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], Kayoko Saito[1,2]*

[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

[5]Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan

*Corresponding Author

Prof. Kayoko Saito, MD, PhD

Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan

10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan

Phone number: +81-3-3353-8111

Fax number: +81-3-5269-7594

Email address: saito.kayoko@twmu.ac.jp

ABSTRACT

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, while 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.

KEY WORDS: hybrid *SMN* gene/ intragenic mutation/ sequencing/ spinal muscular atrophy/ survival motor neuron 1 (*SMN1*)

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 6,000–10,000 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 plays a role in pre-messenger RNA splicing in the anterior horn cells in the spinal cord.[7] Although transcription of *SMN1* produces full-length messenger RNA (mRNA), transcription of *SMN2* yields only 15% full-length mRNA, while 85% of the mRNA is incomplete (lacking exon 7).[4]

SMN1 is the SMA-determining gene; approximately 95% of patients have homozygous disruptions of *SMN1* due 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 (qPCR) and multiplex ligation-dependent probe amplification (MLPA) 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 or long-range PCR of genomic

DNA, both of which have limitations.[6,14,17,18] It can be difficult to construct SMN1 cDNA 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′-UTR 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 these 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) and adjusted to a final concentration of 100 ng/μL. The *SMN1* exon 7 deletion was detected by PCR-restriction fragment length polymorphism (PCR-RFLP).[6,20]

***SMN* copy number analysis using the MLPA method**

We used the SALSA MLPA KIT P021-A1 SMA (MRC-Holland) 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* (e.g. the *NAIP* and *H4F5* (*SERF1*) genes); other chromosomes; and reference probes. After MLPA, DNA fragments were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems) with GeneMapper software v4.1 (Applied Biosystems).

Specific amplification of *SMN1* by long-range PCR

Conventional long-range PCR (cLR-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) 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 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 2-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 3-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 (LOVD) 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 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 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 8 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.

DISCUSSION

We developed an efficient and broadly applicable LR-PCR method to detect intragenic mutations in *SMN1* (Figure 1). Without the need for cDNA cloning, this new method makes it possible to analyze all exons and introns of *SMN1*, the 5′- and 3′-UTRs, 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). 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). 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, while 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, 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, Thomas 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 have no conflicts of interest to declare.

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 (SMA), the Ministry of Health, Labour and Welfare of Japan (to K.S.).

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 Y.K. and K.S.).

REFERENCES

1. Crawford, T. O., Pardo, C. A. The neurobiology of childhood spinal muscular atrophy. *Neurobiol. Dis.* **3,** 97–110 (1996).

2. Ogino, S., Wilson, R. B. Genetic testing and risk assessment for spinal muscular atrophy (SMA). *Hum. Genet.* **111,** 477–500 (2002).

3. Prior, T. W., Snyder, P. J., Rink, B. D., Pearl, D. K., Pyatt, R. E., Mihal, D. C. *et al.* Newborn and carrier screening for spinal muscular atrophy. *Am. J. Med. Genet. A.* **152A,** 1608–1616 (2010).

4. Kolb, S. J., Kissel, J. T. Spinal muscular atrophy: a timely review. *Arch. Neurol.* **68,** 979–984 (2011).

5. Zerres, K., Davies, K. E. 59th ENMC International Workshop: Spinal Muscular Atrophies: recent progress and revised diagnostic criteria 17-19 April 1998, Soestduinen, The Netherlands. *Neuromuscul. Disord.* **9,** 272–278 (1999).

6. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L. *et al.* Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* **80,** 155–165 (1995).

7. Pellizzoni, L., Charroux, B., Dreyfuss, G. SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins. *Proc. Natl. Acad.*

Sci. U S A. **96,** 11167–11172 (1999).

8. Burghes, A. H. When is a deletion not a deletion? When it is converted. *Am. J. Hum. Genet.* **61,** 9–15 (1997).

9. Wirth, B. An update of the mutation spectrum of the survival motor neuron gene (*SMN1*) in autosomal recessive spinal muscular atrophy (SMA). *Hum. Mutat.* **15,** 228–237 (2000).

