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ORIGINAL PAPER

Universal fluorescent multiplex PCR and capillary electrophoresis for evaluation of gene conversion between *SMN1* and *SMN2* in spinal muscular atrophy

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Abstract We have developed a capillary electrophoresis (CE) method with universal fluorescent multiplex PCR to simultaneously detect the *SMN1* and *SMN2* genes in exons 7 and 8. Spinal muscular atrophy (SMA) is a very frequent inherited disease caused by the absence of the *SMN1* gene in approximately 94% of patients. Those patients have deletion of the *SMN1* gene or gene conversion between *SMN1* and *SMN2*. However, most methods only focus on the analysis of whole gene deletion, and ignore gene conversion. Simultaneous quantification of *SMN1* and *SMN2* in exons 7 and 8 is a good strategy for estimating *SMN1* deletion or *SMN1* to *SMN2* gene conversion. This study established a CE separation allowing differentiation of all copy ratios of *SMN1* to *SMN2* in exons 7 and 8. Among 212 detected individuals, there were 23 SMA patients, 45 carriers, and 144 normal subjects. Three individuals had different ratios of *SMN1* to *SMN2* in two exons, including an SMA patient having two *SMN2* copies in exon 7 but one *SMN1* copy in exon 8. This method could provide more information about *SMN1* deletion or *SMN1* to *SMN2* gene conversion for SMA genotyping and diagnosis.

Keywords Gene conversion · Spinal muscular atrophy · *SMN1*/*SMN2* · Universal fluorescent multiplex PCR · Capillary electrophoresis

Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive disorder with a high frequency of one in 10,000 live births, and one in 40 to one in 50 individuals is a carrier. This severe disorder is characterized by the degeneration of α-motor neurons in the anterior horn cells of spinal cord which results in progressive atrophy of proximal muscles, paralysis, respiratory failure, and infant death [1–3]. According to the clinical severity, patients with SMA are classified into four types [4–6]. The SMA-

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determining gene group, survival motor neuron (*SMN*) gene, has been found in a region of chromosome 5q13. Two highly homologous genes, telomeric *SMN* (*SMN1*) and centromeric *SMN* (*SMN2*), have been identified. These two genes possess only two differences in the complementary DNAs: substitution of single nucleotides in exon 7 (c.840 C > T) and exon 8 (G > A). The consequence of these nucleotide substitutions is that lower amounts of full-length transcript are produced from *SMN2* than from *SMN1* [7, 8].

Absence of the *SMN1* gene has been reported in approximately 94% of clinically typical SMA-affected patients and results in the absence of more functional protein [9, 10]. The absence of *SMN1* indicates the deletion of a whole *SMN1* gene or gene conversion between *SMN1* and *SMN2* [10]. There are six possible gene conversions between *SMN1* and *SMN2* genes (as shown in Fig. 1). Some affected individuals might lack both copies of *SMN1* in exon 7, but retain at least one copy of *SMN1* in exon 8 [8, 10]. In some individuals, exon 8 with *SMN1* was found to be physically juxtaposed to exon 7 with *SMN2*, consistent with an *SMN1* to *SMN2* gene conversion event [10, 11]. In addition, gene conversion has also been investigated with a relationship to severity. Previous studies suggest the loss of the *SMN1* gene by either gene deletion resulting in a severe-SMA allele or gene conversion of *SMN1* to *SMN2* producing a mild-SMA allele [12–15]. That emphasizes the potential role of gene conversion in SMA research, which has not been discussed very much. Simultaneous quantification of *SMN1* and *SMN2* genes in exons 7 and 8 is a good strategy to distinguish gene deletion or conversion, but there are some difficulties owing to high similarity between these two genes. Most techniques for diagnosis of SMA in clinical medicine depend on the detection of whole *SMN1* deletion, such as quantitative real-time PCR [1, 16, 17], denatured high-performance liquid chromatography [18–21], and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [22]. Recently, a method utilizing multiplex ligation-dependent probe amplification (MLPA) was used to simultaneously analyze *SMN1* and *SMN2* genes in exons 7 and 8 [23, 24]. However, this method is expensive and time-consuming in hybridizing *SMN1* and *SMN2* genes. Additionally, the specificity of probe hybridization was poor and failed to truly differentiate *SMN1* and *SMN2* peaks. These drawbacks would hinder the diagnosis of SMA in clinical medicine.

