Swoboda et al., 2009; Tiziano et al., 2010; Kissel et al., 2011). However, all clinical trials reported so far failed to show significant effectiveness of the therapeutic approaches, which may indicate the difficulties of designing clinical trials for this disorder. An adequate design should take into account the rarity of the patients, clinical disease heterogeneity (subtypes, onset age, sex, stage of disease progress, timing of enrollment, and intervention relative to disease progression), treatment plans (selection of the drug with possible ameliorating effects on the clinical symptoms, sufficient dose, and duration to see some measurable effects) and outcome measures [laboratory biomarkers including *SMN* transcript and SMN protein amounts, muscle mass and strength, motor function testing, respiratory function testing, MUNE, questionnaires for quality of life (QOL)] (Swoboda et al., 2007; Kissel et al., 2011). Clinical endpoints, i.e., the target outcome of the clinical trials: such as extension of the survival period in the patients with SMA type 1 (which will be discussed again below), improvement of motor function in the patients with SMA type 2, and extension of the walking period in the patients with SMA type 3, need to be specified. However, great subtlety may be required for the accurate evaluation of these outcomes. Even if a therapeutic approach could ameliorate the symptoms in some patients, these outcomes may not be detected if the trials are not adequately designed.

To address the challenges due to the rarity of SMA, Mercuri's group (2012) called for clinical trials to be carried out as large multicenter international trials. Such large-scale collaborations would increase the numbers of patients enrolled and would enable randomized placebo studies to be carried out. This approach could also overcome the problems due to clinical heterogeneity as a stratification method could be used to provide a fair evaluation of the treatments (Mercuri et al., 2012).

The selection of appropriate outcome measures to test the efficacy of a therapy remains one of the most difficult problems to be resolved. As for laboratory biomarkers, only *SMN* transcript or SMN protein levels have been established. However, determination of *SMN* transcript or SMN protein levels may not be enough, because these cannot be used to evaluate treatments targeting biochemical reactions downstream of SMN-related signaling (Crawford et al., 2012). Recently, metabolomics studies have suggested that some proteins and metabolites can be used as laboratory biomarkers to reflect responsiveness to treatment (Finkel et al., 2012). Further studies are still required for future clinical usage.

The Hammersmith Functional Motor Scale (HFMS) (Main et al., 2003), Modified HFMS (MHFMS) (Krosschell et al., 2006), and gross motor function measure (GMFM) (Nelson et al., 2006) have been established as standard measures of functional ability in children with SMA types 2 and 3 for use in longitudinal multicenter clinical trials. The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) may also be used for the evaluation of children with SMA type 1 (Glanzman et al., 2010). However, it is difficult to evaluate the actual change in motor scales in SMA patients with any motor function measurements. Thus, it is necessary for investigators in multicenter networks to share the test skills and scoring criteria in order to improve inter-rater reliability and objectivity. For that purpose, training of test skills and collaboration in the scoring criteria should be implemented across centers with different expertise (Mercuri et al., 2012).

In an SMA mouse model, extension of lifespan has been considered to reflect the effectiveness of therapeutic approaches. However, lifespan cannot be simply applied to evaluate the therapeutic approaches in human SMA patients because not only the administered therapy, but the type of supportive care including respiratory management can also change the lifespan of patients. In addition, the use of an artificial respirator in SMA type 1 management is still controversial. Such differences in clinical care may hamper simple comparison using lifespan outcomes in international clinical trials. The occurrence of death and the requirement for an artificial respirator may be considered as equivalent events when evaluating the efficacy of clinical trials in patients with SMA type 1 because improvement of motor scale cannot be expected from these patients (Oskoui et al., 2007; Mercuri et al., 2012). Currently, using lifespan as the only available outcome measure, is not ideal anymore. If it is possible to measure improvements in respiratory function or restoration of motor function, alternative outcome measures for SMA type 1 may become achievable. Highly effective therapies which will improve motor scale of patients with SMA type 1 can then be sought.

Conclusions

SMA is an incurable motor neuron disease with autosomal recessive inheritance. Molecular biology studies of SMA have been greatly advanced in two directions, namely diagnostic applications and pathophysiological studies, since the discovery of the SMN genes in 1995. Molecular diagnostics has enabled us not only to diagnose SMA in patients, but has also provided the ability to carry out carrier and newborn screening of SMA for populations. Pathophysiological studies have provided an improved understanding of the underlying pathogenesis of SMA, including alternative splicing of *SMN2*, aberrant splicing due to the defect of snRNPs, impairment of motor circuit formation and/or NMJ development, and dysregulation of cytoskeleton dynamics. To date, there has been no successful therapy for SMA, but an in-depth understanding of the pathophysiology underlying the disease

can offer useful insights for development of effective treatment approaches. Some therapeutic strategies have already been devised based on current pathophysiological knowledge of the disease, namely *SMN2*-targeting, *SMN1*-introduction and non-*SMN* targeting strategies. With multiple approaches in therapeutic strategies for SMA being pursued, some of which are already in clinical trials, it is expected that some candidate compounds may emerge as potential therapeutic agents in the near future. These exciting developments offer promising outcomes for SMA patients in overcoming this debilitating disease.

