplification of *SMN1* was performed using forward primer hybridization—654 bp from the transcription initiation site and a specific *SMN1* exon 8 reverse primer to amplify a 28.2-kb region that includes exons 1–8 of *SMN1* (Figure 1). The reaction was performed with KOD FX Neo polymerase (TOYOBO, Osaka, Japan) by step-down cycle PCR in a 50 μl reaction volume, with 25 μl of 2× PCR Buffer, 0.4 mM of each dNTP, 0.15 μM of each primer (SMN_FL_(ex1-654)_F and SMN_FL_ex8_R), 1 U of polymerase and 100 ng of genomic DNA (Supplementary Table 1). nLR-PCR was performed as follows: initial denaturation at 94 °C for 2 min, followed by 5 cycles of denaturation at 98 °C for 10 s, annealing and extension at 71.2 °C for 15 min, followed by 5 cycles of denaturation at 98 °C for 10 s, annealing and extension at 69.2 °C for 15 min, followed by 5 cycles of denaturation at 98 °C for 10 s, annealing and extension at 67.2 °C for 15 min, and 20 cycles of denaturation at 98 °C for 10 s, annealing and extension at 65.2 °C for 15 min and a final extension at 65.2 °C for 7 min. Expected 28.2-kb products were confirmed by 0.7% agarose gel electrophoresis. Amplified nLR-PCR products were excised, extracted with the QIAEX II Gel

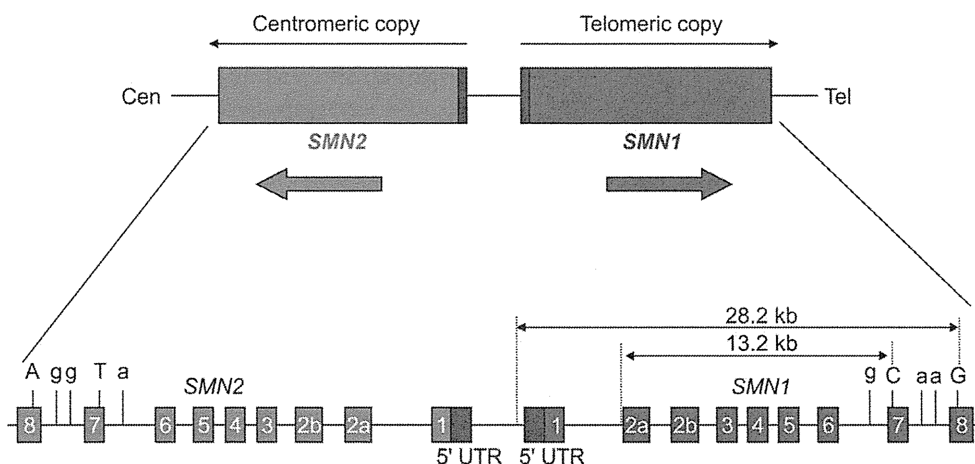

Figure 1 Strategy for specific amplification of *SMN1* by long-range PCR. *SMN1* and *SMN2* lie, respectively, on the telomeric and centromeric halves of an inverted duplication in chromosome region 5q13. Long-range PCR (13.2 kb) of the region including exons 2a–7 of *SMN1* was reported by Clermont *et al.*[17] The new long-range PCR (28.2 kb) encompasses the region including exons 1(-654)–8 of *SMN1*. We specifically amplified *SMN1* using the 1-base difference in exon 8. A full color version of this figure is available at the *Journal of Human Genetics* online.

Extraction Kit (Qiagen) and eluted in 20 μl of elution buffer. The nLR-PCR products were quantified using the ImageJ (NIH) software.

Intragenic mutations and hybrid *SMN* gene analysis by sequencing
We used 1 μl of the purified nLR-PCR product as a template to amplify each *SMN1* exon by nested PCR. Supplementary Table 1 lists the sequencing PCR primers and their annealing temperatures. Amplification of exon 1 was performed with KOD FX polymerase (TOYOBO) by two-step cycle PCR in a 25 μl reaction volume, with 12.5 μl of 2× PCR Buffer, 0.4 mM of each dNTP, 0.4 μM of each primer, 0.5 U of polymerase and 1 μl of template (Supplementary Table 1). PCR was performed under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s and annealing and extension at 68 °C for 45 s. Other targets were amplified using the Ex Taq polymerase (TAKARA) by three-step cycle PCR in a 25 μl reaction volume with 2.5 μl of 10× Ex Taq Buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 1.25 U of polymerase and 1 μl of template (Supplementary Table 1). PCR was performed under the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. Each *SMN1* exon product was purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit. Mutations reported here have been submitted to a Leiden Open Variation Database (http://www.LOVD.nl/SMN1).

Family analysis
Family members 1–1 and 1–2 were the mother and younger brother of Patient 10, respectively. Copy number and sequencing analyses were performed for all family members of Patient 10.

***In silico* analysis**
The Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and Align-GVGD (http://agvgd.iarc.fr/) classification tools were used to determine the amino-acid changes that were most likely to be responsible for the loss of protein function.[21,22] The dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genome Project databases (http://www.1000genomes.org) and Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html) were used to determine whether the identified variants are polymorphisms.

RESULTS

SMN1 deletion test and *SMN* copy number analysis
The *SMN1* deletion test and *SMN1* copy number analysis in 20 patients with SMA type I, SMA type III or SMA type IV revealed the absence of *SMN1* exon 7 in all of these patients (Supplementary Table 2). The overall distribution of deletion types was as follows: eight patients with homozygous deletion of *SMN1* exons 7 and 8 (patients 1–8); four patients with heterozygous deletion of *SMN1* exons 7 and 8 (patients 9–12); and eight patients with homozygous absence of *SMN1* exon 7 but not exon 8 (patients 13–20). Regarding the *NAIP* and *H4F5* (*SERF1*) genes located near *SMN*, the overall distribution of deletion types was as follows: one patient with homozygous deletion of *NAIP* exon 5 (patient 1); eight patients with heterozygous deletion of *NAIP* exon 5 (patients 2, 6, 8, 10–12, 15 and 16); three patients with heterozygous deletion of *H4F5* (*SERF1* exon 1) (patients 10–12).

Specific *SMN1* analysis by long-range PCR
Eight control subjects (controls 1–8) had two *SMN1* copies and eight patients (patients 1–8) had *SMN1* deletions. Products, 28.2-kb in size, were confirmed for all controls, whereas the bands were faint in the patients (Figure 2a). Band intensity for the controls was four times higher than that for the patients (patients 1 and 2 or patients 3–8 versus controls 6–8; $P<0.05$; Figure 2b). Controls 1 and 2 had the *SMN2* deletion and, therefore, their samples produced the highest-intensity bands (controls 1 and 2 versus controls 6–8; $P<0.05$). *SMN1* intron 6, exon 7 and intron 7 were amplified from the nLR-PCR products by nested PCR using SMN-ex7-F and R primers and sequenced to verify *SMN1* specificity (Figure 2c).