10. Hahnen, E., Forkert, R., Marke, C., Rudnik-Schoneborn, S., Schonling, J., Zerres, K. *et al.* Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homozygous deletions of the *SMN* gene in unaffected individuals. *Hum. Mol. Genet.* **4,** 1927–1933 (1995).

11. van der Steege, G., Grootscholten, P. M., Cobben, J. M., Zappata, S., Scheffer, H., den Dunnen, J. T. *et al.* Apparent gene conversions involving the *SMN* gene in the region of the spinal muscular atrophy locus on chromosome 5. *Am. J. Hum. Genet.* **59,** 834–838 (1996).

12. Alias, L., Bernal, S., Fuentes-Prior, P., Barcelo, M. J., Also, E., Martinez-Hernandez, R. *et al.* Mutation update of spinal muscular atrophy in Spain: molecular characterization of 745 unrelated patients and identification of four

novel mutations in the *SMN1* gene. *Hum. Genet.* **125,** 29–39 (2009).

13. Feldkotter, M., Schwarzer, V., Wirth, R., Wienker, T. F., Wirth, B. Quantitative analyses of *SMN1* and *SMN2* based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am. J. Hum. Genet.* **70,** 358–368 (2002).

14. Harada, Y., Sutomo, R., Sadewa, A. H., Akutsu, T., Takeshima, Y., Wada, H. *et al.* Correlation between *SMN2* copy number and clinical phenotype of spinal muscular atrophy: three *SMN2* copies fail to rescue some patients from the disease severity. *J. Neurol.* **249,** 1211–1219 (2002).

15. Cusco, I., Barcelo, M. J., Rojas-Garcia, R., Illa, I., Gamez, J., Cervera, C. *et al. SMN2* copy number predicts acute or chronic spinal muscular atrophy but does not account for intrafamilial variability in siblings. *J. Neurol.* **253,** 21–25 (2006).

16. Lunn, M. R., Wang, C. H. Spinal muscular atrophy. *Lancet.* **371,** 2120–2133 (2008).

17. Clermont, O., Burlet, P., Benit, P., Chanterau, D., Saugier-Veber, P., Munnich, A. *et al.* Molecular analysis of SMA patients without homozygous *SMN1* deletions using a new strategy for identification of *SMN1* subtle

mutations. *Hum. Mutat.* **24,** 417–427 (2004).

18. Zapletalova, E., Hedvicakova, P., Kozak, L., Vondracek, P., Gaillyova, R., Marikova, T. *et al.* Analysis of point mutations in the *SMN1* gene in SMA patients bearing a single *SMN1* copy. *Neuromuscul. Disord.* **17,** 476–481 (2007).

19. Kotani, T., Sutomo, R., Sasongko, T. H., Sadewa, A. H., Gunadi., Minato, T. *et al.* A novel mutation at the N-terminal of SMN Tudor domain inhibits its interaction with target proteins. *J. Neurol.* **254,** 624–630 (2007).

20. van der Steege, G., Grootscholten, P. M., van der Vlies, P., Draaijers, T. G., Osinga, J., Cobben, J. M. *et al.* PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet.* **345,** 985–986 (1995).

21. Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P. *et al.* A method and server for predicting damaging missense mutations. *Nat. Methods.* **7,** 248–249 (2010).

22. Ng, P. C., Henikoff, S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic. Acids. Res.* **31,** 3812–3814 (2003).

23. Parsons, D. W., McAndrew, P. E., Iannaccone, S. T., Mendell, J. R., Burghes, A. H., Prior, T. W. Intragenic *telSMN* mutations: frequency, distribution, evidence

of a founder effect, and modification of the spinal muscular atrophy phenotype by *cenSMN* copy number. *Am. J. Hum. Genet.* **63,** 1712–1723 (1998).

24. Monani, U. R., Pastore, M. T., Gavrilina, T. O., Jablonka, S., Le, T. T., Andreassi, C. *et al.* A transgene carrying an A2G missense mutation in the *SMN* gene modulates phenotypic severity in mice with severe (type I) spinal muscular atrophy. *J. Cell. Biol.* **160,** 41–52 (2003).

