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Original Article

Clinical phenotypes of spinal muscular atrophy patients with hybrid *SMN* gene

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ABSTRACT

BACKGROUND: Spinal muscular atrophy (SMA) is a neuromuscular disease caused by homozygous deletion of *SMN1* exons 7 and 8. However, exon 8 is retained in some cases, where *SMN2* exon 7 recombines with *SMN1* exon 8, forming a hybrid *SMN* gene. It remains unknown how the hybrid *SMN* gene contribute to the SMA phenotype.

METHOD: We analyzed 515 patients with clinical suspicion for SMA. *SMN1* exons 7 and 8 deletion was detected by PCR followed by enzyme digestion. Hybrid *SMN* genes were further analyzed by nucleotide sequencing. *SMN2* copy number was determined by real-time PCR.

RESULTS: *SMN1* exon 7 was deleted in 228 out of 515 patients, and *SMN1* exon 8 was also deleted in 204 out of the 228 patients. The remaining 24 patients were judged to carry a hybrid *SMN* gene. In the patients with *SMN1* exon 7 deletion, the frequency of the severe phenotype was significantly lower in the patients with hybrid *SMN* gene than in the patients without hybrid *SMN* gene. However, as for the distribution of *SMN2* exon 7 copy number among the clinical phenotypes, there was no significant difference between both groups of SMA patients with or without hybrid *SMN* gene.

CONCLUSION: Hybrid *SMN* genes are not rare in Japanese SMA patients, and it appears to be associated with a less severe phenotype. The phenotype of patients with hybrid *SMN* gene was determined by the copy number of *SMN2* exon 7, as similarly for the patients without hybrid *SMN* gene.

Keywords: Spinal muscular atrophy; hybrid *SMN* gene; *SMN1*; *SMN2*; gene conversion

1. Introduction

Spinal Muscular Atrophy (SMA) is a common autosomal recessive neuromuscular disease defined by defects of lower motor neurons in the spinal cord, resulting in weakness and wasting of voluntary muscles. According to a recent review, SMA has an incidence of 1 in 11,000 live births with an estimated carrier frequency of 1 in 47~72 people [1].

SMA is conventionally classified into three clinical subtypes (I, II, III) based on the age of onset and accomplished motor milestones. SMA type I is the severe form with symptoms appearing before 6 months of age. The patients can never sit unsupported and, without proper intervention, they usually succumb to respiratory failure before their second birthday. Symptoms in patients with SMA type II, appear within the first 18 months of life. The patients can sit but never walk unaided, although they can survive until adulthood. Individuals with SMA type III, a mild form, exhibit symptoms after18 months of life. The patients can stand and walk without support [2]. Recently, a more detailed clinical classification has been defined including type 0 (the severest form with respiratory failure at birth) and IV (the mildest form with adult onset) [1].

In 1995, the *survival motor neuron* (*SMN*) genes were identified in chromosome 5q13 as being responsible for SMA [3]. This gene is present in two highly homologous copies in humans. The telomeric copy, *SMN1*, produces full-length transcript that encodes for the functional protein. *SMN1* is now termed the disease-causing gene because it is deleted in about 95% of SMA patients, and mutated in 5% of cases [3]. Contrarily, the centromeric copy, *SMN2*, is recognized to be the disease-modifying gene because its copy number is related to the clinical phenotype. High number of *SMN2* copies may improve the survival outcomes and maintenance of motor function. *SMN2* copy number is inversely proportional to the clinical phenotype [4-7]. Patients with SMA type 0 usually possess only one copy of the *SMN2* gene, meanwhile some studies have reported individuals with SMA type IV as having four *SMN2* copies or more [1].

SMN1 can be distinguished from *SMN2* by only five nucleotide differences within the region covering intron 6 to exon 8; one in intron 6, one in exon 7, two in intron 7, and one in exon 8 [3, 8]. The nucleotides at position +6 of exon 7 are C in *SMN1* and T in *SMN2*. The *C-to-T* transition in *SMN2* exon 7 leads to the production of a large amount of Δ7-*SMN2* transcript (or *SMN2* exon 7-skipped transcript) and Δ7-SMN2 protein, and a small amount of full-length *SMN2* transcript and full-length SMN2 protein [9]. Subsequently, two research groups reported different machineries of "abnormal" splicing of *SMN2* exon 7. One is that the C-to-T transition disrupts an exonic splicing enhancer (ESE) [10], and the other is that the C-to-T transition creates an exonic splicing silencer (ESS) [11]. Exon 7 splicing in *SMN1* and *SMN2* may be regulated by a fine balance between positive and negative determinants of exon identity and alternative splicing [12]. On the contrary, the one nucleotide difference in exon 8 lies within the 3′ untranslated region (3'-UTR) and may have no effect on the translated protein sequence [9].

The nucleotide differences between *SMN1* and *SMN2* in exons 7 and 8 are used for the molecular diagnosis of SMA [3,13]. Most SMA patients show homozygous deletion of *SMN1*, involving exon 7 and exon 8. However, some patients show exon 7 deletion, but retain exon 8 [3, 14-21]. Such patients carry a gene recombination involving *SMN2* exon 7 and *SMN1* exon 8 forming a hybrid *SMN* gene. To date, hybrid *SMN* genes have been reported in patients from at least six different ethnic groups [3, 15, 16, 18, 20, 21]. However, the contribution of the hybrid *SMN* gene to the clinical phenotype of SMA has not been elucidated.