Direct sequencing for patient 9, who had a known intragenic mutation (c.275G>C), revealed an abnormal heteroduplex signal (blue: Cytosine, black: Guanine) in exon 3 of *SMN1* and *SMN2* (Figure 3). Only *SMN1* regions were isolated by nLR-PCR; *SMN1* exon 3 was amplified by nested PCR from nLR-PCR products; sequencing revealed increased cytosine and decreased guanine signal intensity (Figure 3). These findings suggested that the cytosine was derived from *SMN1* and that the mutation was present in *SMN1* exon 3. *SMN1* intron 6, exon 7 and intron 7 were also sequenced from nLR-PCR products to verify *SMN1* specificity (data not shown).

Novel intragenic mutations and family analysis
We screened all exons of *SMN* for novel intragenic mutations by direct sequencing of genomic DNA. Patient 10, with SMA type III, produced an abnormal heteroduplex signal (blue: Cytosine, red: Thymine) in exon 1 of *SMN1* and *SMN2* (Figure 4a), indicating an intragenic mutation in exon 1 of *SMN1* or *SMN2*. To determine which gene carried the mutation, *SMN1* nLR-PCR products were sequenced. A single signal (red: Thymine) was detected in *SMN1* exon 1, indicating that the mutation was present in *SMN1* exon 1 (Figure 4a). This C-to-T mutation at position 5 (c.5C>T) causes an alanine-to-valine substitution at amino acid 2 (p.A2V). This mutation was also identified in patients 11 and 12 (Table 1).

Copy number and sequencing analyses were performed for relatives (family members 1–1 and 1–2) of patient 10 (II–1; Figure 4b). The mother (family member 1–1; I–2) carried one *SMN1* copy and two *SMN2* copies; the brother (family member 1–2; II–2) carried two *SMN1* copies and two *SMN2* copies. The intragenic mutation in patient 10 (II–1) was absent in both of the family members tested (I–2 and II–2).

***In silico* analysis**
The c.5C>T mutation was not observed in 100 normal Japanese control samples. This mutation has not been documented in dbSNP, the 1000 Genome Project database or the Human Genetic Variation Database. Functional significance was evaluated by referring to Polyphen-2, SIFT and Align-GVGD. The mutation was assumed to lead to a hazardous change in protein function because all three programs returned evaluations of 'DAMAGING (PolyPhen-2 score: 0.939, SIFT score: 0.01)' and 'Class C65.' Thus, in SMA type III patients 10–12, the disease was attributed to a compound heterozygous mutation, including one *SMN1* allele deletion and a c.5C>T mutation in the other *SMN1* allele.

Hybrid *SMN* gene analysis by long-range PCR and sequencing
Patients 13–20, carrying a homozygous absence of *SMN1* exon 7 but not exon 8, were assessed for the presence of the hybrid *SMN* gene by nLR-PCR amplification of a region that includes exons 1–8 of *SMN1* and by sequencing of intron 6, exon 7 and intron 7 (Table 2). We identified three hybrid *SMN* gene types (Table 2 and Figure 5). The sequences of hybrid *SMN* intron 6, exon 7, intron 7 and exon 8 were as follows: patient 13, aTagG; patients 14 and 16–20, aTggG; and patient 15, gTaaG.

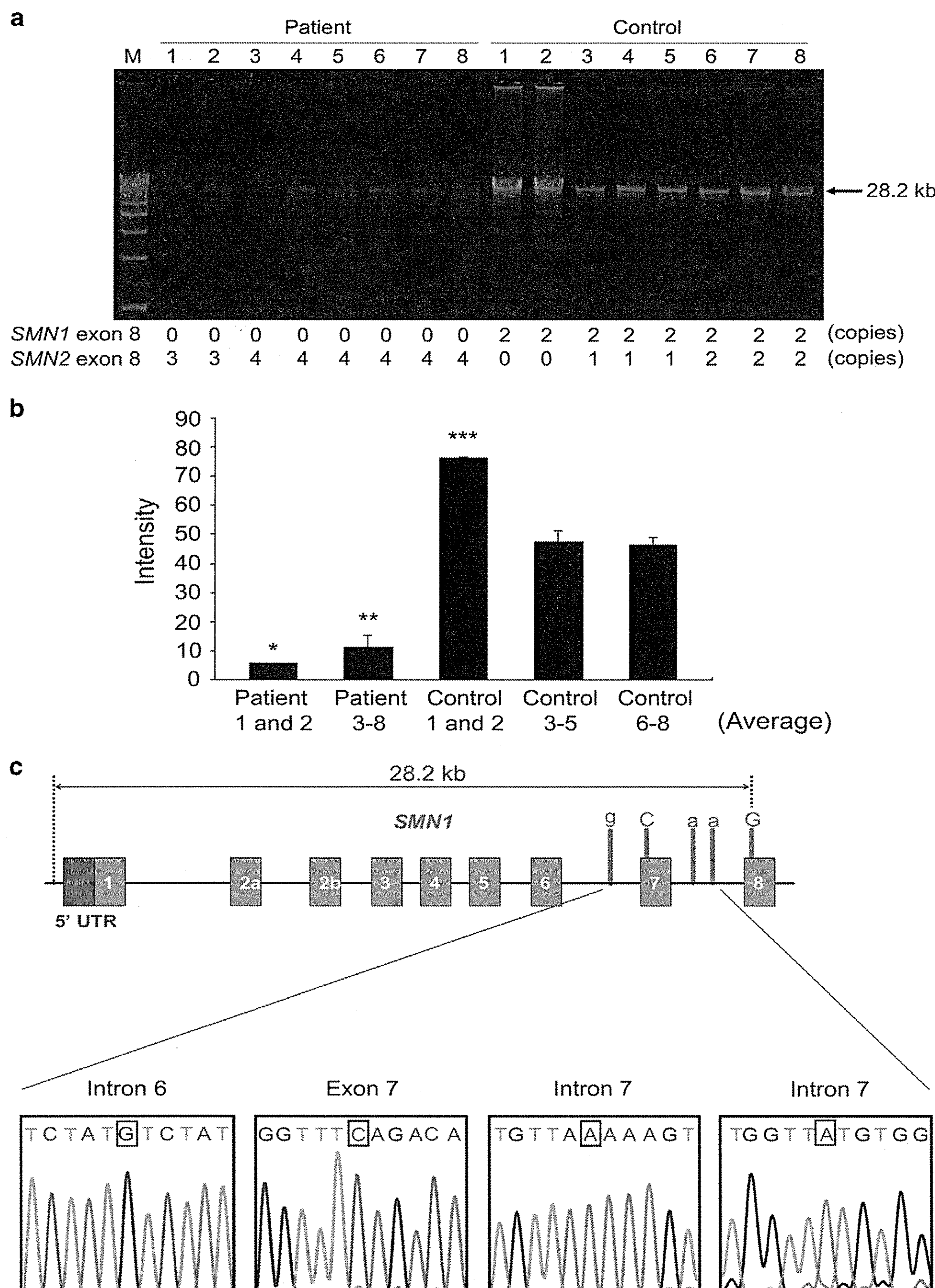

Figure 2 Evaluation of new method. *SMN1*-specific amplifications from exon 1(-654) to exon 8 (28.2 kb) are shown. (**a**) Controls 1–8 yielded 28.2-kb amplicons, whereas there were few signs of amplification in patients 1–8. Copy numbers of *SMN1* and *SMN2* exon 8 determined by MLPA are shown at the bottom of each line. M, molecular weight marker (TAKARA 2.5-kb DNA Ladder). (**b**) Quantification of nLR-PCR products. Average intensities of samples with the same *SMN2* exon 8 copy number are presented. *P*-value: Student's *t*-test. *Patients 1 and 2 versus controls 6–8; $P=0.001$, **Patients 3–8 versus controls 6–8; $P=0.000$, ***controls 1 and 2 versus controls 6–8; $P=0.002$. (**c**) *SMN1* specificity was confirmed by the presence of intron 6, exon 7 and intron 7 sequences. A full color version of this figure is available at the *Journal of Human Genetics* online.