Capillary electrophoresis (CE) is a powerful tool for genotyping owing to its high resolution. Up to now, several researchers have quantified the copy numbers of the *SMN* gene without resolution of *SMN1* and *SMN2* by CE [21, 22]. In our previous papers, we established the first CE methods for quantification of *SMN1* and *SMN2* genes in exon 7 [25, 26]. However, the gene conversion cases were not diagnosed by such a method. Therefore, we focused not only on exon 7, but also on exon 8 in this research. One universal fluorescent multiplex PCR was designed to achieve the fluorescent properties of all desired genes with only one "universal FAM primer." If we tried to monitor these two exons simultaneously, we could get information on their copy ratios and gene conversion. This could

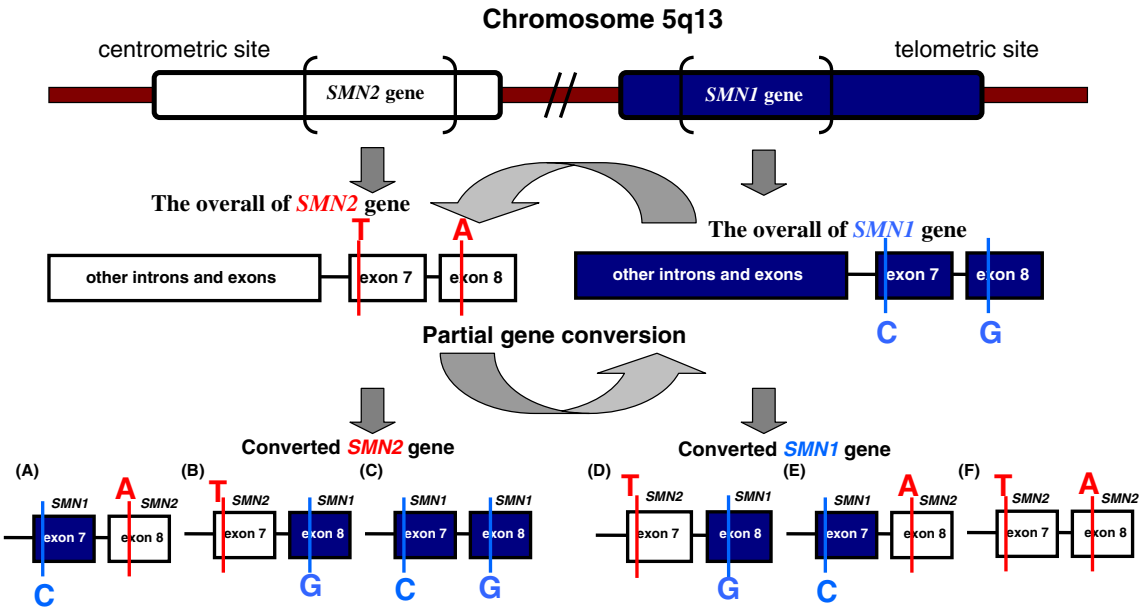

Fig. 1 The six possible gene conversions between *SMN1* and *SMN2* genes in exons 7 and 8. The situation of *A*, *B*, *D*, and *E* would result in different ratios of *SMN1* to *SMN2* in exons 7 and 8, respectively

provide an indication of patients with different gene ratios between exons 7 and 8, and this might have some contribution in aristogenesis. The method did not have to employ probe hybridization [23] or an additional technique such as single base extension [20, 21], and only used PCR products and CE for clinical screening. Meanwhile, the copy numbers and relative ratios of the *SMN1* and *SMN2* genes in exons 7 and 8 could be used for further identifying *SMN1* deletion or *SMN1* to *SMN2* gene conversion in SMA.

Experimental

Chemicals and reagents

Hydroxyethyl cellulose (HEC; 1% in H_2O, about 145 mPa s) was purchased from Fluka Chemie (Buchs, Switzerland). Hydroxypropyl cellulose (HPC; molecular weight about 80,000) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol and urea were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl) aminomethane (Tris)–borate–EDTA (TBE) buffer (5×) was purchased from Protech Technology Enterprise and was diluted to an identical concentration with double-distilled water before use. The double-distilled water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA).

Universal fluorescent multiplex PCR

In this experiment an efficient technique, universal-primer multiplex PCR, was used to simultaneously amplify the fragments of *SMN*, *β-globin*, and *KRIT1* genes (primers shown in Table 1). The amplicons of *β-globin* and *KRIT1* genes were severed as controls for determining the relative gene dose of *SMN1* and *SMN2* in exon 7 as well as exon 8. These primers were designed to have similar melting temperatures and different PCR product lengths, as optimal for the universal-primer multiplex PCR system. The final reaction volume was 25 μL and contained 100 ng of genomic DNA, the proper concentration of each primer (as shown in Table 1), 200 μM dNTPs, 1.0 unit of TaKaRa Taq™ enzyme (TaKaRa Biotechnology), and 2.5 μL of 10× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) as provided by the manufacturer. The PCR amplification was performed in a Px2 thermocycler (Thermo Electron) with an initial denaturing step at 95 °C for 10 min, followed by three cycles of denaturing at 95 °C for 45 s, annealing and elongation at 60 °C for 2 min, and then 25 cycles consisting of denaturation at 95 °C for 45 s, annealing at 50 °C for 90 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. After PCR cycling had been completed, the product was diluted to one quarter concentration by adding 3 vol of double-distilled water, then electrokinetically injected into the capillary, and subsequently analyzed by the CE instrument.