Until now, we have clarified the genotype-phenotype correlation in Japanese SMA patients [22, 23]. We also observed that *SMN2* copy number was inversely proportional to the clinical phenotype in *SMN1*-deleted SMA patients in Japan [22]. However, such an inverse correlation was not found in non-*SMN1*-deleted SMA patients, who carried an intragenic mutation in *SMN1* [23]. In this study, we analyzed genotype-phenotype correlation in the *SMN1*-deleted SMA patients carrying a hybrid *SMN* gene. We have analyzed a total of 515 patients with symptoms of SMA, who had been referred to our

laboratory from 1996 to 2019, and diagnosed 228 *SMN1*-deleted patients as having SMA among them. In the course of molecular diagnosis of SMA, we identified 24 cases with hybrid *SMN* gene with a structure of *SMN2* exon 7-*SMN1* exon 8.

2. Patients, materials and methods

2.1. Patients

We included a total of 515 Japanese patients with symptoms of SMA, who had been referred to our laboratory from 1996 to 2019. All of them presented with delayed developmental motor milestones, muscle weakness, respiratory failures or difficulties with walking. In this study, we adopted the conventional classification (types I, II, III) to express the clinical phenotype of the patients as having SMA [2].

Prior to molecular analysis, written informed consent was obtained from the patients or their parents. This study was approved by the Ethics Committee of Kobe University Graduate School of Medicine (reference 1089, approved on 5 October 2018).

2.2. Detection of SMN1 and SMN2 deletion by PCR and enzyme-digestion analysis

Genomic DNA was extracted from 3-5 ml of whole blood using a DNA extraction kit, SepaGene® (EIDIA, Tokyo, Japan).

PCR amplification of the *SMN1* and *SMN2* genes was performed by the PCR method of van der Steege *et al.* [13]. The oligonucleotide primers for exon 7 of the *SMN1* and *SMN2* genes were R111 [3] and X7-Dra [13], and those for exon 8 of the *SMN1* and *SMN2* genes were 541C960 and 541C1120 [3]. The restriction enzymes *Dra I* and *Dde I* cleaved the PCR-amplified fragments from *SMN2* exons 7 and 8, respectively [13]. The PCR products were digested with *Dra I* for exon 7 and *Dde I* for exon 8, and the digested products were electrophoresed in agarose gels and visualized by DNA staining reagents.

Exon 5 of the *NAIP* gene, which is also located in the SMA locus of chromosome 5q13, was detected by the PCR method of Roy *et al.* [24]. The oligonucleotide primers used in this study were “1864” and “1863” [24]. The PCR products were electrophoresed in agarose gels and visualized by DNA staining reagents.

2.3. Analysis of SMN gene copy number

The copy numbers of *SMN2* exon 7 were determined by a quantitative real-time PCR method of Tran et al. [25].

2.4. PCR amplification & sequencing of the fragments covering SMN intron 6 to exon 8

Genomic DNA fragment covering the *SMN1* (or *SMN2*) intron 6 to exon 8 was PCR-amplified with oligonucleotide primers R111 and 541C1120 [3]. The PCR product was then purified and submitted for direct sequencing. All sequencing analyses were conducted by Greiner Bio-One Co. Ltd. (Tokyo, Japan).

2.5. Statistical analysis

To compare the distribution of clinical phenotypes (SMA subtypes) between patient groups with/without hybrid *SMN* gene, we did Fisher's exact probability test (2 x 2 contingency table). To compare the differences in *SMN2* exon 7 copy number between the two groups, we did Chi-square for independence test (m x n contingency table). To compare the differences in *SMN2* exon 7 copy number among the clinical phenotypes (subtypes) of the two groups, we did Welch's t-test. For these analyses, we used Microsoft Excel with an add-in software, Statcel 3 (The Publisher OMS Ltd., Tokyo, Japan). A *p*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Detection of a hybrid SMN gene in SMA patients

Out of the 515 patients, 228 patients showed complete deletion (homozygous deletion) of *SMN1* exon 7, while 287 patients showed its retention (Table 1). Thus, the *SMN1* exon 7-deleted patients were diagnosed as having SMA. Subsequent examination indicated deletion of *SMN1* exon 8 in 204 of the *SMN1* exon 7-deleted patients and retention of *SMN1* exon 8 in 24 patients. The 24 patients were defined as carrying a hybrid *SMN* gene (*SMN2 exon 7* + *SMN1* exon 8). Thus, the patients were classified into two groups, "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)".

The *SMN1*-deleted patients with hybrid *SMN* gene exhibited a peculiar deletion pattern of *SMN1* on agarose gels, deleting *SMN1* exon 7 while retaining *SMN1* exon 8 [Fig. 1, hybrid *SMN* gene (+)]. *SMN1*-deleted patients without hybrid *SMN* gene showed deletion of both *SMN1* exon 7 and 8 [Fig.1, hybrid *SMN* gene (-)].

The 24 *SMN1*-deleted patients with hybrid *SMN* gene consisted of 13 females and 11 males with age ranging from 3 months to 74 years. SMA subtypes of these patients will be discussed later in section 3.3. It should be noted here that none of the patients with hybrid *SMN* gene showed deletion of *NAIP* exon 5, which occurred frequently in patients with SMA type I [20].

3.2. Nucleotide sequencing confirmation of hybrid SMN gene

PCR amplification followed by nucleotide sequencing was performed to clarify the genomic structure in 11 *SMN1*-deleted patients carrying the hybrid *SMN* gene. In the sequence of a healthy control sample, we confirmed the presence of double peaks presenting *SMN1*- and *SMN2*- specific nucleotides in exon 7 and exon 8 (Fig. 2A).

In *SMN1*-deleted patients without hybrid *SMN* gene, only single peaks presenting *SMN2* specific nucleotides was observed in exon 7 and exon 8 (Fig. 2B). However, in *SMN1*-deleted patients with hybrid *SMN* gene, only a single peak presenting an *SMN2*-specific nucleotide was observed in exon 7,

but a double peak presenting *SMN1*- and *SMN2*-specific nucleotides in exon 8 (Fig. 2C). These findings confirmed the formation of a hybrid *SMN* gene in these patients with *SMN2* exon 7 joined to *SMN1* exon 8 (Fig. 2C).