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Original article

Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy (SMA) patients

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Abstract

Background: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by deletion or intragenic mutation of *SMN1*. SMA is classified into several subtypes based on clinical severity. It has been reported that the copy number of *SMN2*, a highly homologous gene to *SMN1*, is associated with clinical severity among SMA patients with homozygous deletion of *SMN1*. The purpose of this study was to clarify the genotype-phenotype relationship among the patients without homozygous deletion of *SMN1*. *Methods:* We performed molecular genetic analyses of *SMN1* and *SMN2* in 112 Japanese patients diagnosed as having SMA based on the clinical findings. For the patients retaining *SMN1*, the PCR or RT-PCR products of *SMN1* were sequenced to identify the mutation. *Results:* Out of the 112 patients, 106 patients were homozygous for deletion of *SMN1*, and six patients were compound heterozygous for deletion of one *SMN1* allele and intragenic mutation in the retained *SMN1* allele. Four intragenic mutations were identified in the six patients: p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys. To the best of our knowledge, all mutations except p.Trp92Ser were novel mutations which had never been previously reported. According to our observation, clinical severity of the six patients was determined by the type and location of the mutation rather than *SMN2* copy number. *Conclusion: SMN2* copy number is not always associated with clinical severity of SMA patients, especially SMA patients retaining one *SMN1* allele.

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1. Introduction

Spinal muscular atrophy (SMA) is a common neuromuscular disease characterized by degeneration of lower motor neurons, leading to the axial and limb weakness associated with muscle atrophy. The incidence of the disease has been estimated at 1 in 10,000 newborns, with an expected carrier frequency of 1 in 50 [1]. Based on molecular epidemiological analysis using *SMN1* copy number, the worldwide carrier frequency of SMA is 1 in 40–70, suggesting a disease incidence of 1 in 6000–20,000 [2].

SMA is classified into four subtypes depending on the age of disease onset and the achievement of motor milestones [3]: namely, type 1 (severe form; onset age of 0–6 months old, unable to sit unaided), type 2 (intermediate form; onset age of <18 months old, unable to stand or walk unaided), type 3 (mild form; onset age of >18 months old, able to stand or walk unaided), and type 4 (milder form; onset age of >21 years old, able to stand or walk unaided).

All SMA subtypes have been mapped to chromosomal region 5q11.2–13.3 [4–7] and the survival motor neuron gene (*SMN*) and neuronal apoptosis-inhibitory protein gene (*NAIP*) were cloned as SMA-causing gene candidates [8,9]. The *SMN* gene exists as two highly homologous copies, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [8]. It is now established that SMA is caused by deletions or intragenic mutations of *SMN1*. *SMN1* is homozygously deleted in more than 90% of SMA patients [8,10], and deleteriously mutated in the remaining patients [8,11]. On the other hand, *NAIP*-deletion has been found only in 50% of type 1 patients, and much less frequently in type 2 and 3 patients. The presence or absence of *NAIP* may be associated with the clinical severity of SMA [9,10].

Increased *SMN2* copy number is related to improved survival outcomes and maintenance of motor function [12–16]. Both *SMN* genes, *SMN1* and *SMN2*, differ by only five nucleotides [8]. Of the five nucleotide differences between the two *SMN* genes, only one is present in the coding region at position +6 of exon 7 in *SMN1* (c.840C) and *SMN2* (c.840T). Although this mutation is translationally silent, the C-to-T transition alters the splicing pattern in *SMN2* exon 7 [17]. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% of exon7-lacking (△7) *SMN2* transcripts and ~10% of FL-*SMN2* transcripts [18]. It is expected that high *SMN2* copy number may produce a large amount of FL-*SMN2* to compensate for the loss of *SMN1* to some degree.

However, most phenotype-genotype correlation studies have been conducted only in SMA patients with a complete loss of *SMN1*. The relationship between *SMN2* copy number and clinical severity are yet to be clarified in SMA patients retaining one *SMN1* allele. In this study, to understand the modifying factors in determining the clinical phenotype of SMA patients retaining one *SMN1* allele, we conducted a mutation analysis and investigated the contribution of *SMN2* copy number to the clinical severity in such patients.

2. Patients and methods

2.1. Patients

All 112 Japanese patients (51 males and 61 females) fulfilled the diagnostic criteria defined by the International SMA Consortium [19]. Here, patients with onset before 20 years old was classified into type 3, and those with onset after 21 years old was classified into type 4 [3]. Informed consent was obtained from these patients and/or their parents. This study project including genetic analysis was approved by the Ethical Committee of the Kobe University Graduate School of Medicine, Japan.