Genomic DNA

DNA samples from SMA patients, carriers, and normal individuals were obtained from one hospital. Genomic DNA was collected from peripheral whole blood using a GFX™ genomic blood DNA purification kit according to

Table 1 The primers used for β-*globin*, *KRIT1*, and *SMN* genes

Gene	Primer name	Sequence (5′-3′)	Primer length	DNA length (bp)	Amount (μM)
SMN (exon 7)	uni-5′-exon7	ATAAGTGACGTACTAGCAACGTCG AACTCCTGAGCTCAGGT	43	540	0.016
	3′-exon7	AAAAGTAAGATTCACTTTCA	20		0.160
SMN (exon 8)	uni-5′-exon8	ATAAGTGACGTACTAGCAACGGAA CATTTAAAAAGTTCAGATGTTA	46	792	0.016
	3′-exon8	TTTAAGACACTCTAACACTT	20		0.240
β-globin	uni-5′-globin	ATAAGTGACGTACTAGCAACGACT GACTCTCTCTGCCTATT	41	260	0.004
	3′-globin	AAGTTCTCAGGATCCACGTG	20		0.040
KRIT1	uni-5′-KRIT1	ATAAGTGACGTACTAGCAACGTTC GAATGGCTACTTCTACCTG	43	343	0.006
	3′-KRIT1	AAAACGTCTTTTAAATCAGAGC	22		0.060
	5′-FAM	FAM-GTGACGTACTAGCAACG	17		0.320

FAM (6-carboxyfluorescein) was the fluorescent dye for laser-induced-fluorescence detection

the instructions. A total of 212 DNA samples were analyzed in this study, including those from 23 patients with SMA, 45 carriers, and 144 subjects from the general population.

CE system

The mutation screening was performed with a Beckman (Fullerton, CA, USA) P/ACE MDQ system equipped with a laser-induced-fluorescence detector (excitation wavelength 488 nm; emission wavelength 520 nm). Separations were performed in a coated DB-17 capillary (Agilent Technologies) with 100-μm internal diameter and 30-cm effective length. A detection window was fabricated 10.2 cm from the outlet. A new capillary was preconditioned with methanol for 10 min and double-distilled water for 20 min; before the first run, a 50-min rinse with the polymer mixture was performed, with a 10-min separation buffer rinse before subsequent injections. After the final injection, the polymer matrix was washed out by methanol and double-distilled water. Sample injection was carried out at 10 kV (250 V/cm) with the anode at the outlet for 30 s, then the separation was accomplished at 8 kV (200 V/cm) in reverse-polarity mode. The temperature was normally set at 15 °C, but the effect of the capillary temperature on the separation was also investigated as reported in "Results and discussion."

To enhance the separation efficiency, we used a polymer mixture as the separation matrix. This gel mixture of HEC/HPC was prepared in 1.5× TBE buffer at designated concentrations. For the purpose of comparison, pure HEC and HPC were also prepared under the same conditions. In this study, identical amounts of urea were added to each gel solution to magnify the variation of DNA conformation that allowed separation of the single nucleotide polymorphism directly from the PCR amplified products.

Results and discussion

Universal fluorescent multiplex PCR

To reduce the time and cost for the synthesis of each fluorescent primer, one "universal fluorescent multiplex PCR" [26] was applied to label all desired genes with fluorescent sensitivity (as shown in Table 1). In such a way, we simultaneously amplified the β-*globin*, *KRIT1*, and *SMN* genes in exons 7 and 8. Both β-*globin* and *KRIT1* were severed as controls to quantify *SMN1* and *SMN2* genes in exons 7 and 8. All fluorescent gene fragments could be obtained using one "universal FAM primer." The final products could be directly analyzed by our CE method without probe hybridization [23] or an additional technique such as single base extension [20, 21]. In addition, the factors affecting the universal multiplex PCR were also examined and validated. The optimal condition was determined to be with the minimum amount of forward specific primer containing a universal section and a large difference in the annealing temperatures between the first and second PCR cycles (50–60 °C).