DISCUSSION

We developed an efficient and broadly applicable LR-PCR method to detect intragenic mutations in *SMN1* (Figure 1). Without the need for complementary DNA cloning, this new method makes it possible to analyze all exons and introns of *SMN1*, the 5′- and 3′-untranslated regions, the promoter region, small or large insertions and deletions and hybrid *SMN* genes. Differences between controls and patients were clear ($P<0.05$), and the specificity was verified (Figure 2b). The absence of *SMN2*, which inhibits *SMN1*-specific PCR, yielded an increase in nLR-PCR products (controls 1 and 2). Even when there are more copies of *SMN2* than of *SMN1*, specific *SMN1* regions can be amplified using our nLR-PCR method (Figure 3).

We identified a novel mutation in exon 1 of *SMN1*, c.5C>T, in three unrelated patients (patients 10–12) with SMA type III (Table 1).

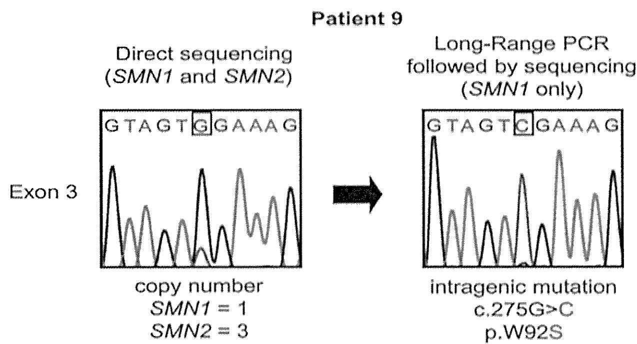

Figure 3 Detection of an intragenic mutation in a patient with type I SMA. Patient 9 was compound heterozygous for *SMN1*, with one deleted *SMN1* allele and an intragenic mutation (c.275G>C, p.W92S) in the other allele.[19] This patient had three *SMN2* copies. (Left) Direct sequencing of *SMN1* and *SMN2*; (Right) Sequencing of *SMN1* exon 3 isolated by the new long-range PCR technique is shown.

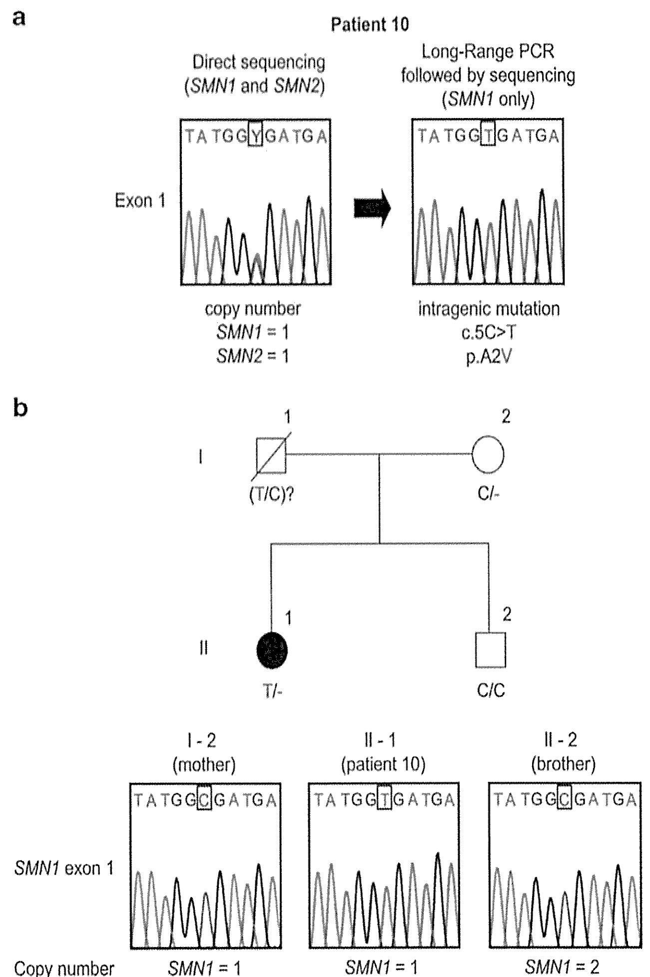

Figure 4 Identification of an intragenic mutation in *SMN1*. (**a**) Patient 10 had one copy each of *SMN1* and *SMN2*. (Left) Direct sequencing for *SMN1* and *SMN2* results are shown; (Right) direct sequencing of *SMN1* (right) exon 1 isolated by the new long-range PCR technique. The sequence revealed a c.5C>T mutation (red signal), leading to an alanine-to-valine substitution (p.A2V). (**b**) Patient 10 family analysis. The mutation in patient 10 (II–1) was absent from I–2 and II–2.

With the currently available methods, it was difficult to isolate only *SMN1* mRNA from the peripheral blood leukocytes of patient 11 (data not shown). We attribute this to low *SMN1* mRNA expression in these cells. Although family members of patient 10 (II–1) were shown by sequencing analysis to have the c.5C>T mutation, the intragenic mutation in patient 10 (II–1) was absent in both her mother (I–2) and her brother (II–2; Figure 4b). Patient 10 (II–1) had inherited the allele deletion from her mother, whereas the intragenic mutation had either been inherited from her father or occurred *de novo*.

The c.5C>T mutation was evaluated as a hazardous change based on *in silico* analysis results. The c.5C>T mutation was not registered in dbSNP, the 1000 Genome Project database or the Human Genetic Variation Database and might be a Japanese-specific variant. Consistent with these results, one patient with SMA type II and two with SMA type III with c.5C>G (p.A2G, dbSNP: rs75030631) mutations were reported previously.[23] These patients had only one *SMN2* copy and presented with similar mild symptoms. There are also reports of SMA associated with the c.5C>G mutation. Although *SMN* knockout mice with low *SMN2* copy numbers have severe SMA, phenotype rescue could be achieved in a transgene SMN A2G missense mutant.[24] Although SMN knockout is lethal in mouse embryos,[25] SMN(A2G) SMA mice exhibit the onset of motor neuron loss, resulting in mild SMA. The SMN A2G mutation inhibits self-association and affects SMN binding, probably by disrupting the formation of SMN oligomers. Because the effect of p.A2G is mild, it is associated with a later age of onset and relatively mild symptoms. The p.A2V variation is likely similar to p.A2G in its phenotypic effect.