Since *SMN1* and *SMN2* differ by five nucleotides in intron 6, exon 7, intron 7 and exon 8, there are two specific haplotypes in a normal situation, namely G-C-A-A-G (*SMN1*) and A-T-G-G-A (*SMN2*). Our sequencing results show that 3 types of the hybrid *SMN* gene were observed arising from different patterns of fusion of *SMN1/SMN2* genes. Partial conversion of *SMN1* resulted in hybrid haplotypes: A-T-G-G-G (9 patients), A-T-G-A-G (1 patient) and A-T-A-A-G (1 patient) (Fig. 3).

3.3. SMA subtypes in hybrid SMN gene (+) / (-) groups

To evaluate the effect of the hybrid *SMN* gene on clinical severity, we determined the distribution of clinical phenotypes (SMA subtypes) in "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)" groups.

We grouped the patients into type I and non-type I subtypes. Consequently, in "hybrid *SMN* gene (+)" group, only 3 patients (13%) were type I and 21 patients (87%) were non-type I (Fig. 4A). In the "hybrid *SMN* gene (-)" group, 93 patients (45.6%) were type I and 111 patients (54.4%) were non-type I (Fig. 4B). Fisher's exact probability test in the 2 x 2 contingency table analysis proved that these differences were statistically significant (Fisher's exact probability test, $p<0.01$).

For more details, in "hybrid *SMN* gene (+)" group ($n=24$), the subtype distribution was as follows: type I ($n=3$, 12.5%), type II ($n=14$, 58.3%), and type III ($n=7$, 29.2%) (Table 2). In "hybrid *SMN* gene (-)" group ($n=204$), the subtype distribution was as follows: type I ($n=93$, 45.6%), type II ($n=57$, 27.9%), and type III ($n=54$, 26.5%) (Table 2). Chi-square test in the m x n contingency table analysis showed that the differences between the two groups were statistically significant ($x^2=12.0$, $p<0.01$).

3.4. SMN2 exon 7 copy number in hybrid SMN gene (+) / (-) groups

The distribution of *SMN2* exon 7 copy number among the "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)" groups were compared (Table 3). Here, the native *SMN2* gene (*SMN2* exon 7 + *SMN2* exon 8) and the hybrid *SMN* gene (*SMN2* exon 7 + *SMN1* exon 8) were not discriminated. Chi square test in the m x n contingency table analysis proved that there were no significant differences (x^2=3.9, p=0.27).

The distributions of *SMN2* exon 7 copy number were also compared in each subtype between "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)" groups (Table 4). Welch's t-test proved that there were no significant differences in each subtype between "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)" groups. In the "hybrid *SMN* gene (+)" group (n=23), the copy numbers of *SMN2* exon 7 (mean±SD) were as follows: type I (n=3, 2.33±0.41), type II (n=14, 2.93±0.01) and type III (n=6, 3.50±0.40). In the "hybrid *SMN* gene (-)" group (n=177), the copy numbers of *SMN2* exon 7 (mean±SD) were as follows: type I (n=80, 2.18±0.60), type II (n=51, 2.96±0.07) and type III (n=46, 3.39±0.11).

Fig. 5 revealed the same tendencies of genotype (*SMN2* exon 7 copy number)-phenotype relationship between "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)" groups. In both groups, the mean value of the *SMN2* copy number increased with milder phenotype.

4. Discussion

4.1. Frequency of hybrid SMN gene in SMA patients

In this study, we examined the characteristics of SMA patients with hybrid *SMN* genes in the Japanese population. Overall, we found that 10.8% (24/228) of SMA patients with an *SMN1* deletion contained a hybrid *SMN* gene.

Previous studies have been shown that the frequencies of hybrid *SMN* gene in SMA patients vary among different ethnicities, ranging from 5-30% of all SMA patients. According to Hahnen *et al.* (German group), the frequency of hybrid *SMN* gene in SMA patients of German origin was ~12% while the frequency in SMA patients with Czech or Polish background was ~30% [15]. However, other research groups reported a lower frequency of hybrid *SMN* gene in SMA patients. Lefebvre *et al.* (French group) reported that the frequency of hybrid *SMN* gene in SMA patients with various ethnic origins was 5.6% [3]. Rodrigues *et al.* (UK group), reported a hybrid *SMN* gene frequency of 5.1% [16]. The subjects in their study were drawn from ethnically diverse populations in the UK and Europe, including at least 70% of the cases ever diagnosed in Finland. Cusco *et al.* (Spanish group) reported a hybrid *SMN* gene frequency of 5.2% in the Spanish population [18]. Omrani *et al.* (Iranian group) and Qu *et al.* (Chinese group) reported hybrid *SMN* gene frequencies of 5.3% and 5.5%, respectively, in the populations from Iran and China [20, 21].

Thus, the frequency (f) of hybrid *SMN* gene in SMA patients may be classified into three types, high frequency type ($f \geq 20\%$), medium frequency type ($10\% \leq f < 20\%$) and low frequency type ($f < 10\%$). Based on the present data, hybrid *SMN* genes are present in Japanese SMA patients at a medium frequency level, suggesting that hybrid *SMN* gene formation is not a rare event in Japanese SMA patients. Our results suggest that the frequency of genomic structure variants of the SMA locus on chromosome 5 differs depending on ethnicity.