CE method optimization

The polymer is a very significant element for DNA analysis in CE. To obtain the best separation efficiency, the choice of polymer was investigated. In our previous research [25, 26], we found a copolymer solution could be more efficient than a single polymer for analysis of the *SMN* genes. This was demonstrated by combining two or more polymers to form new separation matrices, which provided quasi-interpenetrating networks and showed better results [27–30]. Therefore, we utilized a polymer mixture of HEC (1.5%) and HPC (2.0%) and successfully resolved *SMN1* and *SMN*2 genes in the exon 7 and 8 fragments in this study (Fig. 2a). The polymer mixture was compared with the

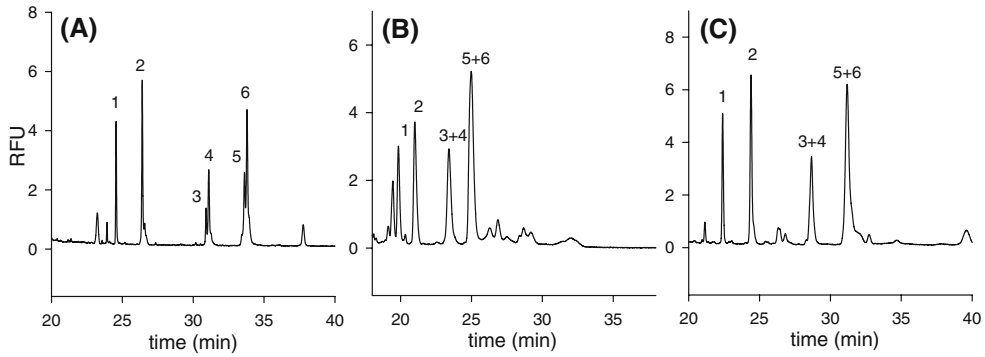

Fig. 2 Electropherograms of a sample with an *SMN1 to SMN2* ratio of 1:2 in exons 7 and 8 using different separation matrices in 1.5× tris (hydroxymethyl)aminomethane–borate–EDTA buffer: **a** 1.5% hydroxyethyl cellulose (HEC) + 2.0% hydroxypropyl cellulose (HPC) + 5 M urea; **b** 2.0% HPC + 5 M urea; **c** 1.5% HEC + 5 M urea. Peaks: *1*, β-*globin*; *2*, *KRIT1*; *3*, *SMN2* (exon 7); *4*, *SMN1* (exon 7); *5*, *SMN2* (exon 8); *6*, *SMN1* (exon 8). The conditions were as follows: sample loading, -10 kV (-250 V/cm) for 30 s; separation voltage, -8 kV (-200 V/cm); capillary temperature, 15 °C; DB-17 coated capillary, 30.0 cm (effective length) × 100-μm inner diameter

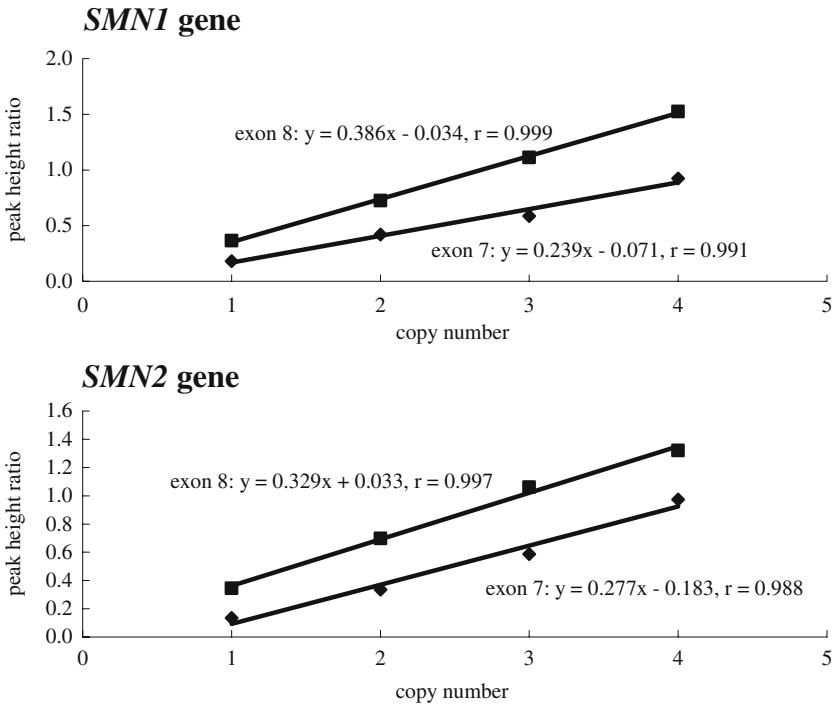

Fig. 3 Calibration curves of peak height ratios versus copy numbers of *SMN1* and *SMN2* in exons 7 and 8

single polymers, 2.0% HPC or 1.5% HEC, for separation of the same DNA sample. The *SMN1* and *SMN2* genes in exons 7 and 8 were coupled (Fig. 2b, c). Finally, the mixture solution of HEC (1.5%) and HPC (2.0%) was selected as the separation medium.