Phenotypic effects might differ among intragenic mutation positions. For example, despite patient 9, with W92S(c.275G>C) and *SMN1* deletion, having three copies of *SMN2*, the relatively severe SMA type I phenotype was evident.[19] This mutation was located in exon 3, corresponding to the Tudor domain, an essential region for interaction of SMN with fundamental components of multiple nuclear RNA-protein complexes. This mutation impaired the interaction of SMN with various proteins. Therefore, mutations of this type may have a critical impact on SMN function.

Furthermore, the positions of intragenic mutations seemed to have more profound effects on phenotype than the size of the deletion in one allele. Although patients 10–12 had a large deletion including *NAIP* and *H4F5* in one allele (Supplementary Table 2), their phenotype was mild. On the other hand, although patient 9 had a small deletion including only *SMN1*, the SMA phenotype was severe.

We identified three hybrid *SMN* gene types in eight patients. Our method enables the direct isolation and sequencing of the entire hybrid *SMN* gene. We identified large (Type A), complex (Type B) and small conversions (Type C; Figure 5). SMA in patients 13–17 was associated with a deletion in *SMN1* exon 7 combined with an *SMN1-to-SMN2* conversion. SMA in patients 17–20 was associated with a homozygous *SMN1-to-SMN2* conversion. Cusco *et al.*[26] reported milder symptoms in patients with a homozygous conversion than in those with a combination of deletion and conversion. An association between disease severity and conversion has been described[27] but other reports suggest no such association.[28] Increased copy numbers of hybrid *SMN* genes and *SMN2* have also been reported to be associated with disease severity.[26] In this study, similar to a report by *Cusco et al.*,[26] symptoms were found to be milder in patients 18–20, who carry a homozygous conversion. Patient 15 had late onset of disease compared with patients 13, 14, 16 and 17, and could walk,

Table 1 Detected mutations, genotypes and phenotypes

Patient	SMA type	Onset (year)	Mutation	Site of mutation	*SMN2* copy number	Phenotype	Reference
9	I	<6 m	c.275G>C, p.W92S	Exon 3	3	Japanese male severely floppy infant, muscular hypotonia, depression of tendon reflexes. At 5 months, he exhibited poor sucking. At 8 months, ventilator support was required.	Kotani *et al.*[19]
10	III	12	c.5C>T, p. A2V	Exon 1	1	Japanese female showing motor function regression with symmetrical muscle weakness in the limbs. Walked until age 32; wheelchair-bound since age 32. Positive Gowers sign and waddling gait; muscle biopsy showed neurogenic changes.	—
11	III	11	c.5C>T, p. A2V	Exon 1	1	Japanese male with muscular atrophy and muscle weakness of the quadriceps. Walking at age 11; easily tired by non-strenuous exercise. Progressive muscle weakness of the limbs starting at age 13. Electromyography showed a neurogenic pattern. Muscle biopsy showed neurogenic changes.	Yamamoto *et al.*[30]
12	III	13	c.5C>T, p. A2V	Exon 1	1	Japanese female with mild proximal lower limb weakness and plantar muscular atrophy. Walking and swimming at age 13. Waddling gait; gradually lost ability to run. Electromyography showed a neurogenic pattern; muscle biopsy showed neurogenic changes.	Yamamoto *et al.*[30]

Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron.

Table 2 Hybrid *SMN* gene analysis in eight SMA patients with homozygous deletion of *SMN1* exon 7 but not exon 8

Patient	SMA Type	Onset (year)	Highest function	Copy number				Hybrid SMN gene sequence I6, E7, I7, E8	Hybrid type
				SMN2 E7	SMN2 E8	SMN1 E7	SMN1 E8		
13	III	6 m[a]<	Walk	3	2	0	1	aTagG	B
14	III	12 m[a]	Stand	3	2	0	1	aTggG	A
15	III	8	Walk	3	2	0	1	gTaaG	C
16	III	14 m[a]	Stand	3	2	0	1	aTggG	A
17	III	9 m[a]	Stand	3	2	0	1	aTggG	A
18	III	3	Walk	4	3	0	1	aTggG	A
19	III	15	Walk	4	2	0	2	aTggG	A
20	IV	40	Walk	4	2	0	2	aTggG	A

Bold face: sequence (**gCaaG**) derived from *SMN1*; italics: sequence (*aTggA*) derived from *SMN2*.
[a]We assigned SMA type by giving priority to evaluating each patient's highest function over age of onset.

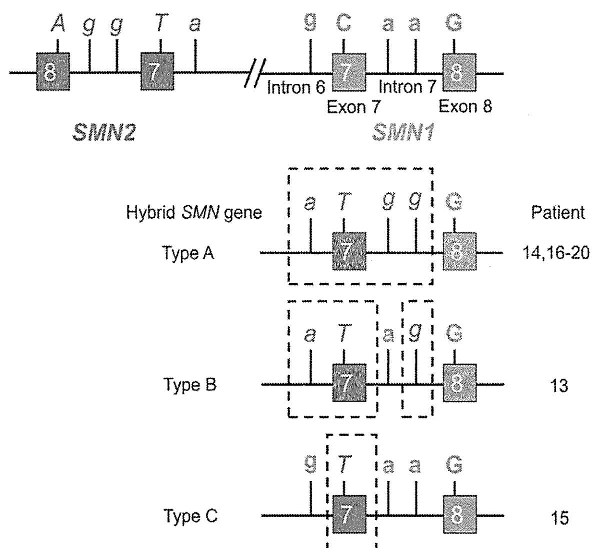

Figure 5 Schematic illustration of the three hybrid *SMN* gene types. Dotted line frames indicate *SMN2* sequences and show the *SMN1-to-SMN2* gene conversion. The type A hybrid was most common. The sequences of intron 6, exon 7 and intron 7 were of *SMN2* origin, whereas that of exon 8 was of *SMN1* origin. Type B was a complex form. The sequences of intron 6, exon 7 and intron 7 (only one base) were of *SMN2* origin, whereas those of intron 7 (the other base) and exon 8 were of *SMN1* origin. Type C had the fewest changes: the exon 8 sequence was of *SMN2* origin, whereas intron 6, intron 7 and exon 8 were of *SMN1* origin. A full color version of this figure is available at the *Journal of Human Genetics* online.

thereby showing disease severity similar to that of patients 18–20. We speculate that milder symptoms might correspond to small conversion regions, like Type C.

Patients with a missense mutation or hybrid *SMN* gene, identified in this study, showed relatively mild SMA symptoms. As to possible mechanisms underlying such mild symptoms, Prior *et al.*[29] reported that the c.859G>C substitution in the *SMN2* gene is a positive modifier of the SMA phenotype. Although we tested for the c.859G>C change in the *SMN2* gene, neither the missense mutation nor the hybrid *SMN* gene (patients 9–20) carried this change.

Our method for detecting intragenic mutations of *SMN1* by nLR-PCR (28.2 kb) is more efficient and has broader applications than the currently available methods. In three patients for whom current methods yielded no results, we identified a c.5C>T mutation in *SMN1* exon 1. In eight patients with a hybrid *SMN* gene, we identified three hybrid types. This new method allows analysis of previously undetectable regions, including all introns and exons of *SMN1* and all *SMN* genes. Furthermore, we identified three distinct hybrids.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to the patients who agreed to participate in this study. We thank Drs T Yamamoto, S Morikawa, S Sawai, H Nakajima, T Oshita and T Kurashige for their valuable contributions to this study. This work was supported by Grants-in-Aid from the Research Committee of Spinal Muscular Atrophy, the Ministry of Health, Labour and Welfare of Japan (to KS). This

work was also supported by the Global COE program, Multidisciplinary Education and Research Center for Regenerative Medicine (MERCREM) from the Ministry of Education, Culture, Sports Science, and Technology (MEXT), Japan (to YK and KS).