25. Schrank, B., Gotz, R., Gunnersen, J. M., Ure, J. M., Toyka, K. V., Smith, A. G. *et al.* Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc. Natl. Acad. Sci. U S A.* **94,** 9920–9925 (1997).

26. Cusco, I., Barcelo, M. J., del Rio, E., Martin, Y., Hernandez-Chico, C., Bussaglia, E. *et al.* Characterisation of *SMN* hybrid genes in Spanish SMA patients: de novo, homozygous and compound heterozygous cases. *Hum. Genet.* **108,** 222–229 (2001).

27. DiDonato, C. J., Ingraham, S. E., Mendell, J. R., Prior, T. W., Lenard, S., Moxley, R. T. 3rd. *et al.* Deletion and conversion in spinal muscular atrophy patients: is there a relationship to severity? *Ann. Neurol.* **41,** 230–237 (1997).

28. Hahnen, E., Schonling, J., Rudnik-Schoneborn, S., Zerres, K., Wirth, B.

Hybrid survival motor neuron genes in patients with autosomal recessive spinal muscular atrophy: new insights into molecular mechanisms responsible for the disease. *Am. J. Hum. Genet.* **59,** 1057–1065 (1996).

29. Prior, T. W., Krainer, A. R., Hua, Y., Swoboda, K. J., Snyder, P. C., Bridgeman, S. J. *et al.* A positive modifier of spinal muscular atrophy in the *SMN2* gene. *Am. J. Hum. Genet.* **85,** 408–413 (2009).

30. Yamamoto, T., Sato, H., Lai, P. S., Nurputra, D. K., Harahap, NI., Morikawa, S. *et al.* Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy (SMA) patients. *Brain. Dev.* (in Press).

Titles and legends to figures

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.

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.

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.

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.

Supplementary Information

Supplementary Table 1. Primers used for long-range polymerase chain reaction and sequencing

Supplementary Table 2. *SMN* copy numbers in 10 controls and 20 unrelated SMA patients

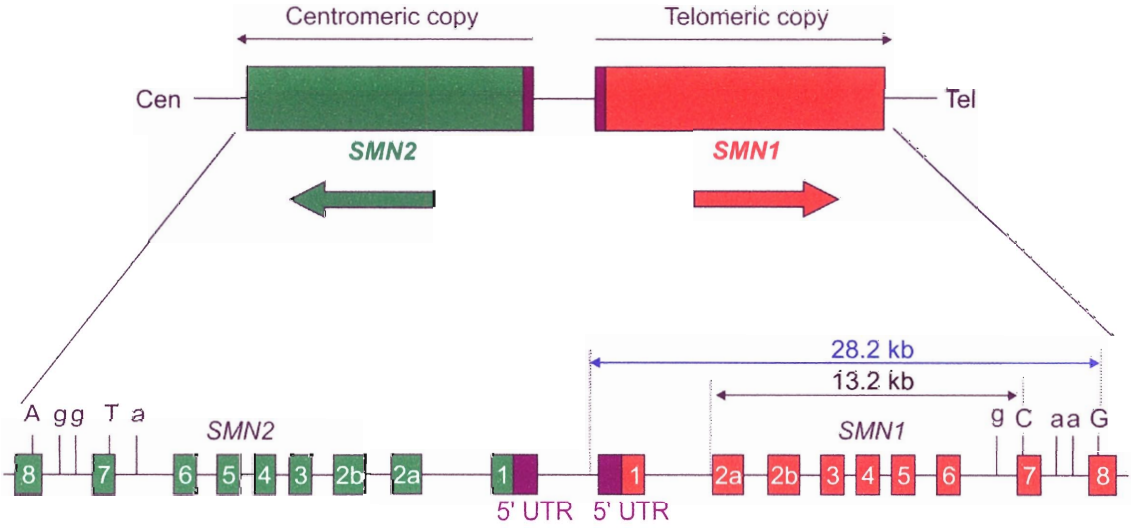
Centromeric copy
Telomeric copy
Cen
Tel
SMN2
SMN1
28.2 kb
13.2 kb
A gg T a
SMN2
SMN1
g C aa G
8 7 6 5 4 3 2b 2a 1 1 2a 2b 3 4 5 6 7 8
5' UTR 5' UTR