DNA can be denatured at higher temperature [31, 32] and the temperature changes also altered the viscosity of the polymer sieving matrix solution. For these reasons, the effect of temperature on this separation method was investigated. The DNA separation was performed at 15, 20, 25, and 30 °C to determine the effect on resolution. The resolution was calculated by Eq. 1:

$$\mathrm{Rs} = 2(t_2 - t_1)/(W_1 + W_2), \quad (1)$$

where t is migration time and W is the peak width. At 25 and 30 °C, the separations of *SMN1* and *SMN2* genes in exons 7

Table 2 The different *SMN1* to *SMN2* gene ratios in exons 7 and 8 among apparently healthy individuals, carriers, and spinal muscular atrophy (*SMA*) patients

Group	*SMN1*:*SMN2* (exon7)	*SMN1*:*SMN2* (exon8)	Number of subjects
SMA	0:2	0:2	6
	0:2[a]	1:1[a]	1[a]
	0:3	0:3	11
	0:4	0:4	5
Carrier	1:1	1:1	5
	1:2	1:2	24
	1:3	1:3	13
	1:4	1:4	3
Normal controls	2:0	2:0	3
	2:0[a]	1:1[a]	1[a]
	3:0	3:0	3
	4:0	4:0	2
	3:1	3:1	6
	2:1	2:1	44
	2:1[a]	1:2[a]	1[a]
	2:2	2:2	83
	3:2	3:2	1
Total			212

[a] The ratios of *SMN1* to *SMN2* in exon 7 were different from those in exon 8.

and 8 were poor (Rs=0). The resolution was better at lower temperature (Rs=0.44 in exon 7 and Rs=0.34 in exon 8 at 20 °C; Rs=0.60 in exon 7 and Rs=0.43 in exon 8 at 15 °C). At lower temperature, the mesh size became smaller, as demonstrated by previous research in the presence of HEC and HPC, resulting in the *SMN1* and *SMN2* genes being well separated [29]. The temperature was set at 15 °C.

A higher voltage could be used in CE than in slab gel electrophoresis, which resulted in its better performance, speed, and high efficiency. The higher voltage makes the DNA sample move rapidly; however, the resolution might become poor. Several electric field strengths (-100, -150, -200, and -250 V/cm) were investigated. *SMN1* and *SMN2* fragments could be resolved at four applied voltages. When -100 V/cm was applied, 80 min was needed to finish the separation. Considering the speed and current, we set the voltage at -200 V/cm.

The reproducibility of this CE method was determined by analysis of the migration time in five runs. The means of the migration time of β-*globin*, *KRIT1*, *SMN1* (exon 7), *SMN2* (exon 7), *SMN1* (exon 8), and *SMN1* (exon 8) were 24.54±0.22, 26.45±0.23, 31.25±0.29, 31.06±0.27, 34.06±0.36, and 33.87±0.35 min, respectively, for five successive runs. All the relative standard deviations were below 1.1%, which demonstrated that this CE system could provide good reproducibility.