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

日本遺伝カウンセリング学会誌
Jpn J Genet Counsel
35 : 99-104, 2014

【原著】

脊髄性筋萎縮症における*SMN*遺伝子のコピー数解析と遺伝カウンセリングへの応用

久保祐二[1) 2) 3)], 伊藤万由里[2)], 青木亮子[2)], 斎藤加代子[1) 2)]

Copy number analysis of the *SMN* gene and genetic counseling using the results in spinal muscular atrophy

Yuji Kubo[1) 2) 3)], Mayuri Ito[2)], Ryoko Aoki[2)], Kayoko Saito[1) 2)]

【要旨】

脊髄性筋萎縮症（SMA：spinal muscular atrophy）は脊髄前角細胞の変性による筋萎縮と進行性筋力低下を特徴とする常染色体劣性遺伝性疾患である。SMA の原因遺伝子は *survival motor neuron 1*（*SMN1*）遺伝子であり，ホモ接合性欠失により発症する。同じように *SMN1* 遺伝子欠失（0 コピー）を示す症例でも，発症年齢や臨床的重症度に差が生じる。本研究では，臨床的重症度の差がなぜ生じるかを解明するために，SMA Ⅰ～Ⅳ型患者 33 例において *SMN1* 遺伝子と *SMN2* 遺伝子のコピー数を調べた。その結果，臨床的に軽症なほど *SMN1* 遺伝子の欠失範囲が小さくなり，*SMN2* 遺伝子のコピー数が増加した。*SMN1* 遺伝子欠失を示した症例では，*SMN1* 遺伝子の欠失範囲と *SMN2* 遺伝子のコピー数が重症度の差を生む要因であった。*SMN* 遺伝子のコピー数情報は症状の重さや進行の予測などに利用できる可能性を示した。

キーワード：脊髄性筋萎縮症 spinal muscular atrophy（SMA），*SMN* 遺伝子 *survival motor neuron*（*SMN*）gene，コピー数解析 copy number analysis，遺伝カウンセリング genetic counseling，発症年齢と臨床的重症度 onset and clinical severity

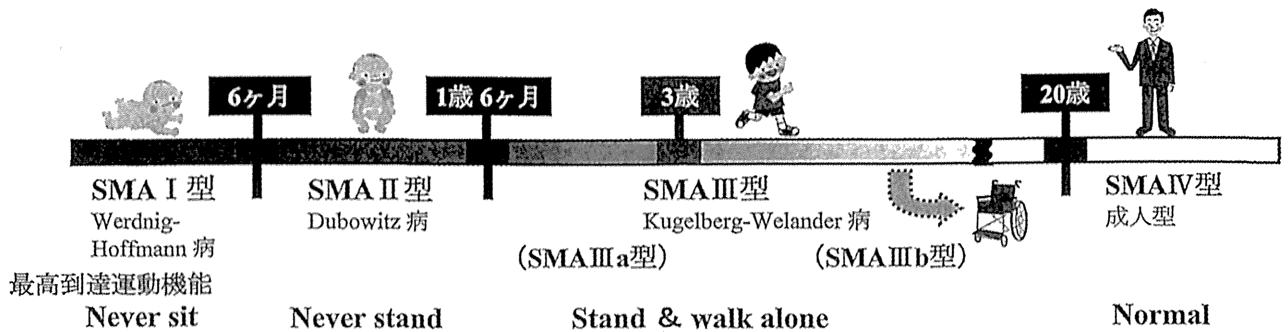
1) 東京女子医科大学大学院先端生命医科学系専攻遺伝子医学分野
Branch of Genetic Medicine, Advanced Biomedical Engineering and Science, Graduate School of Medicine, Tokyo Women's Medical University
2) 東京女子医科大学附属遺伝子医療センター
Institute of Medical Genetics, Tokyo Women's Medical University
3) 凸版印刷株式会社総合研究所
Technical Research Institute, Toppan Printing Co., Ltd,

平成 26 年 2 月 3 日受付
平成 26 年 6 月 23 日修正受付
平成 26 年 8 月 4 日受理

緒言

脊髄性筋萎縮症（SMA; OMIM 253300）は脊髄前角細胞の変性による筋萎縮と進行性筋力低下を特徴とする疾患である[1)]。SMA の頻度はおよそ出生児 6,000 ～ 10,000 人に 1 人であり，諸外国の保因者頻度は 40 ～ 60 人に 1 人である[2) 3)]。日本では 2006 年の全国調査により SMA は約 1,000 人の患者数が推定される。小児期発症はⅠ型，Ⅱ型，Ⅲ型に，成人発症はⅣ型に分類される下位運動ニューロン病である[3 4)]。Ⅰ型（Werdnig-Hoffmann 病）は 6 ヶ月までに発症し，生涯座位保持不可能である。2 歳以降の生存のためには人工呼吸管理を必要とする。Ⅱ型（Dubowitz 病）は 1 歳 6 ヶ月までに発症し，生涯起立・歩行の獲得が不可能である。Ⅲ型（Kugelberg-Welander 病）は 1 歳 6 ヶ月以降に

図1　SMA の型と臨床症状

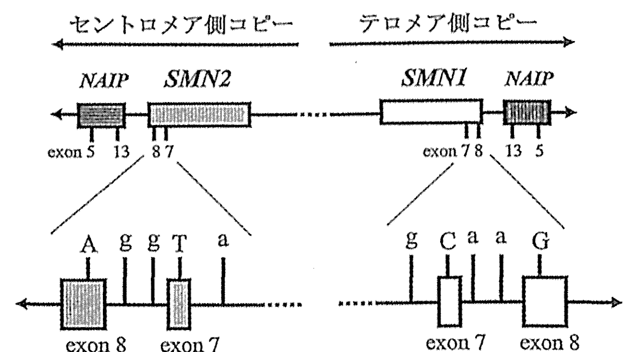


発症し，自立歩行が可能だが症状が進むにつれ不可能となる[3)-5)]。Ⅳ型は弧発性が多く，20歳以降に発症する[4)]（図1）。

小児期発症のSMAの原因遺伝子は *survival motor neuron 1*（*SMN1*）遺伝子であり，ホモ接合性欠失により発症する[6)]。*SMN1* 遺伝子は第5染色体長腕5q13に存在し，同領域に向反性に重複した配列の *SMN2* 遺伝子も存在する（図2）。テロメア側に位置する *SMN1* 遺伝子とセントロメア側に位置する *SMN2* 遺伝子の間には5つの塩基の違いがあり[6)]，翻訳領域には exon 7 に存在する1塩基の違い（*SMN1* 遺伝子は c.840C, *SMN2* 遺伝子は c.840T）しか存在しない[6) 7)]。1塩基の違いが exon 7 でのスプライシングパターンに変化をもたらし，*SMN1* 遺伝子は全長の *SMN1* 転写産物を産生し，*SMN2* 遺伝子は exon 7 領域を欠いた（Δ7）*SMN2* 転写産物を約85%，全長の *SMN2* 転写産物を約15%産生する[4) 8)]。exon 7 領域を欠いたタンパク質 SMN Δ7 は機能を持たず，すぐに分解される[9)]。全長の転写産物から翻訳された機能的なタンパク質 SMN は脊髄神経細胞の核に存在し，RNA の代謝に関与している[10)]。また，*SMN*（*SMN1*, *SMN2*）遺伝子の下流には細胞のアポトーシスを抑制する蛋白質をコードする *neuronal apoptosis inhibitory protein*（*NAIP*）遺伝子が存在し，SMA の重症度に関連があると考えられている[6) 11)]。