In this study, six patients (Patients 1–6) retaining one allele of *SMN1* exon 7, were found to carry intragenic mutations in *SMN1*. Patients 1 (female) and 2 (male) were type 1 patients reported previously to have one *SMN1* allele [20]. Patient 3 was a 19-day-old male with SMA type 1, referred to us because of respiratory insufficiency and swallowing difficulties. Patient 4 was a 7-year-old female with type 2 SMA. She was first diagnosed as having SMA type 2 close to type 3 because she could sit unaided and stand while holding onto something (such as a wall or table) for support. However, she rapidly lost such abilities at 2 years old. Finally, she was bound to artificial ventilator because of respiratory insufficiency at 3 years old. Patient 5 was a 13-year-old male with type 3 SMA, who had pain and heaviness in legs during exercise since the age of 11 years. He later developed symptoms including waddling gait, muscle weakness and atrophy in quadriceps, and attenuated patellar tendon reflex. Patient 6 was a 19-year-old female with type 3 SMA, who had noticed muscle weakness during swimming exercise at the age of 13 years. She gradually lost her running ability and could no

longer run as fast as the other classmates in her high school days.

2.2. SMN and NAIP deletion test

Genomic DNA was extracted from 3 ml of whole blood using a DNA extraction kit, SepaGene (Sanko Junyaku, Tokyo, Japan). For the *SMN* and *NAIP* deletion test, PCR and enzyme digestion reactions were performed according to the method of van der Steege et al. [21]. Exon 5 of the *NAIP* gene was detected using the PCR method of Roy et al. [9]. Here we adopted "exon 5" as a widely accepted exon number, although this exon has been denoted as "exon 4" by Chen et al. [22].

2.3. Copy number analysis of the SMN genes using real time PCR method

We determined the copy numbers of the *SMN* genes based on the real-time PCR method of Tran et al. [23]. Cystic fibrosis trans-membrane regulator gene (*CFTR* gene) was used as a reference gene for the relative quantification of copy numbers.

2.4. Messenger RNA analysis

For the assignment of the mutation to *SMN1* or *SMN2*, mRNA analysis was performed. Total RNA was extracted from leukocytes using the acid guanidiumthiocyanate-phenol–chloroform method. *SMN1* and *SMN2* mRNA species were amplified by reverse transcriptase (RT)-PCR method [16,24]. A new primer, ex1-F (5′-TGC GCA CCC GCG GGT TTG CT-3′), was designed for this study. The mRNA species encompassing exons 1–8 were amplified using primers ex1-F and 541C1120 [8], and the mRNA species encompassing exons 1–7 were amplified using primers ex1-F and 541C770 [8].

2.5. Nucleotide sequencing

The amplified PCR or RT-PCR products of *SMN* exons were purified and sequenced directly or after subcloning. The sequencing reaction was performed using a dye terminator cycle-sequencing kit (Life Technologies Corporation, Carlsbad, CA). The reaction product was electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA).

2.6. Computational algorithms

We predicted the mutation effects on the protein function using three computational algorithms: Sorting Intolerant from Tolerant amino acid substitutions (SIFT) [25], Polymorphism Phenotyping-2 (PolyPhen-2) [26], and Grantham score difference (Align-GVGD) [27].

2.7. Statistics

The correlation of copy number of *SMN2* with the clinical subtypes was compared by chi-square test and *t*-test. *P*-value of less than 0.05 was considered to indicate a significant difference. The software used for statistical analysis was Statistical Program for Social Science (SPSS) Version 16 (IBM Corporation, Paulo Alto, US).

3. Results

3.1. SMN1 and NAIP deletion test

SMN1 exon 7-deletion (herein after referred to as *SMN1*-deletion) was found in almost all SMA patients, regardless of clinical subtypes: 106 out of 112 (95%) patients with SMA in this study had *SMN1*-deletion and 6 patients (5%) had subtle mutations in *SMN1*. Out of 106 *SMN1*-deleted patients, 48 (45%) were type 1, 35 (33%) were type 2, 19 (18%) were type 3, and 4 (4%) were type 4 (Table 1).

In our study, 96 of 106 (91%) *SMN1*-deleted patients had deletion of *SMN1* exon 8. However, the other 10 patients (9.0%) retained *SMN1* exon 8. We confirmed that these patients had at least one copy of the hybrid gene with *SMN2* exon 7 and *SMN1* exon 8 using direct sequencing analysis of the PCR fragment amplified with the common primers for *SMN1* and *SMN2*.

NAIP exon 5-deletion (herein after referred to as *NAIP*-deletion) was always accompanied by *SMN1*-deletion (Table 1). In addition, *NAIP*-deletion was much more frequent in SMA type 1 than SMA non-type 1. *NAIP*-deletion was found in 29 out of 48 (60%) patients with *SMN1*-deleted SMA type 1, while it was found in only 8 out of 58 (14%) patients with *SMN1*-deleted SMA types 2, 3 and 4.

3.2. SMN2 copy number and clinical severity in patients with SMN1-deletion

We determined the *SMN2* copy numbers of all the patients enrolled in this study using the real-time PCR method. For the analysis of *SMN2* copy number and clinical severity, the"*SMN2* exon 7-*SMN1* exon 8 hybrid" gene is regarded as *SMN2*.

A significant relationship between *SMN2* copy number and clinical severity was observed in this study (Table 2). 38 out of 48 (79%) patients with *SMN1*-deleted SMA type 1 showed one copy or two copies of *SMN2*, 34 out of 35 (97%) patients with *SMN1*-deleted SMA type 2 showed three copies of *SMN2*, 18 out of 19 (95%) patients with *SMN1*-deleted SMA type 3 showed three or four copies of *SMN2*, and 3 out of 4 (75%) patients with *SMN1*-deleted SMA type 4 showed four copies of *SMN2*.