4.2. Clinical phenotype of the patients with hybrid SMN gene

In our study, the frequency of SMA type I patients in the "hybrid *SMN* gene (+)" group was much lower than that in the "hybrid *SMN* gene (-)" group (Fig. 4). The number of SMA type I patients was only 3 out of 24 patients in the "hybrid *SMN* gene (+)" group (12.5%). The majority of the patients in the "hybrid *SMN* gene (+)" group showed milder symptoms, and were diagnosed as having SMA type II or type III. On the other hand, the number of SMA type I patients was 93 out of 204 patients in the "hybrid *SMN* gene (-)" group (45.5%), accounting for almost half of the patients. When the distribution of the patient number in each clinical subtype was compared between "hybrid *SMN* gene (+)" and "hybrid *SMN* gene (-)" groups: type II>type III>type I in hybrid *SMN* gene (+) group and type I>type II>type III in "hybrid *SMN* gene (-)" group (Table 2). The findings in our study may give an impression that the presence of hybrid *SMN* gene may ameliorate the clinical phenotype. However, Hahnen *et al.* found a roughly equal distribution in all clinical subtypes: type I (*n*=14, 33.3%), type II (*n*=13, 30.9%), and type III (*n*=15, 35.7%) [15]. Their finding suggested that the presence of hybrid *SMN* gene may have no correlation with the clinical phenotype.

Here, we propose a hypothesis that copy number analysis of *SMN2* exon 7 may provide some clues to the understanding of the role of the hybrid *SMN* gene in the modification of clinical phenotype of SMA. One nucleotide difference in exon 8 (G in *SMN1* and A in *SMN2*) lies within the 3'-UTR, which implies that the hybrid *SMN* gene and the native *SMN2* gene produce the same proteins（Δ7-SMN2 and full-length SMN2 protein). However, if the hybrid *SMN* gene did affect the clinical phenotype, it would be as a result of increased production of full-length SMN protein. If this were the case, the mean copy number of *SMN2* exon 7 in each clinical subtype of the "hybrid *SMN* gene (+)" patients would be smaller than that of the "hybrid *SMN* gene (-)" patients. This is discussed in more detail below.

4.3. SMN2 exon 7 copy number of patients with hybrid SMN gene.

SMN2 copy number is an important modifier of SMA clinical phenotype, being inversely proportional to the SMA subtypes [4-7]. However, it remains unknown whether the hybrid *SMN* gene produces more full-length SMN2 protein or less SMN2 protein compared to the native *SMN2* gene.

In this study, the copy number of *SMN2* exon 7 including native *SMN2* genes and hybrid *SMN* genes was used for the analysis. When the native *SMN2* genes and the hybrid *SMN* genes were not discriminated, the *SMN2* copy number distributions in each clinical subtype of the "hybrid *SMN* gene (+)" patients were the same as that of the "hybrid *SMN* gene (-)" patients (Fig. 5, Table 4). This finding suggested that the hybrid *SMN* gene may produce a similar level of full-length SMN2 protein as the native *SMN2* genes.

According to Lorson *et al.* [9], cells transfected with engineered hybrid *SMN* genes showed high-level *Δ 7-SMN* transcript expression and reduced full-length *SMN* transcript expression, which was consistent with exon 7 splicing pattern of the native *SMN2* gene in SMA patients. Their finding also suggested that the properties of the hybrid *SMN* gene and the native *SMN2* genes are similar to each other in transcription.

Taken together, these findings suggest that there may be little functional difference between the hybrid *SMN* and native *SMN2* genes. However, because we did not determine SMN protein levels in the current study, we cannot draw a definitive conclusion. Regardless, based on the available evidence, it is reasonable to suggest that the two genes produce similar amounts of full-length SMN protein.

4.4. Mechanisms of hybrid SMN gene formation

Hybrid *SMN* gene formation of *SMN2* exon 7-*SMN1* exon 8 is regarded as a fusion between the flanking region of *SMN2* exon 7 and the flanking region of *SMN1* exon 8. Hahnen *et al.* hypothesized that the mechanisms responsible for the hybrid *SMN* genes may be unequal

recombination, intrachromosomal deletion and gene conversion from *SMN1* to *SMN2* [15]. They also identified an unaffected individual who carried a homozygous deletion of *SMN2* exon 7 only. This case carried a hybrid *SMN* gene with a structure of *SMN1* exon 7-*SMN2* exon 8, showing the reciprocal state on a non-SMA chromosome. Ogino *et al.* also provided evidence that *SMN1* converted from *SMN2* is present in the general population [26]. Hybrid exon 8 genes converted from *SMN1* to *SMN2* and vice-versa have also been reported among healthy individuals by Fang *et al.* [27].

Nucleotide sequencing analyses of hybrid *SMN* genes in SMA patients have revealed that various genomic structure types are present [15,17, 19, 21, this study]. The most common type, A-T-G-G-C (Fig. 3, Supplementary Fig. 1) in this study of Japanese patients also appears to be similar to the observations of Hahnen *et al*, Kubo *et al* and Qu *et al*. [15, 19, 21]. We identified two other less frequent types in this study; A-T-G-A-G, and A-T-A-A-G (Fig. 3). As shown in Supplementary Fig. 1, there are at least six known genomic structure types of hybrid *SMN* genes.

The variety of hybrid *SMN* gene types led us to the idea that the downstream sequences of the *SMN* genes might be very susceptible to gene conversion or other events such that they could occur two or more times in the same region. Recently, *SMN1/SMN2* variants lacking exons 7 and 8 have been found in up to 20% of individuals in several Caucasian populations [28]. The presence of hybrid *SMN* genes and *SMN1/SMN2* variants lacking exons 7 and 8 suggest the vulnerability of this region to the genomic structure-changing events.

This begs the question of the identity of the vulnerability factor that underlies hybrid *SMN* gene formation. Presence of the most abundant type of short interspaced elements (SINES), *Alu* elements, may be a candidate vulnerability factor of the *SMN1/2* genes. *Alu* elements are major contributors to non-allelic homologous recombination events causing genome diversity, copy number variation and many diseases [29]. Ottesen *et al.* noted that about 41% of the human *SMN* gene including the promoter region is occupied by more than 60 *Alu*-like sequences [30]. *Alu*-rich

regions were also identified in intron 6 and were implicated in the deletion of exons 7 and 8 in SMA patients [30]. Actually, Wirth et al. reported an SMA patient with an *Alu*-mediated deletion from intron 4 to intron 6 [6]. One would thus expect sequence insertion/deletion or conversion event due to the presence of *Alu* elements in *SMN* intron 6. However, further studies are necessary to fully understand the mechanisms of hybrid *SMN* gene formation.