Figure 1

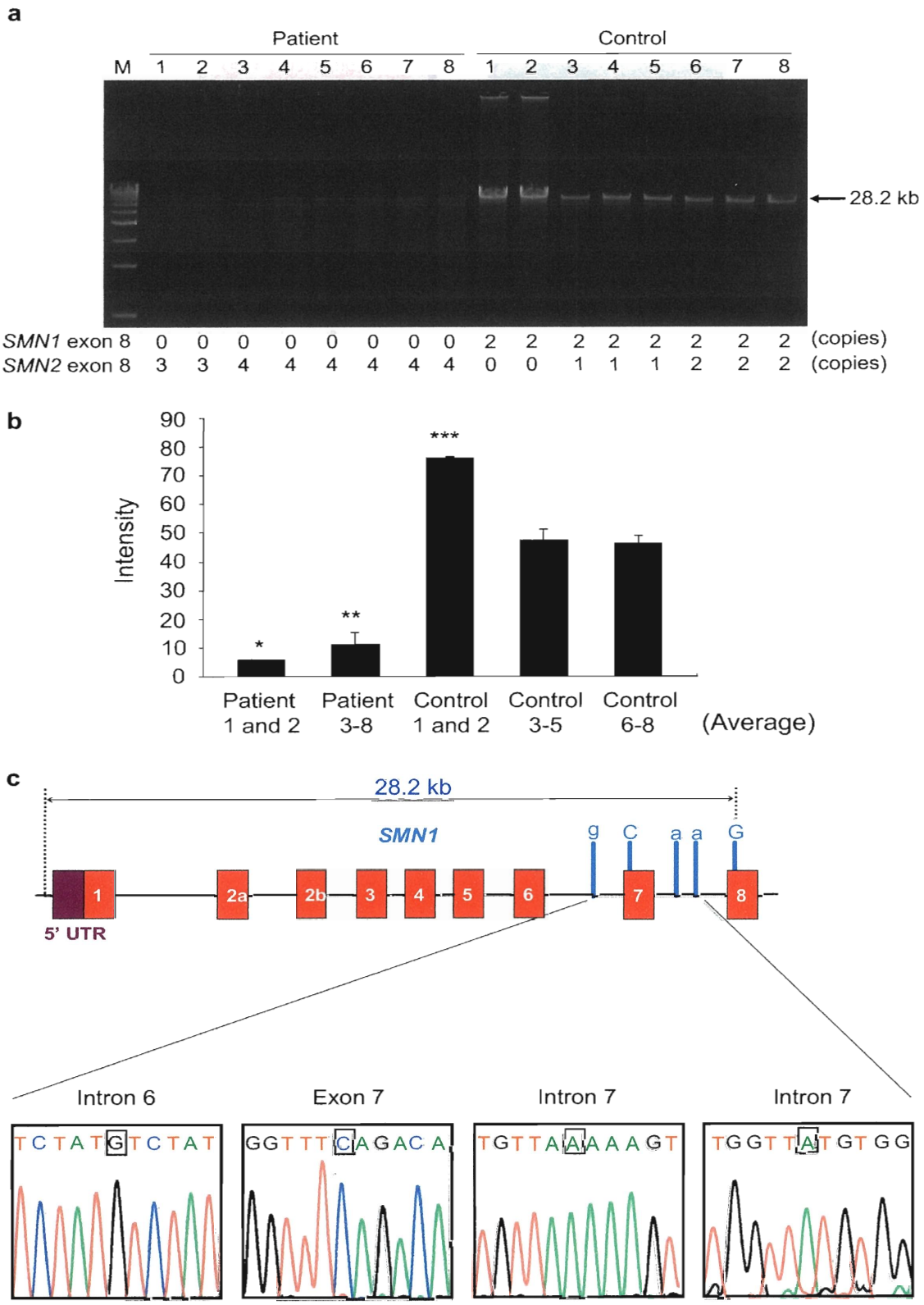
a
Patient
Control
M 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
28.2 kb
SMN1 exon 8 0 0 0 0 0 0 0 0 2 2 2 2 2 2 2 2 (copies)
SMN2 exon 8 3 3 4 4 4 4 4 4 0 0 1 1 1 2 2 2 (copies)
b
Intensity
90
80
70
60
50
40
30
20
10
0
*
**
***
Patient 1 and 2
Patient 3-8
Control 1 and 2
Control 3-5
Control 6-8
(Average)
c
28.2 kb
SMN1
g C a a G
1 2a 2b 3 4 5 6 7 8
5' UTR
Intron 6
Exon 7
Intron 7
Intron 7
TCTATGTCTAT
GGTTTCAGAC A
TGTTAAAAAGT
TGGTTATGTGG