Table 1
SMN1 and *NAIP* deletion test ($n = 112$).

SMN1		*NAIP*	Type 1	Type 2	Type 3	Type 4	Total
Exon 7	Exon 8	Exon 5					
Del	Del	Del	29	6	1	1	37
Del	Del	Non-del	17	24	15	3	59
Del	Non-del	Non-del	2	5	3	0	10
Non-del	Non-del	Non-del	3	1	2	0	6
Total			51	36	21	4	112

Table 2
Clinical severity and *SMN2* copy number in patients with homozygous *SMN1*-deletion ($n = 106$).

	1 copy	2 copies	3 copies	4 copies	Mean	(SD)
Type 1	1	37	10	0	2.18	(0.44)
Type 2	0	1	34	0	2.97	(0.17)
Type 3	0	1	13	5	3.18	(0.51)
Type 4	0	0	1	3	3.80	(0.40)
Total	1	39	58	8		

Table 3
Clinical severity and *SMN2* copy number in patients retaining one *SMN1* allele ($n = 6$).

	Sex	Onset	Type	*SMN2* copy number	Nucleotide change (exon)	Amino acid change	Domain	References
Patient 1	F	5m	1	3	c.275 G > C (exon 3)	p.Trp92Ser	Tudor	[20]
Patient 2	M	6m	1	3	c.275 G > C (exon 3)	p.Trp92Ser	Tudor	[20]
Patient 3	M	0m	1	2	c.819_820 insT (exon 6)	p.Thr274Tyr fsX32	C-terminal	This study
Patient 4	F	12m	2	1	c.830 A > G (exon 6)	p.Tyr277Cys	C-terminal	This study
Patient 5	M	11y	3	1	c.5 C > T (exon 1)	p.Ala2Val	N-terminal	This study
Patient 6	F	13y	3	1	c.5 C > T (exon 1)	p.Ala2Val	N-terminal	This study

3.3. SMN2 copy number and clinical severity in patients retaining one SMN1 allele

In this study, we identified four different intragenic mutations in *SMN1* of six patients without *SMN1*-deletion (Patients 1–6) (Table 3). All of them were compound heterozygous for deletion of one *SMN1* allele and an intragenic point mutation of the other *SMN1* allele. The intragenic mutations included three missense mutations and one frame-shift mutation: c. 5C > T (p.Ala2Val) in exon 1, c. 275G > C (p.Trp92Ser) in exon 3, c.819_820insT (p.Thr274TyrfsX32) in exon 6, and c.830 A > G (p.Tyr277Cys) in exon 6. Three of the mutations, p.Ala2Val, p.Thr274TyrfsX32 and p.Tyr277Cys, are novel ones which have never been previously reported.

We predicted the effect of the missense mutations on the protein function using three computational algorithms: SIFT [25], PolyPhen-2 [26], and Align-GVGD [27]. All three types of missense mutation were predicted to damage the protein function.

Interestingly, the observed phenotype of patients carrying an intragenic mutation deviated from the expected correlations with the *SMN2* copy number (Table 3 and Fig. 1): type 3 patients with p.Ala2Val (Patients 5 and 6) carried only a single copy of *SMN2*, while type 1 patients with p.Trp92Ser (Patients 1 and 2) carried as many as 3 copies of *SMN2*. These findings suggested that intragenic mutations in *SMN1* influence the clinical phenotype more significantly than *SMN2* copy numbers in some patients.

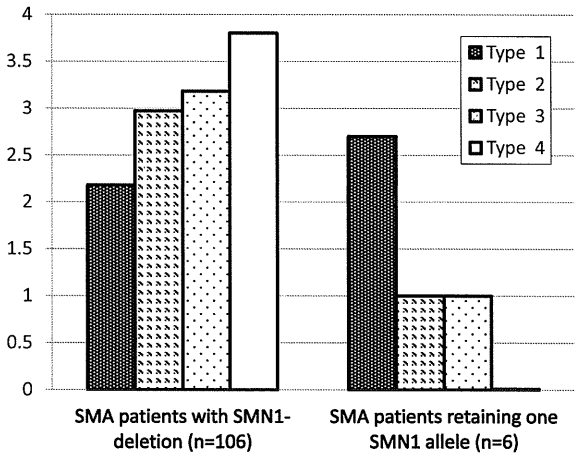

Fig. 1. Mean *SMN2* copy numbers in SMA patients. Patients with *SMN1*-deletion ($n = 106$) carried zero copies of *SMN1*. Patients retaining one *SMN1* allele ($n = 6$) which harbored intragenic mutations: p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys.

4. Discussion

The identification of intragenic mutations, especially missense mutations, may help us to further elucidate the function of SMN and the pathogenic mechanism of SMA. In this study, we identified four different intragenic *SMN1* mutations in six SMA patients without *SMN1*-deletion. These intragenic mutations were p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32, and p.Tyr277Cys.