Lastly, we must mention our observations on the *NAIP* gene. The gene was also identified in the SMA locus of chromosome 5q13 [24]. This gene is present in two highly homologous copies, telomeric and centromeric copies in humans, similarly to the *SMN* genes [24]. *NAIP* exon 5 is a telomeric copy-specific sequence. The frequency of homozygous deletion of *NAIP* exon 5 in the severe phenotype (SMA type I) is higher than that in the milder phenotype (SMA types II and III) [24]. In this study, we observed no deletion of *NAIP* exon 5 in the patients with hybrid *SMN* gene. Hahnen *et al*. showed the same observation as ours [15]. However, according to Rodrigues *et al*., formation of the hybrid *SMN* gene can occur in the presence or absence of *NAIP* exon 5 [16]. These findings suggest that various events changing the genomic structure can occur with the various involvement of SMA locus in chromosome 5q13.

5. Conclusion

Our study demonstrated that hybrid *SMN* gene formation is not a rare event in Japanese SMA population. Nucleotide sequencing analyses of the hybrid *SMN* genes showed that there are different genomic structure types among them. Hybrid *SMN* gene formation occurred in all the clinical subtypes of SMA, but more frequently in types II and III (milder forms). When hybrid *SMN* genes and the native *SMN2* genes were not discriminated, the *SMN2* exon 7 copy number distribution in each clinical subtype in the “hybrid *SMN* gene (+)” group was almost same as that in the “hybrid *SMN* gene (-)” group. Based on this finding, the properties of hybrid *SMN* genes were expected to be similar to native *SMN2* genes.

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Table 1: *SMN1* deletion test

All patients that participated in this study (*n*=515)			
SMN1 exon 7 (+) (*n*=287)		*SMN1* exon 7 (-) (*n*=228)	
SMN1 exon 8(+) (*n*=287)	*SMN1* exon 8(-) (*n*=0)	*SMN1* exon 8(+) (*n*=24)	*SMN1* exon 8(-) (*n*=204)
		Hybrid *SMN* gene (+)	Hybrid *SMN* gene (-)

Table 2: Frequencies of clinical subtypes among SMA patients with or without hybrid *SMN* gene

	Type I	Type II	Type III	Total
Hybrid *SMN* gene (+)	3	14	7	24
Hybrid *SMN* gene (-)	93	57	54	204
Total	96	71	61	228

(x^2=12.0, p<0.01)

Table 3: *SMN2* copy number in SMA patients with or without hybrid *SMN* gene

	1 copy	2 copies	3 copies	4 copies	Total *
Hybrid *SMN* gene (+)	0	4	15	4	23
Hybrid *SMN* gene (-)	1	66	91	19	177
Total	1	70	106	23	200

(x^2=3.9, p=0.27)

* Copy number was not determined in one patient with hybrid *SMN* gene or 27 patients without hybrid *SMN* gene

Table 4: *SMN2* copy number distribution for each clinical subtype among SMA patients with or without hybrid *SMN* gene

Clinical subtype	*Hybrid *SMN* gene (+)/ (-)	1 copy	2 copies	3 copies	4 copies	Mean (±SD)	*p*-value
Type I	(+)	0	2	1	0	2.33±0.41	0.12
	(-)	1	63	16	0	2.18±0.60	
Type II	(+)	0	1	13	0	2.93±0.01	0.24
	(-)	0	2	49	0	2.96±0.07	
Type III	(+)	0	1	1	4	3.50±0.40	0.09
	(-)	0	1	26	19	3.39±0.11	

* Copy number was not determined in one patient with hybrid *SMN* gene or 27 patients without hybrid *SMN* gene

Fig. Legends

Fig. 1: Detection of *SMN1* and *SMN2* deletion by PCR and enzyme-digestion analysis.

Patients in the "hybrid *SMN* gene (+)" group showed deletion of exon 7 and retention of exon 8, while patients in the "hybrid *SMN* gene (-)" group showed deletion of exon 7 and exon 8.

Fig. 2: Nucleotide sequencing analysis of *SMN1*-deleted patients.

A. Healthy control. The red arrows in exon 7 and exon 8 indicate double peaks of *SMN1*- and *SMN2*-specific nucleotides. **B**. "Hybrid *SMN* gene (-)" patient. The blue arrows in exon 7 and exon 8 indicate single peaks of *SMN2*-specific nucleotides. **C**. "Hybrid *SMN* gene (+)" patient. The blue arrow in exon 7 indicates a single peak of an *SMN2*-specific nucleotide. The red arrow in exon 8 indicates a double peak of *SMN1*- and *SMN2*-specific nucleotides. The yellow and green boxes in the schematic representation contain *SMN1*- and *SMN2*-specific nucleotides, respectively.

Fig. 3: Genomic structures of hybrid *SMN* genes.

A. Genomic structures of normal *SMN* genes. **B.** Genomic structures of hybrid *SMN* genes. Three types of genomic structures of the hybrid *SMN* genes were identified in this study. The yellow and green boxes in the schematic representation contain *SMN1*- and *SMN2*-specific nucleotides, respectively.

Fig. 4: Frequency of hybrid *SMN* gene in SMA type I and non-type I patients.

A. "Hybrid *SMN* gene (+)" group (n=24). **B**. "Hybrid *SMN* gene (-)" group (n=204). The frequency of SMA type I patients was significantly different between the two groups (Fisher's exact probability test, p <0.01).