SMA の遺伝学的検査としては PCR-RFLP 法を用いて *SMN1* 遺伝子欠失を調べる方法が一般的である[6) 12)]。近年は Real time PCR 法や Multiplex Ligation-dependent Probe Amplification（MLPA）法を用いて遺伝子のコピー数解析が行われるようになり，*SMN* 遺伝子のコピー数が疾患の重症度と関連があるという報告がされている[13)-15)]。当センターではこれまでに *SMN1* 遺伝子欠失を示す SMA において，発症年齢や臨床的重症度に差を認める症例を確認している。本研究では，この臨床的スペクトラムの成因解明のために *SMN1* 遺伝子欠失を示す症例のコピー数を調べ，コントロールと比較した。また，*SMN* 遺伝子と *NAIP* 遺伝子のコピー数の解析結果をどのように遺伝カウンセリングに活用できるかについて検討した。

図2　*SMN* 遺伝子の構造



対象・方法

（1）対象

SMA Ⅰ～Ⅳ型症例33例〔Ⅰ型　15例（0～6ヶ月），Ⅱ型　7例（7～18ヶ月），Ⅲ型　9例（19ヶ月～20歳未満），Ⅳ型　2例（20歳以上）〕，コントロール70例（20歳以上）。

20歳以上の対象者についてはインフォームドコンセントにて同意を取得し，20歳未満の対象者についてはインフォームドアセントを得て，同意能力を欠く場合には代諾者から同意を得た。また，本研究は東京女子医科大学倫理委員会の承認を受けて実施した。

（2）*SMN1* 遺伝子 exon 7，*NAIP* 遺伝子 exon 5 欠失解析

ゲノム DNA は全血から QIAamp DNA Blood Mini Kit（QIAGEN）を用いて抽出し，最終濃度が 100 ng / μl になるように調製した。*SMN1* 遺伝子 exon 7 の欠失解析には，Lefebvre らの PCR-RFLP 法を用いて検査を行った[6) 12)]。*NAIP* 遺伝子 exon 5 の欠失解析には，Roy らの PCR 法を用いた[11)]。本方法は SMA の遺伝学的検査として，当センターで実施している。

（3）MLPA 法を用いた *SMN* 遺伝子，*NAIP* 遺伝子コピー数解析

SMN 遺伝子のコピー数解析には SALSA MLPA KIT P021-A1 SMA（MRC-Holland）を用いた。キットには *SMN1* 遺伝子と *SMN2* 遺伝子 exon 7 に特異的なプローブ，

SMN1 遺伝子と *SMN2* 遺伝子 exon 8 に特異的なプローブ, *SMN1* 遺伝子と *SMN2* 遺伝子 exon 1, 4, 6, 8 に特異的なプローブ, *SMN* 遺伝子近傍の *NAIP* 遺伝子に特異的なプローブ, 他の染色体に特異的なプローブ, リファレンスとなるプローブが含まれている。MLPA 反応後の DNA フラグメントは GeneMapper software v4.1 (Applied Biosystems) を用いて ABI 3130 Genetic Analyzer (Applied Biosystems) で解析を行った。

結果

(1) *SMN1* 遺伝子 exon 7, *NAIP* 遺伝子 exon 5 欠失解析

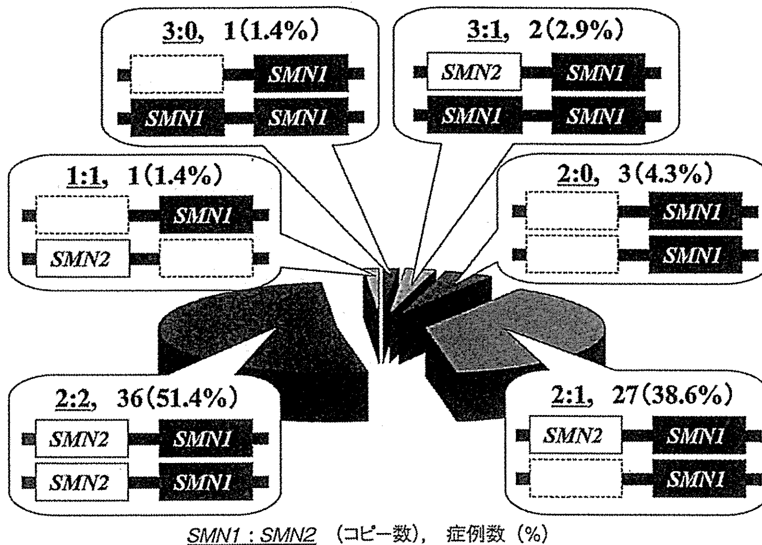
SMA 症例 33 例において *SMN* 遺伝子 exon 7 の欠失解析を行い, 全症例において *SMN1* 遺伝子 exon 7 の欠失を確認した。SMA I 型では 8 例に *NAIP* 遺伝子 exon 5 の欠失を確認した。

(2) MLPA 法を用いた *SMN* 遺伝子コピー数解析

1) コントロールにおける *SMN1* 遺伝子コピー数解析

MLPA 法を用いてコントロールの *SMN* 遺伝子 (exon 1, 4, 6, 7, 8) のコピー数を解析した (図 3)。*SMN1* 遺伝子, *SMN2* 遺伝子をそれぞれ 2 コピーずつもつ症例〔*SMN1* : *SMN2* (コピー数比) = 2 : 2〕が最も多く, 36 例 (51.4%) であった。次に *SMN1* 遺伝子を 2 コピー, *SMN2* 遺伝子を 1 コピーもつ症例(*SMN1* : *SMN2* = 2 : 1) が 27 例(38.6%) であった。その 2 つのタイプが 90%を占めた。*SMN1* 遺伝子を 1 コピー (*SMN1* : *SMN2* = 1 : 1) もしくは 3 コピーもつ症例 (*SMN1* : *SMN2* = 3 : 0 or 1) や, *SMN2* 遺伝子を 0 コピーもつ症例 (*SMN1* : *SMN2* = 2 or 3 : 0) を確認した。

図 3　コントロール 70 例における *SMN* 遺伝子コピー数解析



SMN1 : SMN2 (コピー数), 症例数 (%)

2) SMA における *SMN* 遺伝子, *NAIP* 遺伝子コピー数解析

SMA 症例 33 症例について, MLPA 法により *SMN* 遺伝子, *NAIP* 遺伝子のコピー数を解析した (表 1)。

i　SMA I 型

全症例 (15 例) で *SMN1* 遺伝子 exon 7, exon 8 の欠失 (0 コピー) を確認した。13.3% (2/15) は *SMN2* 遺伝子 exon 7 が 3 コピーであった。53.3 % (8/15) は *NAIP* 遺伝子 exon 5 が欠失していた。

