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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

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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.
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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

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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

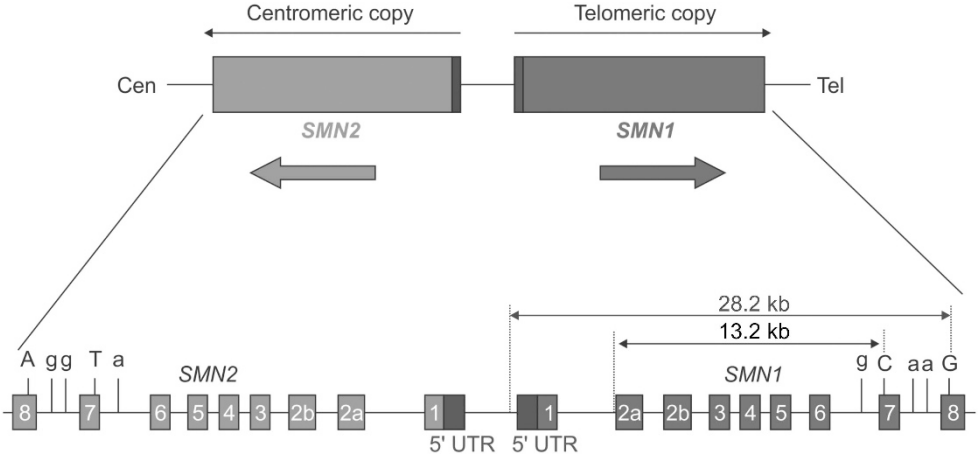

Figure 1 Strategy for specific amplification of *SMN1* by long-range PCR. *SMN1* and *SMN2* lie, respectively, on the telomeric and centromeric halves of an inverted duplication in chromosome region 5q13. Long-range PCR (13.2 kb) of the region including exons 2a–7 of *SMN1* was reported by Clermont *et 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.

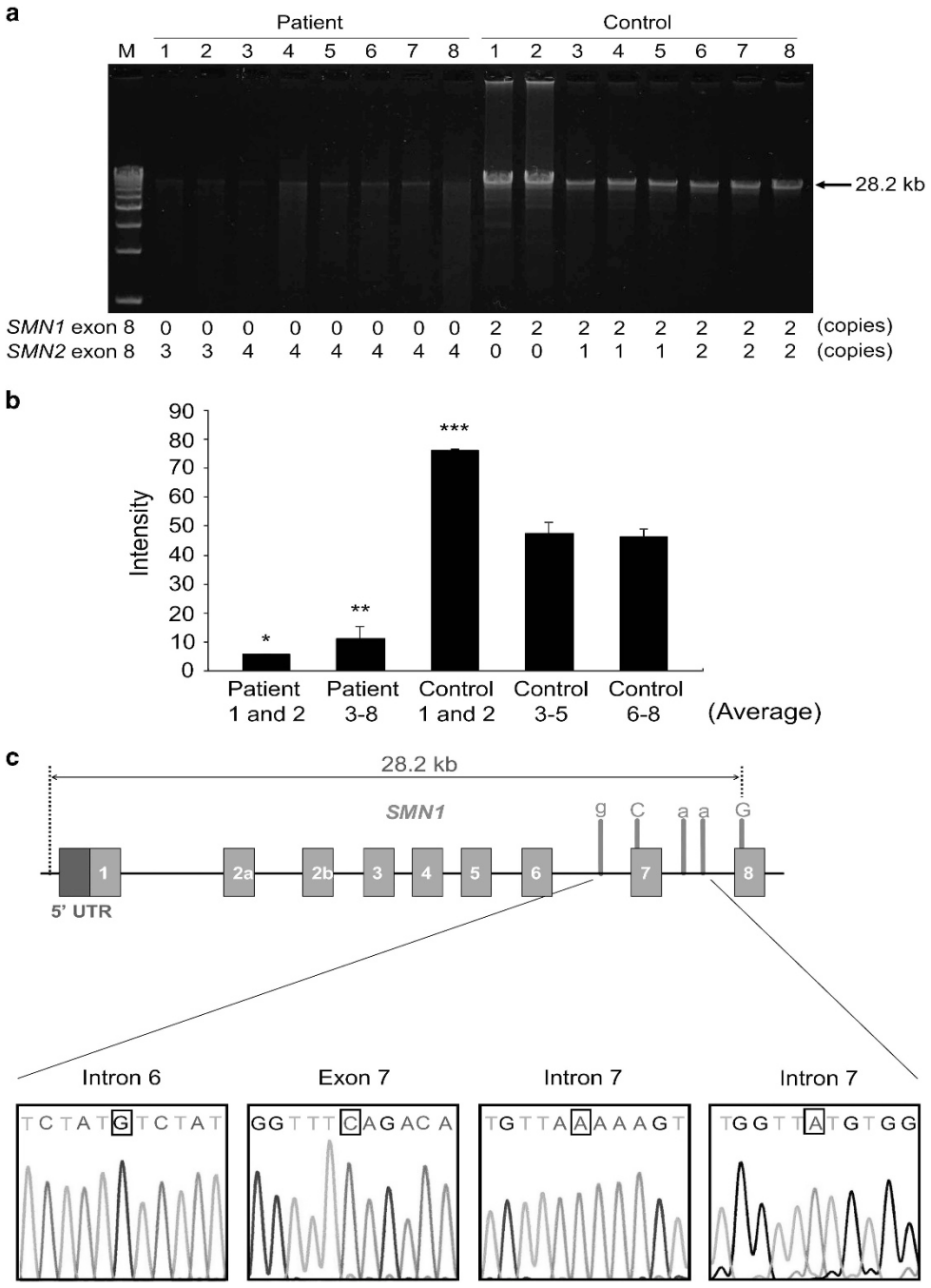

Figure 2 Evaluation of new method. *SMN1*-specific amplifications from exon 1(-654) to exon 8 (28.2 kb) are shown. (**a**) Controls 1–8 yielded 28.2-kb amplicons, whereas there were few signs of amplification in patients 1–8. Copy numbers of *SMN1* and *SMN2* exon 8 determined by MLPA are shown at the 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).