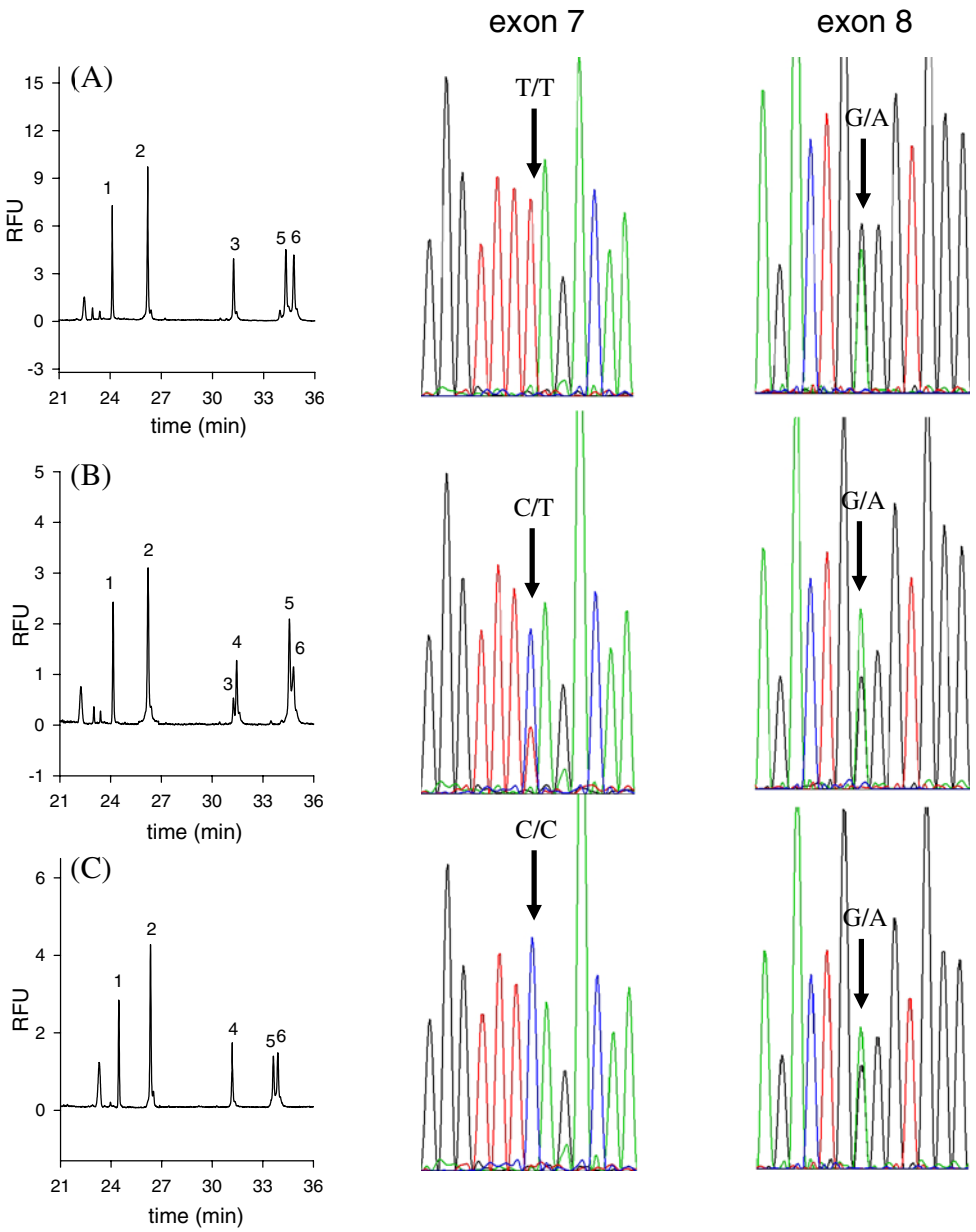

Fig. 4 Electropherograms and sequencing analysis of three individuals with different ratios of *SMN*1 to *SMN2* in the two exons: **a** spinal muscular atrophy (SMA) patient with *SMN1* to *SMN2* ratios of 0:2 in exon 7 and 1:1 in exon 8; **b** a normal control with *SMN1* to *SMN2* ratios of 2:1 in exon 7 and 1:2 in exon 8; **c** a normal control with *SMN1* to *SMN2* ratios of 2:0 in exon 7 and 1:1 in exon 8. All of the sequencing data were matched to those in the electrophoregrams. Peaks: *1*, β-*globin*; *2*, *KRIT1*; *3*, *SMN2* (exon 7); *4*, *SMN1* (exon 7); *5*, *SMN2* (exon 8); *6*, *SMN1* (exon 8)

Quantification of *SMN1* and *SMN2* in exons 7 and 8

Quantification of the *SMN1* to *SMN2* gene ratio is a powerful tool for diagnosis of SMA or related carriers. In previous study [25], we utilized a multiplex PCR method to amplify genes in exon 7. However, it was observed that in some SMA patients *SMN1* was absent from exon 7 but that they retained at least one copy of *SMN1* in exon 8 [8, 10], which could not be detected only by analyzing *SMN1* and *SMN2* in exon 7. Here we developed a method with universal fluorescent multiplex PCR for simultaneous quantification of *SMN1* and *SMN2* in exons 7 and 8 to identify copy numbers and gene conversion between them.

The copy numbers of the *SMN1* and *SMN2* genes in exon 7 as well as exon 8 were obtained by comparison of the peak heights of the β-*globin* and *KRIT1* genes. The calibration curves were established using standards of known-copy-number *SMN* genes (as shown in Fig. 3). The correlation coefficients of all resolved genes were above 0.988. In this investigation, the CE separation allowed differentiation of all ratios of *SMN1* to *SMN2* from 4:0 to 0:4 (data not shown). Among all 212 detected individuals, there were 23 patients with SMA, 45 carriers, and 144 normal controls. The distribution of the copy numbers of *SMN1* and *SMN2* genes in exons 7 and 8 is as shown in Table 2. In the SMA patient group, five of 24