Figure 2

Patient 9

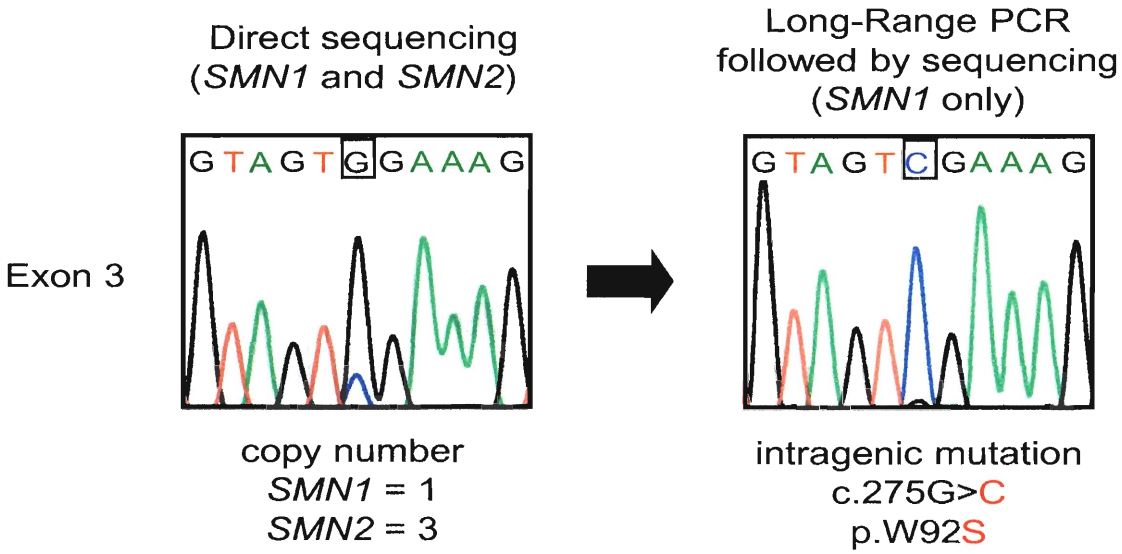

Figure 3

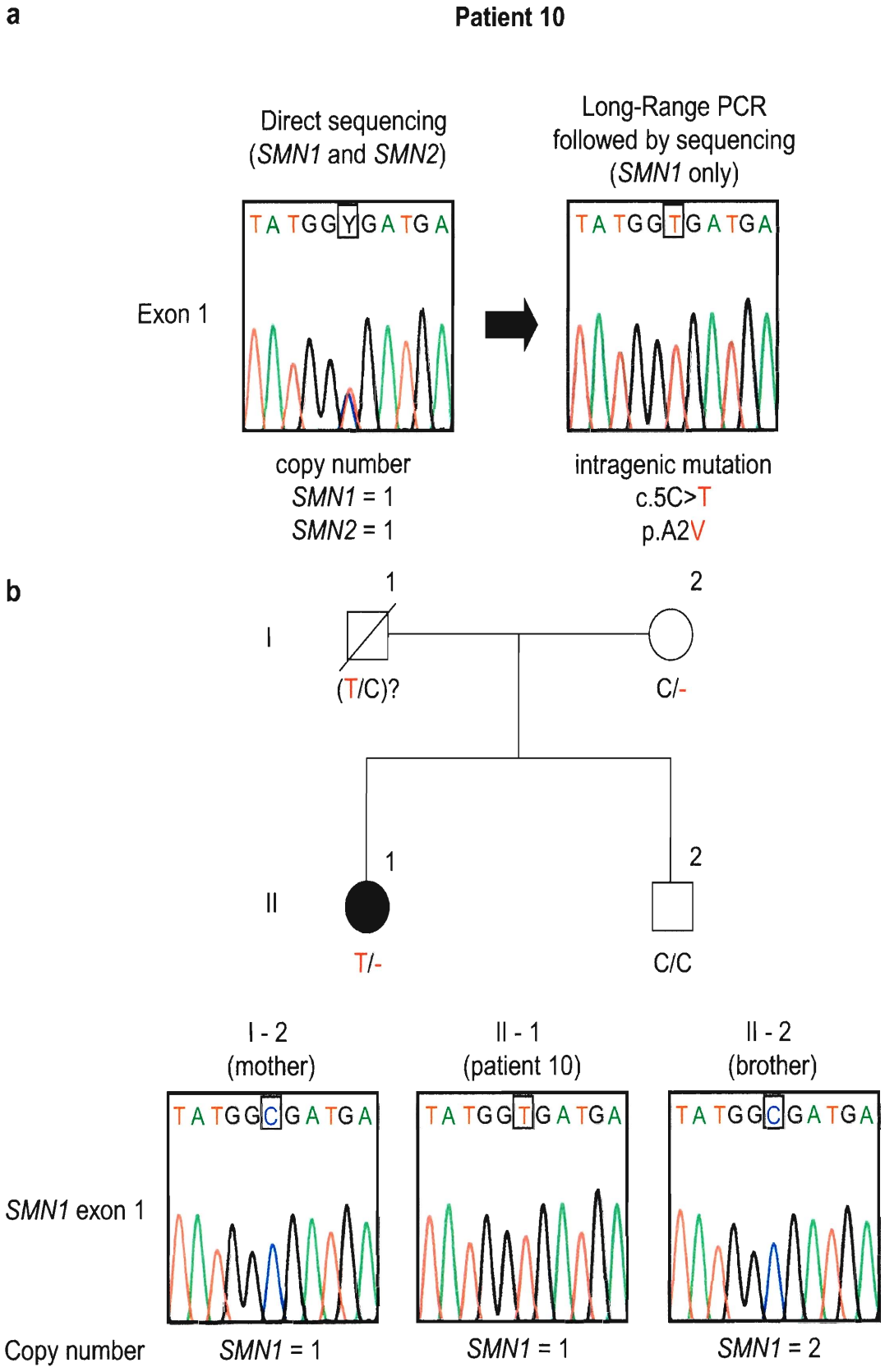
a
Patient 10
Direct sequencing
(SMN1 and SMN2)
Long-Range PCR
followed by sequencing
(SMN1 only)
Exon 1
TATGGYGATGA
TATGGTGATGA
copy number
SMN1 = 1
SMN2 = 1
intragenic mutation
c.5C>T
p.A2V
b
1
2
I
(T/C)?
C/-
1
2
II
T/-
C/C
I - 2
(mother)
II - 1
(patient 10)
II - 2
(brother)
SMN1 exon 1
TATGGCGATGA
TATGGTGATGA
TATGGCGATGA
Copy number
SMN1 = 1
SMN1 = 1
SMN1 = 2