The p.Ala2Val mutation, which is located in the N-terminal domain, has never been reported until now. Our two patients with p.Ala2Val were unrelated. However, another mutation in the same location, p.Ala2Gly, has previously been reported in three SMA patients; these patients were also unrelated individuals, but had the possibility of sharing an ancestral origin [28]. All patients with p.Ala2Gly carried only one *SMN2* copy, and two of them showed mild phenotype (type 3). The mutation effect of p.Ala2Val, as well as p.Ala2Gly, may be much less deleterious than other missense mutations identified in this study. However, *SMN2* may not be dispensable in these patients. The mild SMA mutation, p.Ala2Gly, by itself cannot rescue $Smn^{-/-}$ mice, suggesting that homomer of the mutant SMN is not functional [29]. According to the Workman et al. [30], the heteromer of mutant SMN and FL-SMN from a single copy of *SMN2* must have some function.

We previously reported the p.Trp92Ser mutation in two unrelated patients [20]. This mutation is located in the Tudor domain to which other proteins bind. [31]. Many of them are involved in small nuclear ribonucleoprotein (snRNP) biogenesis. SMN Tudor domain preferentially binds symmetric dimethylated arginine (sDMA) of Sm proteins which constitute Sm core of snRNP [32]. We have already reported that the binding ability of the mutated SMN with p.Trp92Ser to SmB and fibrillarin was reduced to half of normal levels [20]. Most recently, Tripsianes et al. [33] examined the relationship between mutated Tudor domain and the binding capacity to sDMA *in vitro*. According to them, p.Trp92Ser mutant was unfolded, as judged by fingerprint NMR spectra analysis, and did not bind sDMA [33].

The p.Thr274TyrfsX32 mutation is a frameshift mutation arising from a single nucleotide insertion in exon 6 and results in a truncated SMN protein lacking the C-terminal domain of *SMN*. A new isoform of SMN, axonal SMN (a-SMN), is expected to be produced in the patient, because a-SMN is a truncated, alternatively spliced isoform of *SMN1*, originating from the retention of intron 3 [37,38]. Although the role of a-SMN in the pathogenesis of SMA has not been clarified yet, the disease severity of the patient with this mutation suggests that a-SMN functions were not enough to fully compensate for the deleterious mutation.

The p.Tyr277Cys mutation is located in the C-terminal domain of SMN known as the YG box, which is essential for oligomerization or self-association of SMN [31]. Oligomerization defect destroys the function of SMN and correlates with clinical severity of SMA [34]. Many other mutations in the same domain have been frequently reported [35,36], although the p.Tyr277Cys mutation has not been reported up to now.

An interesting question arises as to which factor contributes more significantly to clinical phenotype in SMA, *SMN1* intragenic mutation or *SMN2* copy number. According to our analysis of the patients with homozygous *SMN1*-deletion (Table 2 and Fig. 1), increased *SMN2* copy number was associated with milder phenotype, which was compatible with previous reports [12–16]. However, the phenotype of patients without *SMN1*-deletion was not related to their *SMN2* copy number (Table 3 and Fig. 1). In our study, p.Ala2Val was found in two type 3 patients with one *SMN2* copy, p.Trp92Ser in two type 1 patients with three *SMN2* copies, p.Thr274TyrfsX32 in one type 1 patient with two *SMN2* copies, and p.Tyr277Cys in one type 2 patients with one *SMN2* copy. According to our findings, *SMN1* intragenic mutations appear to contribute much more significantly to clinical severity than *SMN2* copy numbers in some patients.

Since our patients carry various intragenic *SMN1*mutations, the next question is whether *SMN2* copy number effect is present or absent among the patients with the same *SMN1* mutation. Using the data of the SMA patients with missense mutations described in a review paper of Sun et al. [36], we analyzed the relationship between *SMN2* copy number and clinical severity in eleven patients with p.Tyr272Cys in *SMN1*. We observed that higher *SMN2* copy number was correlated with reduced disease severity: patients with three *SMN2* copies showed milder phenotype than the patients with one *SMN2* copy number. Thus, we speculate that *SMN2* copy number effect is present when the *SMN1* mutation is the same in the patients.

In conclusion, *SMN2* copy number is not always associated with clinical severity of SMA patients, especially SMA patients without *SMN1*-deletion. In these patients, clinical severity in SMA caused by *SMN1* mutations may be determined by the type and location of the intragenic mutation. Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy patients.

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Original article

Trinucleotide insertion in the *SMN2* promoter may not be related to the clinical phenotype of SMA

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Abstract

Background: More than 90% of spinal muscular atrophy (SMA) patients show homozygous deletion of *SMN1* (survival motor neuron 1). They retain *SMN2*, a highly homologous gene to *SMN1*, which may partially compensate for deletion of *SMN1*. Although the promoter sequences of these two genes are almost identical, a GCC insertion polymorphism has been identified at c.-320_-321 in the *SMN1* promoter. We have also found this insertion polymorphism in an *SMN2* promoter in an SMA patient (Patient A) who has SMA type 2/3.

Purpose: The aims of this study were to determine the frequency of the GCC insertion polymorphism in SMA patients, and to evaluate its effect on *SMN* transcription efficiency.