表 1　SMA 症例における *SMN* 遺伝子, *NAIP* 遺伝子コピー数解析

型 (合計人数), 該当人数 (%)	コピー数	*SMN2* exon7	*SMN2* exon8	*SMN1* exon7	*SMN1* exon8	*NAIP* exon5	*NAIP* exon13
I (n=15)	0	0	0	15 (100)	15 (100)	8 (53.3)	0
	1	0	0	0	0	7 (46.7)	15 (100)
	2	13 (86.7)	12 (80)	0	0	0	0
	3	2 (13.3)	3 (20)	0	0	0	0
II (n=7)	0	0	0	7 (100)	5 (71.4)	1 (14.3)	0
	1	0	1 (14.3)	0	2 (28.6)	5 (71.4)	6 (85.7)
	2	4 (57.1)	1 (14.3)	0	0	1 (14.3)	1 (14.3)
	3	3 (42.9)	5 (71.4)	0	0	0	0
III (n=9)	0	0	0	9 (100)	5 (55.5)	0	0
	1	0	0	0	0	3 (33.3)	2 (22.2)
	2	0	4 (44.4)	0	4 (44.4)	6 (66.6)	7 (77.7)
	3	9 (100)	2 (22.2)	0	0	0	0
	4	0	3 (33.3)	0	0	0	0
IV (n=2)	0	0	0	2 (100)	2 (100)	0	0
	1	0	0	0	0	0	0
	2	0	0	0	0	1 (50)	2 (100)
	3	2 (100)	0	0	0	1 (50)	0
	4	0	2 (100)	0	0	0	0

SMA I〜IV型患者 33 例 (I型　15 例, II型　7 例, III型　9 例, IV型　2 例) についてコピー数の解析を行った。

ii　SMA Ⅱ型

全症例（7例）で*SMN1*遺伝子exon 7の欠失が確認され，28.6%（2/7）は*SMN1*遺伝子exon 8が1コピーであった。42.9%（3/7）は*SMN2*遺伝子exon 7が3コピーであった。14.3%（1/7）だけが*NAIP*遺伝子exon 5の欠失を示した。

iii　SMA Ⅲ型

全症例（9例）で*SMN1*遺伝子exon 7の欠失が確認され，44.4%（4/9）は*SMN1*遺伝子exon 8が2コピーであった。全症例（9例）で*SMN2*遺伝子exon 7が3コピーを示した。どの症例にも*NAIP*遺伝子exon 5の欠失は認められなかった。

iv　SMA Ⅳ型

全症例（2例）で*SMN1*遺伝子exon 7，exon 8の欠失が確認された。全症例で*SMN2*遺伝子exon 7が3コピーを示した。どの症例にも*NAIP*遺伝子exon 5，exon 13の欠失は認められなかった。

考察

（1）コントロールにおける*SMN*遺伝子コピー数の多様性

コントロールにおける*SMN1*遺伝子コピー数解析の結果より（図3），*SMN1*遺伝子，*SMN2*遺伝子をそれぞれ2コピーもつ症例（*SMN1*：*SMN2*＝2：2）が最も多く（51.4%）観察されたが，*SMN1*遺伝子は1から3コピー，*SMN2*遺伝子は0から2コピーの範囲でバラツキが見られた。コントロールであっても*SMN*遺伝子のコピー数には多様性があり，染色体5q13領域は組換え等の変化が起こりやすい領域であると考えられた。アジアでは台湾や中国でもコントロールにおける*SMN1*遺伝子コピー数解析が大規模に行われている[16)-18)]。本研究と同様に*SMN1*遺伝子，*SMN2*遺伝子をそれぞれ2コピーもつ症例（*SMN1*：*SMN2*＝2：2）が最も多く（台湾56%，中国57.7%）観察され，*SMN1*遺伝子を2コピー，*SMN2*遺伝子を1コピーもつ症例（*SMN1*：*SMN2*＝2：1）が次に多く（台湾28%，中国26.2%）観察され，本研究の結果と同様の割合を示していた。また，*SMN1*遺伝子が1コピーの症例が70例中1例であった（図3）。日本のSMA保因者頻度はこの割合に近い数値であろう。諸外国の保因者頻度は40～60人に1人との報告がある[2)3)]。アジアでは台湾で107,611人における大規模解析が行われており48人に1人[16)]，中国では1,712人について解析が行われ42人に1人と報告されている[18)]。今回の結果からは，日本人における保因者頻度は諸外国に比べると頻度が低いように思われるが，今回の解析は小規模であり，より正確な日本人の保因者頻度を算出するためには，大規模な解析が必要であると考えられた。

（2）遺伝子の欠失範囲と重症度

PCR-RFLP法[6)12)]を用いて*SMN*遺伝子exon 7欠失解析を行い，欠失が認められたSMA症例33例においてMLPA法によるコピー数解析を行った。全症例（33例）で*SMN1*遺伝子exon 7は欠失（0コピー）を示し，PCR-RFLP法による解析と同様の結果を得た。*NAIP*遺伝子exon 5の欠失はSMA Ⅰ型の53.3%（8/15）で認められ，PCR法，MLPA法ともに同様の結果を得た。PCR-RFLP法を用いて，東京女子医科大学附属遺伝子医療センターにおいて遺伝学的検査を実施したSMA症例322例では，*SMN*遺伝子の欠失はⅠ型で98%，Ⅱ型で95%，Ⅲa型で52%，Ⅲb型で42%，Ⅳ型で15%であった[19)]。SMA Ⅰ型では*NAIP*遺伝子exon 5を欠失する割合が41%確認されたが，その他の型では10%にも満たなかった。SMA Ⅰ型における*NAIP*遺伝子exon 5の欠失は本研究結果と近い割合（53.3%）であった（表1）。さらにMLPA法では片アレル欠失の有無の判断が可能であり，Ⅲa型の場合では片アレルだけ欠失の範囲が大きい場合（図4アレルA-2とA-4の組み合わせでもつタイプ）と両アレルとも欠失範囲が小さい場合（図4アレルA-2とA-2の組み合わせでもつタイプ）を比較すると欠失範囲の大きい前者の方が歩行不可能になる時期が早かった。特に10代で発症しているⅢb型は*SMN1*遺伝子exon 7のみの欠失の割合が多く（図4A-1），Ⅲa型との欠失範囲の大きさの差は顕著であった。

*SMN*遺伝子解析だけでなく*NAIP*遺伝子の解析をすることで*SMN*遺伝子近傍の欠失範囲を確認することができた。特にⅠ型では*SMN1*遺伝子だけでなく隣接する*NAIP*遺伝子も欠失している症例が多く見られ，欠失範囲が大きいことを示していた（図4A-4）。Ⅲ型，Ⅳ型では*SMN1*遺伝子の欠失の割合が少ないことは*SMN1*遺伝子以外の別の因子が原因であることも示唆された。