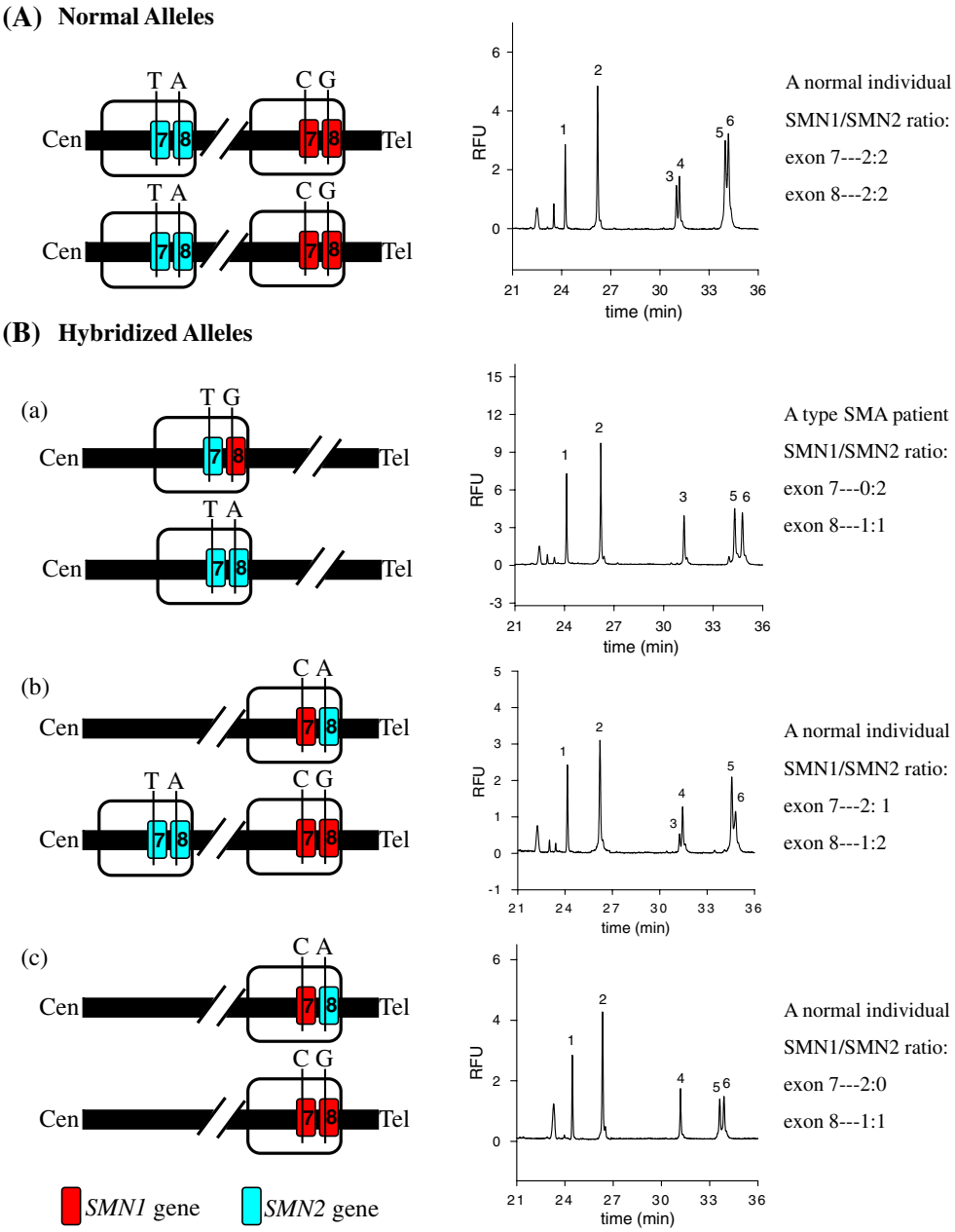

Fig. 5 Normal and hybridized alleles. **a** The normal alleles with a ratio of *SMN1* to *SMN2* of 2:2 in exons 7 and 8. One copy of the *SMN1* and *SMN2* genes is located in telomeric and centromeric sites of a single allele, respectively, without gene conversion. **b** Three subjects with hybridized alleles of *a* a type 1 SMA patient with an *SMN1* to *SMN2* ratio of 0:2 in exon 7 but 1:1 in exon 8, and a gene conversion from *SMN2* to *SMN1* in exon 8, which is identical to the results in previous research [8, 10]; *b* a normal individual with an *SMN1* to *SMN2* ratio of 2:1 in exon 7 but 1:2 in exon 8, suggestive of a hybrid *SMN* gene in exon 8 (from *SMN2* to *SMN1*); and *c* a normal individual with an *SMN1* to *SMN2* ratio of 2:0 in exon 7 and 1:1 in exon 8. Peaks: *1*, β-*globin*; *2*, *KRIT1*; *3*, *SMN2* (exon 7); *4*, *SMN1* (exon 7); *5*, *SMN2* (exon 8); *6*, *SMN1* (exon 8)