Figure 4

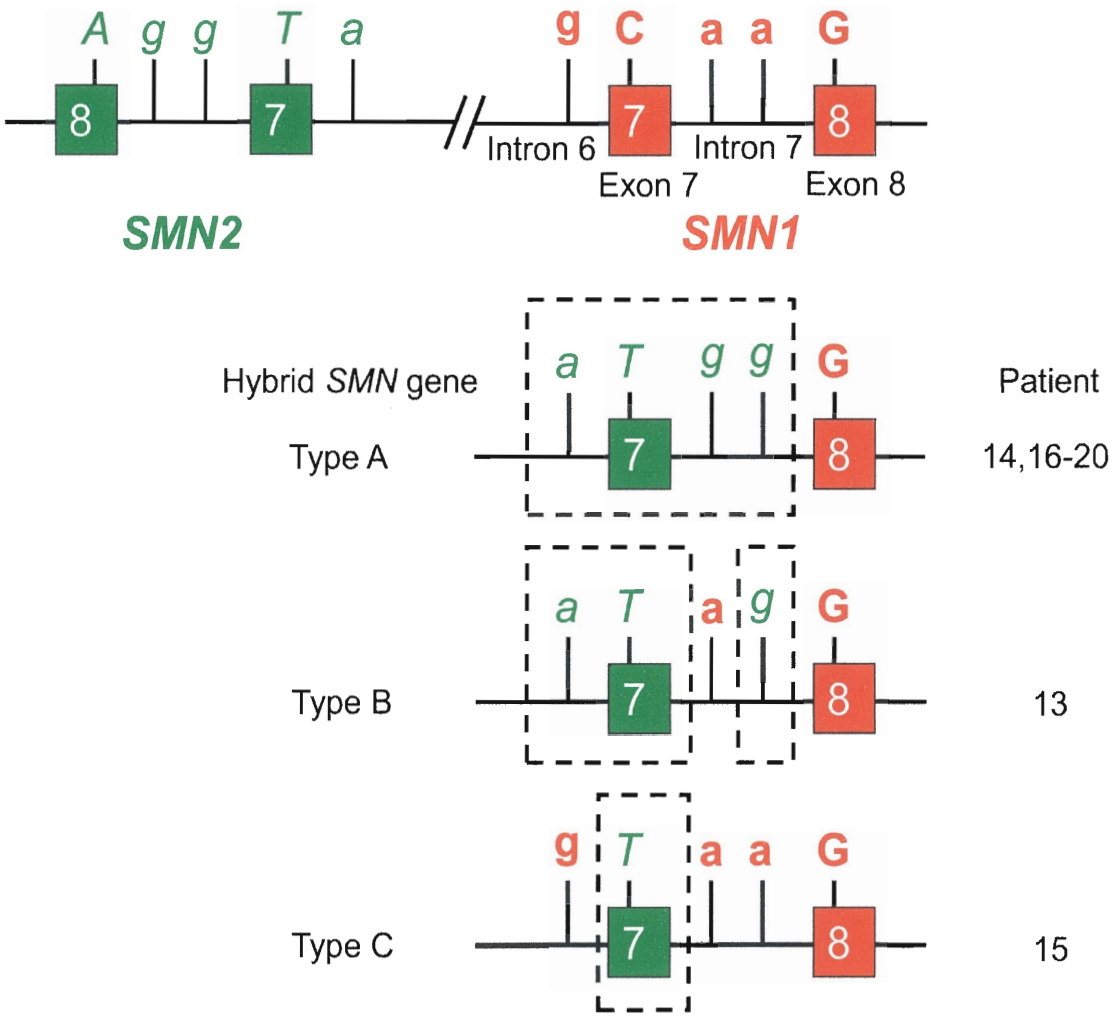
A g g T a
g C a a G
8
7
7
8
Intron 6
Intron 7
Exon 7
Exon 8
SMN2
SMN1
Hybrid SMN gene
Patient
Type A
a T g g G
14,16-20
Type B
a T a g G
13
Type C
g T a a G
15

Figure 5

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]

SMA, spinal muscular atrophy

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*<	Walk	3	2	0	1	*aT***ag****G**	B
14	III	12 m*	Stand	3	2	0	1	*aTgg***G**	A
15	III	8	Walk	3	2	0	1	**g***T***aaG**	C
16	III	14 m*	Stand	3	2	0	1	*aTgg***G**	A
17	III	9 m*	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*.