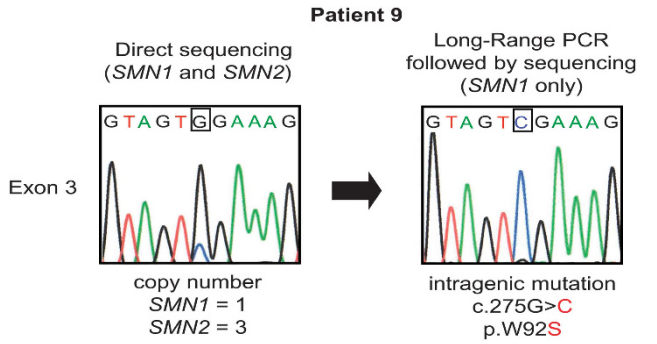

Figure 3 Detection of an intragenic mutation in a patient with type I SMA. Patient 9 was compound heterozygous for *SMN1*, with one deleted *SMN1* allele and an intragenic mutation (c.275G>C, p.W92S) in the other allele.[19] This patient had three *SMN2* copies. (Left) Direct sequencing of *SMN1* and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

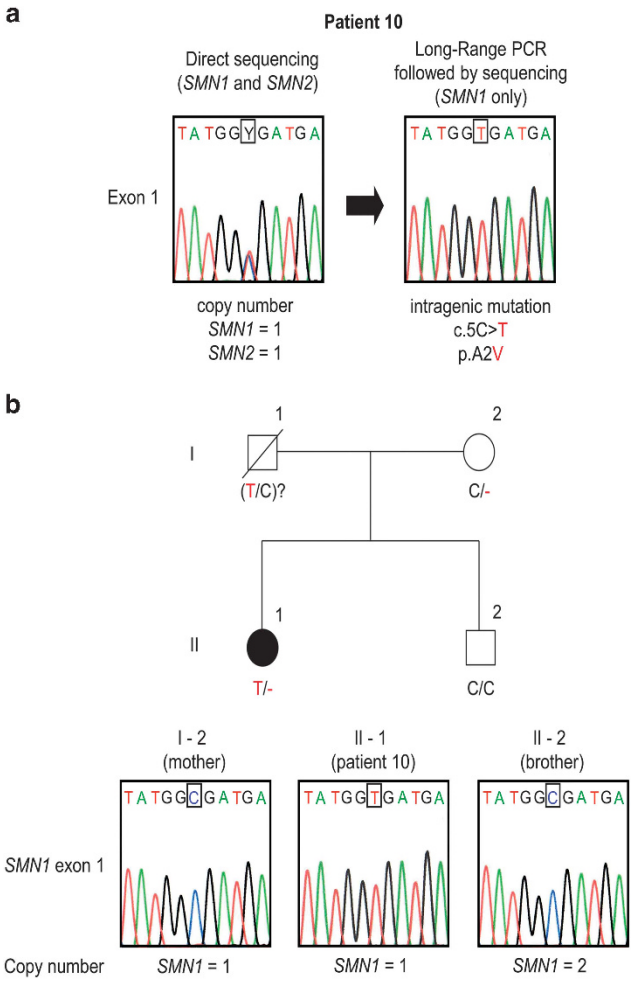

Figure 4 Identification of an intragenic mutation in *SMN1*. (**a**) Patient 10 had one copy each of *SMN1* and *SMN2*. (Left) Direct sequencing for *SMN1* and *SMN2* results are shown; (Right) direct sequencing of *SMN1* (right) exon 1 isolated by the new long-range PCR technique. The sequence revealed 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

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

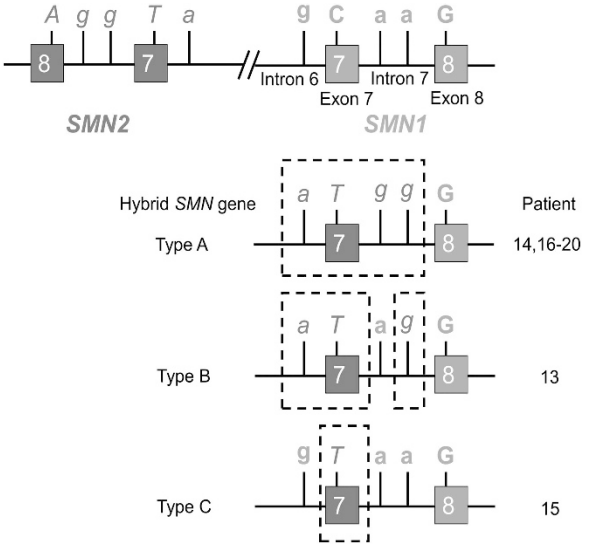

Figure 5 Schematic illustration of the three hybrid *SMN* gene types. Dotted line frames indicate *SMN2* sequences and show the *SMN1-to-SMN2* gene conversion. The type A hybrid was most common. The sequences of intron 6, exon 7 and intron 7 were of *SMN2* origin, whereas that of exon 8 was of *SMN1* 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.

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