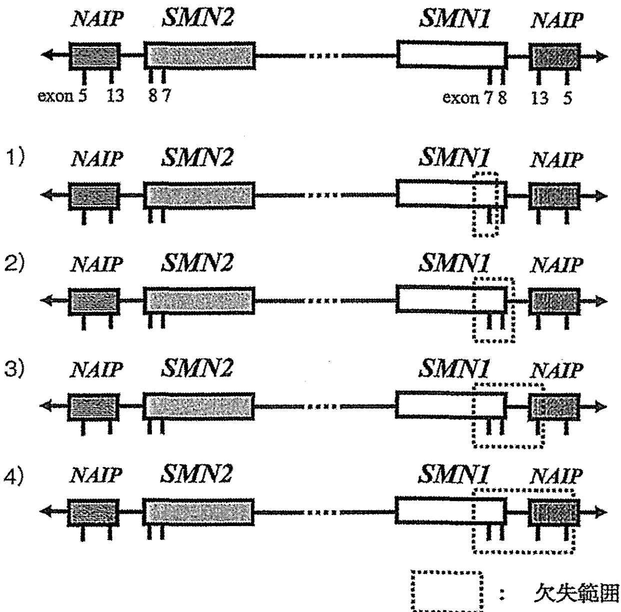
（3）*SMN2*遺伝子のコピー数と重症度

MLPA法解析により，*SMN2*遺伝子のコピー数の変化を確認した（表1，図5）。型別にコピー数の平均値を算出したところ，主に症状が軽くなるに従い，*SMN2*遺伝子のコピー数が増加する傾向にあり（図5），既報告と同様な傾向を示していた[13)-15)]。コピー数の増加は図4Bに示すように遺伝子変換が起こったためと考えられた。

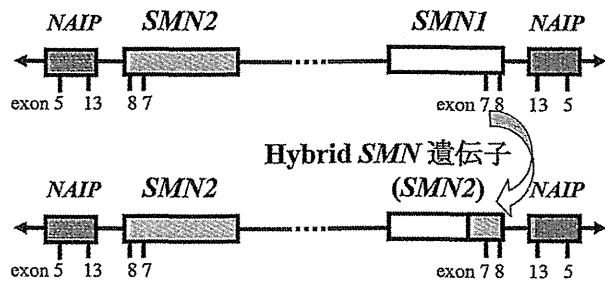
SMAは主に*SMN1*遺伝子を失うことで発症するが，*SMN1*遺伝子が完全に欠失するか，もしくは上述したような*SMN1*遺伝子と*SMN2*遺伝子間での遺伝子変換によるものと考えられる。*SMN1*遺伝子を完全に欠失した症例よりも遺伝子変換により*SMN1*遺伝子が*SMN2*遺伝子に変換した症例のほうが，症状が軽症化する傾向にあった。*SMN2*遺伝子は少ないながらも機能的なSMNタンパク質を産生する。*SMN2*遺伝子のコピー数が増加することで機能的なSMNタンパク質量が増え，症状の軽症化に繋がることが考えられた[20)]。さらには*SMN2*遺伝子からより多くの機能的なSMNタンパク質を産生させることが本症例の軽症化を目

図４　染色体 5q13 領域における変化

A. 欠失範囲の差

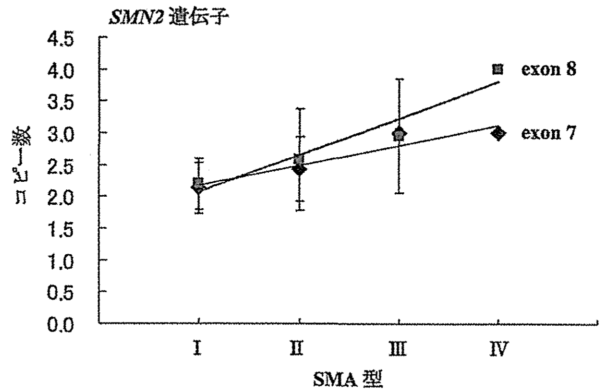


B. 遺伝子変換



A. 1) は *SMN1* 遺伝子 exon 7 のみの欠失，2) は *SMN1* 遺伝子 exon 7, 8 の欠失，3) は *SMN1* 遺伝子 exon 7 から *NAIP* 遺伝子 exon 13 付近までの欠失，4) は *SMN1* 遺伝子 exon 7 から *NAIP* 遺伝子 exon 5 付近までの欠失（もしくは全欠失）を示している。
B. *SMN2* 遺伝子の増加が確認される症例はこのような（*SMN1* から *SMN2* への）遺伝子変換が起こっている可能性が示唆された。

図５　SMA の型と *SMN2* 遺伝子コピー数の関係



型ごとにコピー数の平均値を算出した。I 型からIV型にかけて *SMN2* 遺伝子コピー数の増加を示した。

指す治療に利用できると考えられた。

(4) 遺伝カウンセリング・診療への応用

SMA 症例の遺伝学的検査において，MLPA 法を用いることで *SMN* 遺伝子，*NAIP* 遺伝子のコピー数や欠失領域を知ることが可能になった。さらには，*SMN* 遺伝子，*NAIP* 遺伝のコピー数情報が重症度や進行の早さなどに関連していることが示唆された。

特に発症年齢に差があるIII型の場合にコピー数や欠失領域を診療に利用できると考えている。例えば *SMN2* 遺伝子 (exon 7) のコピー数が同じ 3 症例を比較すると，欠失範囲が小さい（*SMN1* 遺伝子のみの欠失）2 症例は 50 歳を過ぎても歩行可能であったのに対し，欠失範囲が大きい（*SMN1* 遺伝子，*NAIP* 遺伝子ともに欠失）1 症例では 12 歳で歩行不可能になった。後者のような場合には，成長や発達過程を考慮しながら運動機能障害の進展の予測，関節拘縮予防などの理学療法の早期介入などの情報提供が有用であると考えられた。また，遺伝学的検査の解析結果によって，情報提供をする時期や内容を個別に検討し，患者・家族が SMA における運動機能障害の各段階を理解し，受容し，進展過程を少しでも抑制するためのサポートに応用可能であることが示唆された。

また，将来 *SMN2* 遺伝子をターゲットとした薬剤が利用可能になった場合，*SMN2* 遺伝子のコピー数情報がその薬剤の効果予測（例：*SMN2* 遺伝子のコピー数が多いと効果が高い可能性があるなど）に利用できるかもしれない。

結論

SMN 遺伝子欠失を示した SMA 症例では，遺伝子欠失範囲と *SMN2* 遺伝子のコピー数が発症の早さや症状の差を生む要因であった。また，コントロールにおいて *SMN* 遺伝子のコピー数は多様であり，5q13 領域は変化が起こりやすいことを示していた。*SMN* 遺伝子のコピー数情報を活用することで，症状の重さや進行の予測，保因者診断，治療の方向性など様々な情報を提供していくことが可能になった。

この研究は厚生労働科学研究費補助金難治性疾患等克服研究事業（難治性疾患克服研究事業）「脊髄性筋萎縮症の臨床実態の分析，遺伝子解析，治療法開発の研究」(研究代表者　斎藤加代子）の助成によって行われた。本研究は，第 37 回日本遺伝カウンセリング学会において発表し，若手奨励賞を受賞した。

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