*We assigned SMA type by giving priority to evaluating each patient's highest function over age of onset.

Supplementary Table 1 Primers used for long-range polymerase chain reaction (PCR) and sequencing

Target region	*Name*	*Primer sequence (5′–3′)*	*PCR product length (bp)*	*Annealing temp (°C)*
Exon1(−654)–8	SMN_FL_(ex1-654)_F	GTTGGGGGATCAAATATCTTCTAGTGTT	28,233	step down
	SMN_FL_ex8_R	CCCCCACCCCAGTCTTTTACAGATGGT		(71.2→65.2)
Exon 1	SMN-ex1-F	GCGAGGCTCTGTCTCAAAACA	439	68
	SMN-ex1-R	GATCGACTTGATGCTGTCCCGA		
Exon 2a	SMN-ex2a-F	CACATAACCTCTAACCAGGTAA	396	60
	SMN-ex2a-R	GGAGGATATCACCTGATTTAACT		
Exon 2b	SMN-ex2b-F	GGTGTATGATGCCTTTAAGAGCAGTTT	555	60
	SMN-ex2b-R	CTTCTCCCTGCCTTCCATTCACA		
Exon 3	SMN-ex3-F	GCACCATACGCATTTTATCTC	690	60
	SMN-ex3-R	GAAACTTGGCTTTCATTTTCATTC		
Exon 4	SMN-ex4-F	TTCAATTTCTGGAAGCAGAGA	383	60
	SMN-ex4-R	CAAAAGTTTCATGGGAGAGC		
Exon 5	SMN-ex5-F	GACTTCAGGATTTGGTACATGA	354	60
	SMN-ex5-R	CCCAAGGGATGTTCTACAATGAC		
Exon 6	SMN-ex6-F	CAACATAGCAAGACCTCGTCT	431	60

	SMN-ex6-R	TGCAAGAGTAATTTAAGCCTCAGA		
Exon 7	SMN-ex7-F	GCTCCAGGTCTCAAGTGAT	680	60
	SMN-ex7-R	GTGCAGTATGCCTAGGTTAT		

Supplementary Table 2 *SMN* copy numbers in 10 controls and 20 unrelated SMA patients

			Copy numbers by MLPA					
Case	*type*	*Gender*	*SMN2 E7*	*SMN2 E8*	*SMN1 E7*	*SMN1 E8*	*NAIP E5*	*H4F5*
Patient 1	III	F	3	3	0	0	0	2
Patient 2	III	M	3	3	0	0	1	2
Patient 3	III	F	3	4	0	0	2	2
Patient 4	III	F	4	4	0	0	2	2
Patient 5	III	M	4	4	0	0	2	2
Patient 6	III	F	4	4	0	0	1	2
Patient 7	III	M	4	4	0	0	2	2
Patient 8	III	M	4	4	0	0	1	2
Patient 9	I	M	3	3	1	1	2	2
Patient 10	III	F	1	1	1	1	1	1
Patient 11	III	M	1	1	1	1	1	1
Patient 12	III	F	1	1	1	1	1	1
Patient 13	III	F	3	2	0	1	2	2
Patient 14	III	F	3	2	0	1	2	2

Patient 15	III	M	3	2	0	1	1	2
Patient 16	III	F	3	2	0	1	1	2
Patient 17	III	F	3	2	0	1	2	2
Patient 18	III	M	4	3	0	1	2	2
Patient 19	III	M	4	2	0	2	2	2
Patient 20	IV	M	4	2	0	2	2	2
Control 1	—	F	0	0	2	2	3	1
Control 2	—	F	0	0	2	2	2	1
Control 3	—	M	1	1	2	2	3	2
Control 4	—	F	1	1	2	2	2	2
Control 5	—	M	1	1	2	2	3	2
Control 6	—	F	2	2	2	2	2	2
Control 7	—	M	2	2	2	2	2	2
Control 8	—	F	2	2	2	2	3	2
Family 1-1	—	M	2	2	1	1	1	2
Family 1-2	—	F	2	2	2	2	2	2