Fig. 5: Mean values of *SMN2* copy number in *SMN1*-deleted patients.

A. "Hybrid *SMN* gene (+)" group (n=23). **B**. "Hybrid *SMN* gene (-)" group (n=177). Both groups showed a similar distribution of *SMN2* copy number for each clinical subtype.

Figure

Fig. 1

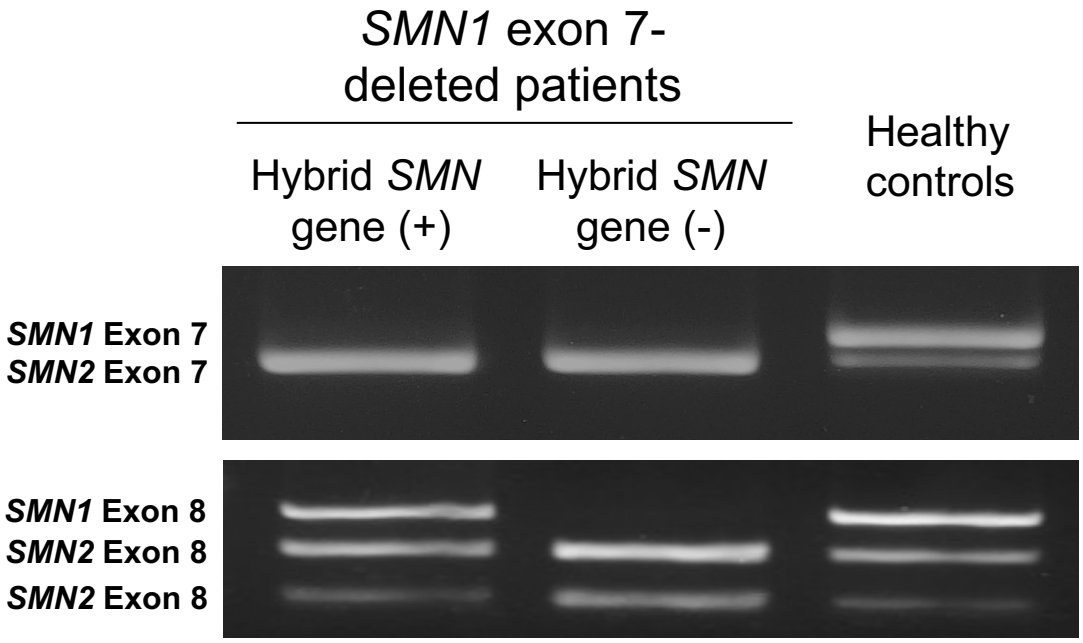
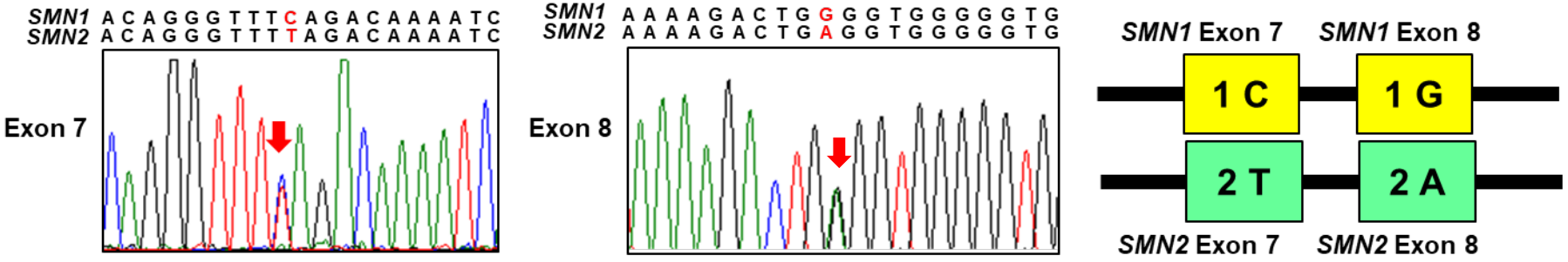
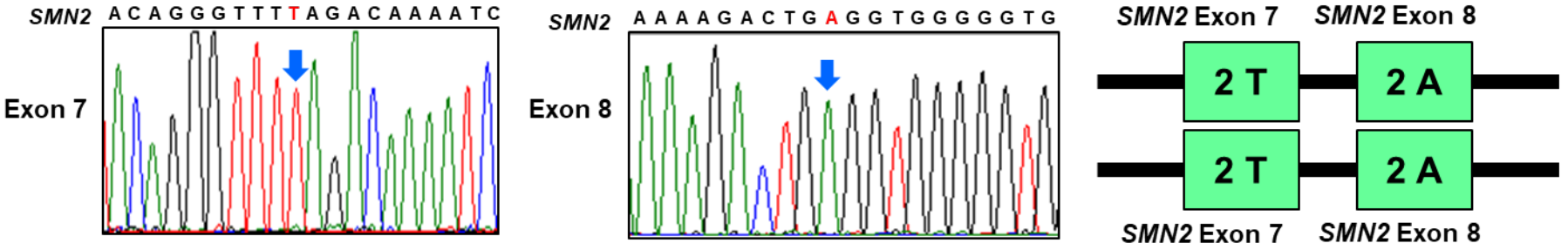

SMN1 exon 7-deleted patients
Hybrid *SMN* gene (+)
Hybrid *SMN* gene (-)
Healthy controls
SMN1 **Exon 7**
SMN2 **Exon 7**
SMN1 **Exon 8**
SMN2 **Exon 8**
SMN2 **Exon 8**