Patients and methods: Fifty-one SMA patients, including Patient A, were involved in this study. *SMN2* transcript levels in white blood cells were measured by real-time polymerase chain reaction. Screening of the GCC insertion polymorphism was performed using denaturing high-pressure liquid chromatography. The transcription efficiency of the promoter with the insertion mutation was evaluated using a reporter-gene assay.

Results: All SMA patients in this study were homozygous for *SMN1* deletion. Patient A retained two copies of *SMN2*, and showed only a small amount of *SMN2* transcript in white blood cells. We detected a GCC insertion polymorphism at c.-320_-321 only in Patient A, and not in 50 other SMA patients. The polymorphism had a slight but significant negative effect on transcription efficiency.

Discussion and conclusion: Patient A was judged to be an exceptional case of SMA, because the GCC insertion polymorphism rarely exists in *SMN1*-deleted SMA patients. The GCC insertion polymorphism did not enhance the transcriptional efficiency of

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SMN2. Thus, this GCC insertion polymorphism in the *SMN2* promoter may not be associated with the milder phenotype of the patient. Patient A suggests that there are other unknown factors modifying the clinical phenotype of SMA.

Keywords: Spinal muscular atrophy; *SMN1*; *SMN2*; Promoter; Polymorphism

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by proximal muscular atrophy of the limbs and trunk, resulting from degeneration of motor neurons in the anterior horn of the spinal cord. The incidence of the disease has been estimated at 1 in 6000–10,000 newborns, with an expected carrier frequency of 1 in 40–50 [1].

SMA is classified into three clinical subtypes depending on the age of disease onset and the achievement of motor milestones [2]: type 1 (severe form, Werdnig-Hoffmann disease; age of onset 0–6 months, unable to sit unaided), type 2 (Dubowitz disease, intermediate form; age of onset <18 months, unable to stand or walk unaided), and type 3 (mild form; Kugelberg–Welander disease; age of onset >18 months, able to stand or walk unaided). Additionally, two other forms of the disease, with the most severe having prenatal onset and the mildest type manifesting after 20 years of age, have been reported as SMA type 0 (prenatal form) and SMA type 4 (adult form) [3].

Using linkage analysis, all clinical subtypes of SMA have been mapped to chromosome 5q11.2–13.3. The survival motor neuron (*SMN*) gene has been identified as a candidate for SMA [4]. *SMN* is in fact two highly homologous genes, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [4]. *SMN1* and *SMN2* encode the same protein; however, *SMN1* is now considered to be responsible for the development of SMA, because its homozygous deletion has been found in >90% of SMA patients, and subtle but deleterious intragenic *SMN1* mutations have been identified in non-deletion patients [4,5]. It has been accepted that *SMN2* may be a modifier gene of SMA. Owing to a single nucleotide difference between *SMN1* and *SMN2*, exon 7 of *SMN2* is alternatively spliced (more precisely, skipped) resulting in the production of an *SMN* transcript lacking exon 7 (Δ7-*SMN* transcript) and an unstable Δ7-SMN protein [6]. The single nucleotide change in *SMN2* exon 7, which is a C-to-T transition located at codon 280, increases Δ7-*SMN* transcript levels and, correspondingly, decreases full-length *SMN* (FL-*SMN*) transcript levels [7]. Even so, *SMN2* is also able to generate a small amount of full-length transcript, and thus it can partially compensate the loss of *SMN1* [8].

Generally, the clinical severity of SMA patients is inversely correlated with *SMN2* copy number. A high copy number of *SMN2* is associated with a milder phenotype, and a low copy number with a more severe phenotype. SMA type 1 patients typically have two copies of *SMN2*, SMA type 2 patients have three copies, and SMA type 3 patients typically have three or more copies [9]. More than four *SMN2* copies are associated with a milder phenotype of SMA type 3 [10]. However, the clinical severity cannot always be determined by the *SMN2* copy number alone.

The expression level of *SMN2* may also be correlated with the clinical severity of the disease and, therefore, analysis of the *SMN2* promoter is important. Echaniz-Laguna et al. and Boda et al. reported that the promoter sequences of *SMN1* and *SMN2* are identical, providing strong evidence for similar transcriptional regulation of these genes [11,12]. However, Monani et al. found more than 10 nucleotide differences between the promoter regions of these two genes [13,14]. One of them, a GCC insertion polymorphism, was specifically identified at c.-320_-321 in the *SMN1* promoter, leading to GCC duplication at c.-324–c.-318. Polymorphisms in the promoter region may have some effect on transcriptional activity.

We found the GCC insertion polymorphism in an *SMN2* promoter in a Japanese boy diagnosed as having SMA type 2/3 (Patient A). The location of the GCC insertion in the *SMN2* promoter in Patient A was corresponding to that of the GCC in the *SMN1* promoter reported by Monani et al. [14]. It is notable that the clinical phenotype of the patient was much milder than expected based on his *SMN2* copy number. In this study, we determined the frequency of the GCC insertion polymorphism in controls and SMA patients. We also evaluated the effect of the GCC insertion polymorphism on *SMN2* transcriptional activity.