patients had an *SMN1* to *SMN2* ratio of 0:4 in exons 7 and 8, 11 had a 0:3 ratio, and six had a ratio of 0:2. However, there was a special individual, type 1 SMA patient, with gene conversion between *SMN1* and *SMN2*. This subject has an *SMN1* to *SMN2* ratio of 0:2 in exon 7, but 1:1 in exon 8. Among the carriers, five of the 45 carriers had a ratio of 1:1, 24 had a 1:2 ratio, 13 had a 1:3 ratio, and three were determined to have a ratio of 1:4. For the 144 healthy individuals, three, three, two, six, 44, 83, and one had ratios of 2:0, 3:0, 4:0, 3:1, 2:1, 2:2, and 3:2, respectively. Gene conversion should be related to disease. Among the normal controls, two individuals were also found to have a gene hybrid in exons 7 and 8. One had an *SMN1* to *SMN2* ratio of 2:1 in exon 7 and a ratio of 1:2 in exon 8. The other had a ratio of 2:0 in exon 7 but 1:1 in exon 8. In the 212 genomic DNA samples, three cases were discovered with the occurrence of gene conversion (1.42%). These findings were further confirmed by sequencing analysis (as shown in Fig. 4). Most important of all, a type 1 SMA individual lacking copies of *SMN1* in exon 7 but retaining one copy of *SMN1* in exon 8 was detected; this finding is similar to results obtained in previous studies [8, 10].

Individuals with gene conversion

SMA is associated with homozygous mutations lacking both copies of the *SMN1* gene, which can indicate a whole *SMN1* deletion or gene conversions between *SMN1* and *SMN2*. Gene conversions have been found in some cases, and the syndrome was milder than for whole gene deletion [12–15]. Hence, the detection of gene conversion is an important issue in the diagnosis of SMA. The C to T conversion in exon 7 from *SMN1* to *SMN2* causes *SMN1* to lose its essential function. Contrarily, the *SMN2* conversion in exon 7 to *SMN1* could serve as a therapeutic strategy for SMA [33]. The conversion of *SMN1* and *SMN2* genes occurring in exon 8 will not influence the production of protein owing to the location of a stop codon at the end of exon 7. These gene conversions would result in different ratios of *SMN1* to *SMN2* in exons 7 and 8, so it is worthwhile determining subjects with these hybrid gene conversions and confirming the gene ratio of exon 7. Therefore, simultaneous quantification of *SMN1* and *SMN2* gene copy numbers in exons 7 and 8 could provide evidence of gene conversion as well as a much needed vision of the molecular basis of the genotype/phenotype relationship in SMA.

The normal alleles with a ratio of *SMN1* to *SMN2* of 2:2 were as shown in Fig. 5a, where one copy of the *SMN1* and *SMN2* genes is often located in telomeric and centromeric sites, respectively, of a single allele. In this study, we found three special individuals had gene conversion of *SMN1* to *SMN2* within exons 7 and 8 (Fig. 5b). One of these was a type 1 SMA patient having an *SMN1* to *SMN2* ratio of 0:2 in exon 7 but 1:1 in exon 8, and we suggest there was a conversion from *SMN2* to *SMN1* in exon 8; this finding is as in previous studies [8, 10]. Nevertheless, the possibility of the conversion from *SMN1* to *SMN2* in exon 7 existed. The other two subjects were normal controls with an *SMN1* to *SMN2* ratio of 2:1 in exon 7 but 1:2 in exon 8, and 2:0 in exon 7 but 1:1 in exon 8, respectively. There might also be a hybrid *SMN* gene from *SMN2* to *SMN1* in exon 8. However, the location of the gene conversion was a conjecture, because the simultaneous analysis of *SMN1* and *SMN2* genes in exons 7 and 8 was only to determine the occurrence of gene conversion.

Conclusions

We developed a novel CE method combined with a universal multiplex PCR for simultaneous quantification of *SMN1* and *SMN2* in exons 7 and 8. Until now, only the MLPA method has been used to do this [23, 24]. However, MLPA is expensive and time-consuming in hybridizing *SMN1* and *SMN2* genes. In addition, the specificity of probe hybridization is poor. Therefore, we established a much simpler and more convenient CE method that could be directly utilized to analyze PCR products without an additional technique, such as single base extension or probe hybridization. The method was used to accurately quantify the copy numbers of *SMN1* and *SMN2* in exons 7 and 8, and also to judge the gene conversion between these two genes. This method could be used for identification of SMA patients, carriers, and normal subjects.

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