Fig. 2

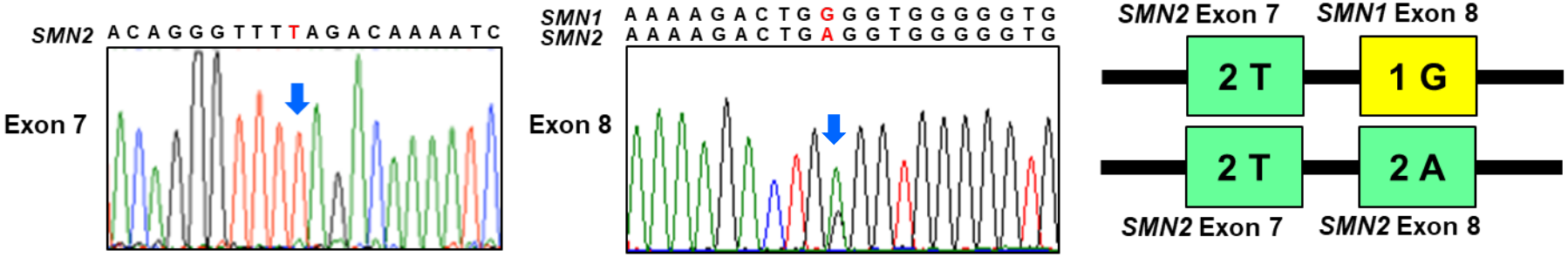
A. Healthy control



B. "Hybrid *SMN* gene (-)" patient



C. "Hybrid *SMN* gene (+)" patient

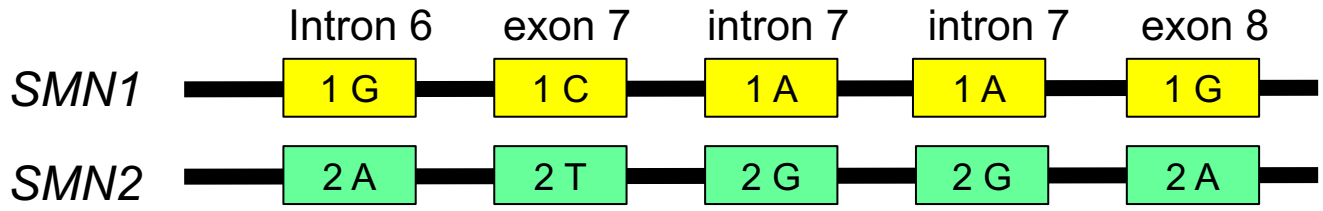


1 @ *SMN1*-specific nucleotide @

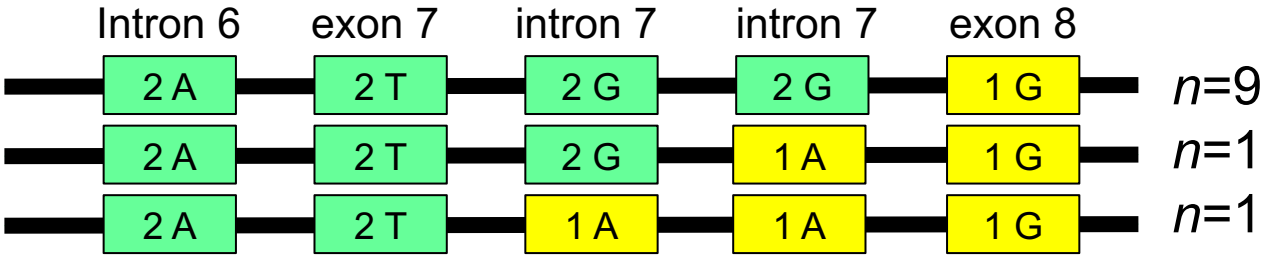
2 @ *SMN2*-specific nucleotide @

Fig. 3

A. Genomic structure of normal *SMN* genes



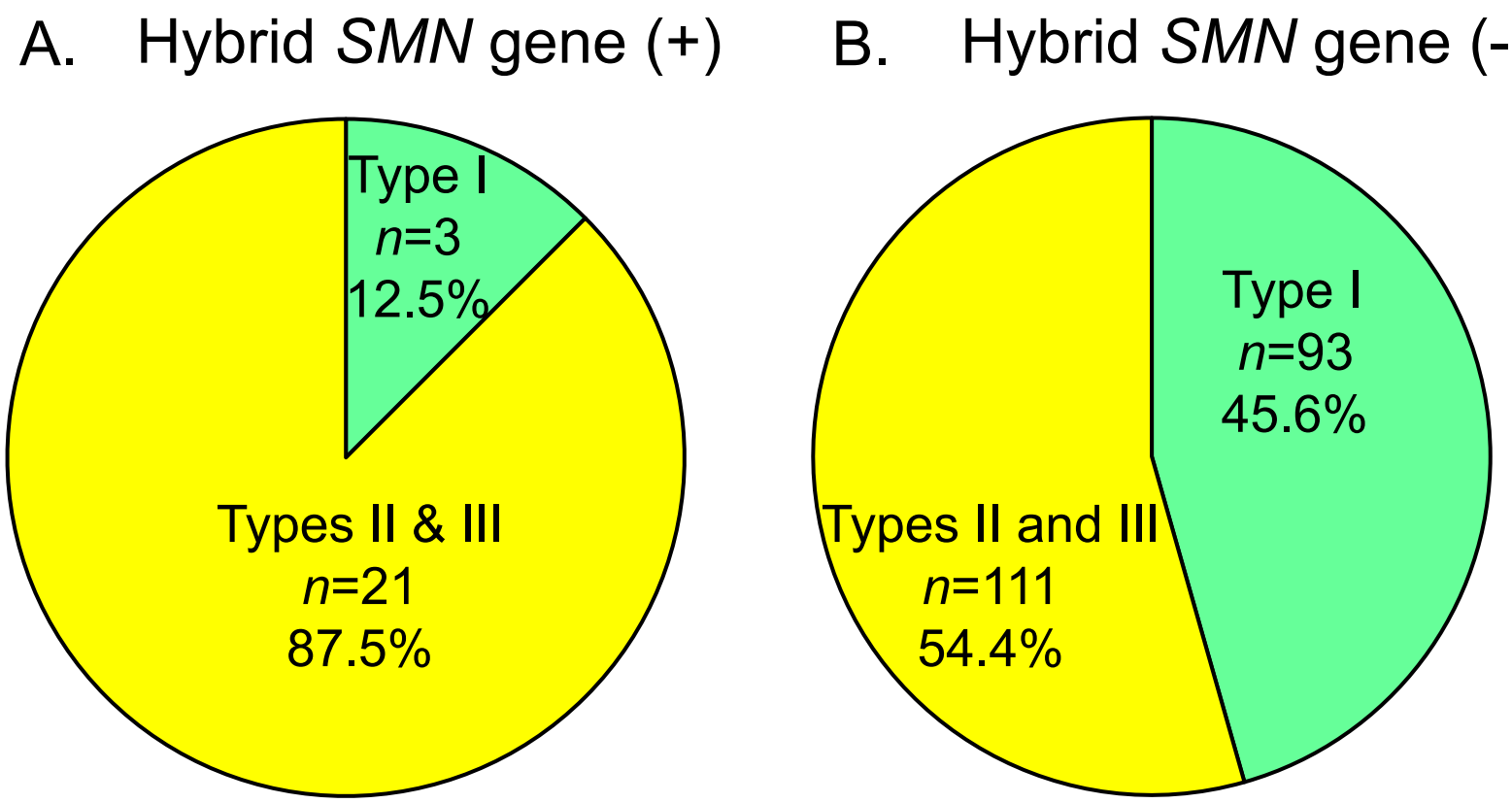
B. Genomic structures of hybrid *SMN* genes