2. Patients and methods

2.1. Patients

All 50 Japanese patients in this study fulfilled the diagnostic criteria defined by the 59th ENMC International Workshop [2]; 26 patients (aged 1–34 years) were type 1, 16 type 2, and eight type 3. The molecular genetic analysis was approved by the Ethical Committee of the Kobe University Graduate School of Medicine, Japan. Informed consent was obtained from the patients or their parents. Fifty healthy Japanese adults (aged 21–

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70 years) volunteered to participate in the study as control subjects.

Patient A was a 2-year-old Japanese boy who was clinically suspected as having a neuromuscular disorder with decreased muscle tonus. He was born as the third child to non-consanguineous and healthy parents. The pregnancy and delivery were non-eventful. Early developmental milestones were slightly delayed: head control was obtained at age 6 months, sitting without support at age 8 months, crawling at age 9 months, and standing and walking with support (ex. handrails) at age 18 months. However, he could never walk without support. He uttered his first word at 18 months, and a simple two-word sentence at 22 months. On admission, his weight and height were 85.5 cm (−0.7 SD) and 11.5 kg (−0.9 SD). His mental status was alert. Apparent facial anomaly was absent, but high-arched palate was present. Lung and heart auscultation revealed no abnormal findings. Abdominal examination was normal. Tongue fasciculation was absent. Muscle tonus was decreased: scarf sign, heel-to-ear sign, and loose-shoulder sign were observed. Muscle strength was also decreased especially in the proximal region of the legs. Deep tendon reflexes were absent or extremely diminished. Laboratory examination revealed no muscular damage (AST 28 IU/L, ALT 10 IU/L, CK 119 IU/L, ALD 7 IU/L, lactate 13 mg/dL, pyruvate 0.8 mg/dL). Muscle biopsy findings were compatible with those of SMA. Based on the muscle biopsy findings, together with the clinical phenotype, he was diagnosed as having SMA type 2/3.

2.2. *SMN1* deletion test and *SMN2* gene dosage analysis

Genomic DNA was extracted from peripheral white blood cells. The *SMN1* exon 7 deletion test was performed by the PCR-restriction fragment length polymorphism method of van der Steege et al. [15]. *SMN2* copy numbers were determined with a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the method of Tran et al. [16].

2.3. RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from peripheral white blood cells. cDNA was synthesized from total RNA with Transcriptor Reverse Transcriptase (Roche Diagnostics) according to the manufacturer's instructions.

Quantitative reverse-transcription-PCR was performed with a LightCycler 1.5 instrument (Roche Diagnostics) using FastStart DNA Master SYBR Green I (Roche Diagnostics). To evaluate transcript levels of the *SMN* genes, we amplified cDNA fragments of exons 1–2b, exons 7 and 8, and exons 5, 6 and 8. The cDNA fragment including exons 1–2b represented total *SMN* transcript, because the sequence of exons 1–2b is commonly included in all transcript isoforms. The cDNA fragment containing exons 7 and 8 represented the FL-*SMN* transcript, because it contained sequence beyond exon 7. The cDNA fragment including *SMN* exons 5, 6 and 8 represented the Δ7-*SMN* transcript, because it did not carry the sequence of exon 7. We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous reference gene, and the levels of *SMN* were normalized relative to those of *GAPDH*. The primers for the total-*SMN*, FL-*SMN*, Δ7-*SMN*, and *GAPDH* transcripts have been described previously [17,18]. Quantitation of the PCR products was performed with the second derivative maximum method of the LightCycler software.

2.4. Denaturing high-pressure liquid chromatography (DHPLC) detection of GCC insertion polymorphism in the SMN promoter

To screen for the GCC insertion polymorphism in SMA patients and controls, DHPLC analysis of PCR products was performed. PCR of the fragment including the polymorphism site was carried out with the primer set: 5′-tgcaatgagccgagatggtg-3′ and 5′-cctccccccttggaaaag-taa-3′. The PCR products were then directly loaded into the autosampler of an automated DHPLC system, the WAVE Nucleic Acid Fragment Analysis System, equipped with a DNASep cartridge (Transgenomic, Omaha, NE, USA). The samples were run under partially denaturing conditions at 54.6 °C (oven temperature). The buffer gradient conditions were the same as previously reported [19].

2.5. Sequencing

Direct and/or subcloned sequencing analyses of PCR-amplified products were performed. Sequencing reactions were performed using a dye terminator cycle-sequencing kit (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA, USA), according to the supplier's instructions. The reaction products were automatically electrophoresed on an ABI PRISM 310 Sequencer (Applied Biosystems) and then analyzed using the Sequencing Software Module provided with the ABI PRISM 310 Sequencer.

2.6. Preparation of expression vectors

The PCR-amplified fragment containing GCCGCC polymorphism or GCC polymorphism was inserted into a firefly luciferase reporter plasmid, pGL2BTK (pGL2-Basic with a minimal herpes virus 1 thymidine kinase promoter). The GCCGCC and GCC fragment-containing plasmids were designated as 'pGCCGCC'

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and 'pGCC', respectively. The construct maps of pGL2BTK, pGCCGCC, and pGCC are shown in Fig. 1.

2.7. Transcription assay

The responses of the test plasmids (pGL2BTK, pGCCGCC, pGCC) to dibutyryl cAMP (dbcAMP; 0.5 mM), forskolin (20 μM), and a combination of dibutyryl cAMP and forskolin were determined in a human neuroblastoma cell line, BE(2)-C cells. The neuroblastoma cell lines have been used as useful experimental models of neuronal differentiation because the morphological, biochemical and electrophysiological properties of neuroblastoma cell lines are similar to those of neurons [20].

Neuroblastoma cells [2×10^5 cells in Minimum Essential Medium (MEM)] were cotransfected with a test plasmid (1.6 μg) and the phRL plasmid (a sea pansy luciferase reporter plasmid; Promega Corporation, Madison, WI, USA) (0.5 ng) using Lipofectamine 2000 (Invitrogen/Life Technologies Corporation). Twenty-four hours after transfection, dibutyryl cAMP, forskolin, or a combination of dibutyryl cAMP and forskolin was added to the MEM. The cells were harvested after culture for an additional 24 h.

Transcriptional activity of the test plasmids was measured using the dual-luciferase reporter assay system, in which sea pansy-luciferase activity was used as a control for the transfection efficiency of the test plasmids. Each transcriptional activity measurement was repeated three times and the data are expressed as the mean ± SD.

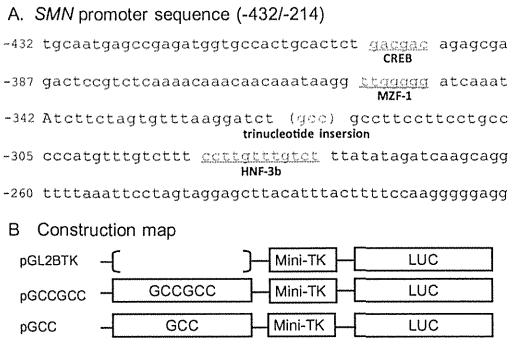

Fig. 1. *SMN* promoter sequence (A) and construction map (B). The *SMN* promoter sequence from c.-432 to c.-214 is shown in the upper part of the figure (A). The numbering of nucleotide in the promoter sequence is based on Monani et al. [14]. Trinucleotide insertion at c.-320_-321 is parenthesized. Putative transcription factor binding sites are underlined. Plasmid construction map is shown in the lower part of the figure (B). All constructs have a firefly-luciferase reporter gene, which is designated as LUC in the map. The pGL2BTK plasmid is a basic plasmid served as control. The GCCGCC and GCC fragment-containing plasmids were designated as 'pGCCGCC' and 'pGCC', respectively.

2.8. Statistics

Statistical analysis of the transcriptional activity data was performed using Microsoft Excel 2003 software and Statistical Package for the Social Sciences (SPSS Inc., Chicago, I, USA). The Student's *t*-test was conducted to evaluate differences between the plasmids. A probability of less than 0.05 was considered statistically significant.

3. Results

3.1. SMN1 deletion test and SMN2 gene dosage analysis

We performed an *SMN1* deletion test on Patient A, who was suspected as having SMA type 2/3. The patient carried zero copies of *SMN1* and two copies of *SMN2*. Based on molecular analysis, he was diagnosed as having SMA.

A nucleotide substitution in *SMN2* exon 7, c.859G>C, has been reported as a positive modifier of the SMA phenotype [21,22]. To check whether the mutation is present in Patient A, we performed a sequencing analysis of the exon 7. However, we did not find any substitutions including c.859G>C.

3.2. SMN2 transcript levels

Our aim of this study was to compare the *SMN2* transcript levels of Patient A to those of other SMA type 2 patients, because we hypothesized that *SMN2* transcript expression was the key determinant of the SMA phenotype. It would have been preferable to compare Patient A with SMA type 2 patients carrying two copies of *SMN2*. However, we did not have cDNA samples from SMA type 2 patients with zero copies of *SMN1* and two copies of *SMN2*. In this study, we determined the baseline transcript levels of total *SMN*, FL-*SMN*, and Δ7-*SMN* in the white blood cells of Patient A, five disease controls (DCs 1–5; they were all SMA type 2 patients with zero copies of *SMN1* and three copies of *SMN2*) and three healthy controls. All of the disease controls were able to sit without support, but could not stand or walk even with any support.

Total *SMN* transcript levels of Patient A, DC1, DC2, DC3, DC4, and DC5 were 38%, 76%, 66%, 181%, 232%, and 166% of the mean value of the healthy controls, respectively. This finding suggested that *SMN2* transcription in Patient A was significantly reduced compared with that of the disease controls.

The FL-*SMN* transcript levels of Patient A, DC1, DC2, DC3, DC4, and DC5 were 53%, 58%, 64%, 44%, 68%, and 95% of the mean value of the healthy controls, respectively. The Δ7-*SMN* transcript levels of Patient A, DC1, DC2, DC3, DC4, and DC5 were 167%, 206%, 130%, 130%, 97%, and 145% of the mean value of the healthy controls, respectively. These findings suggested

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