1 @ *SMN1*-specific nucleotide @

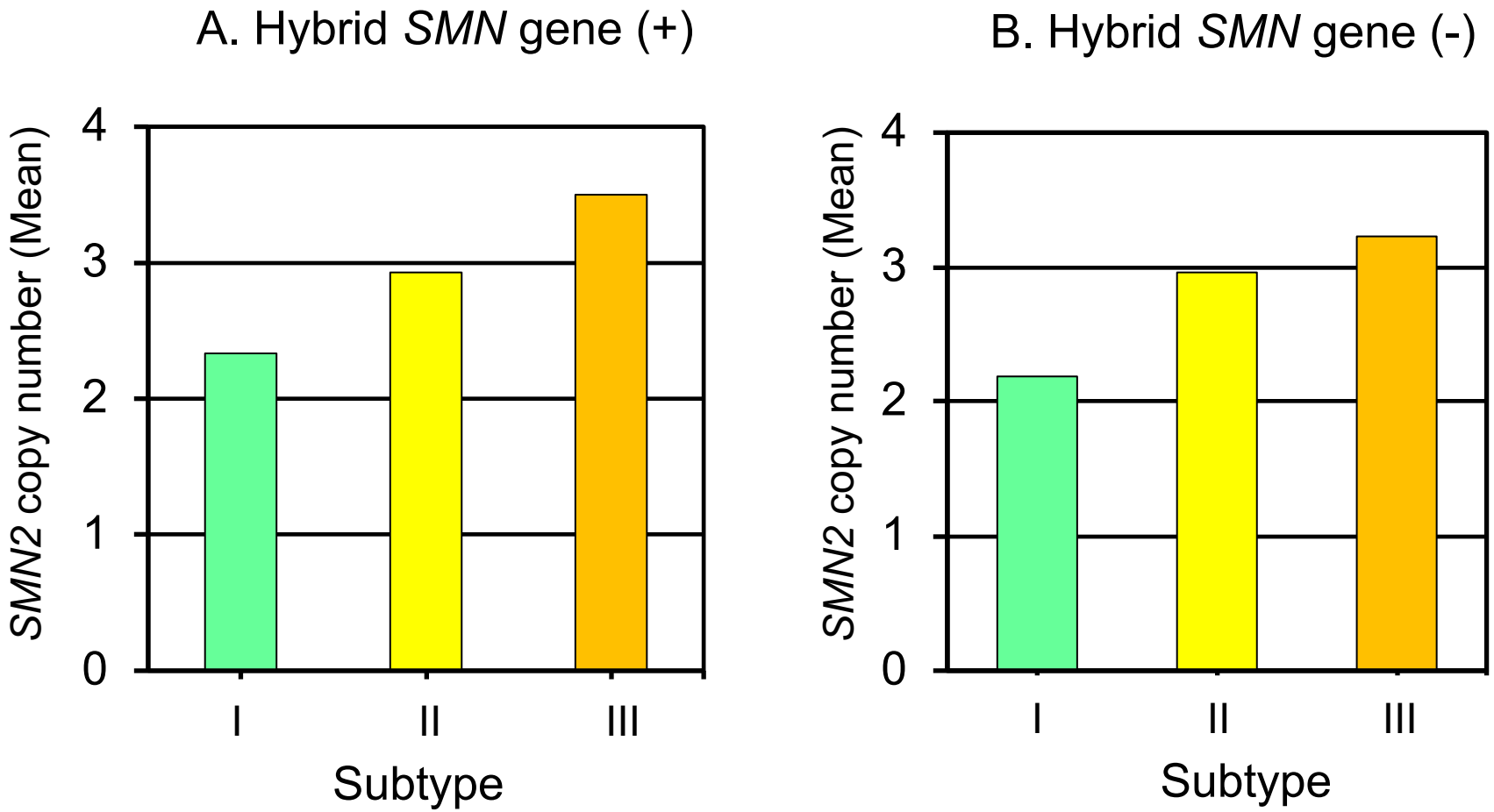
2 @ *SMN2*-specific nucleotide @

Fig. 4

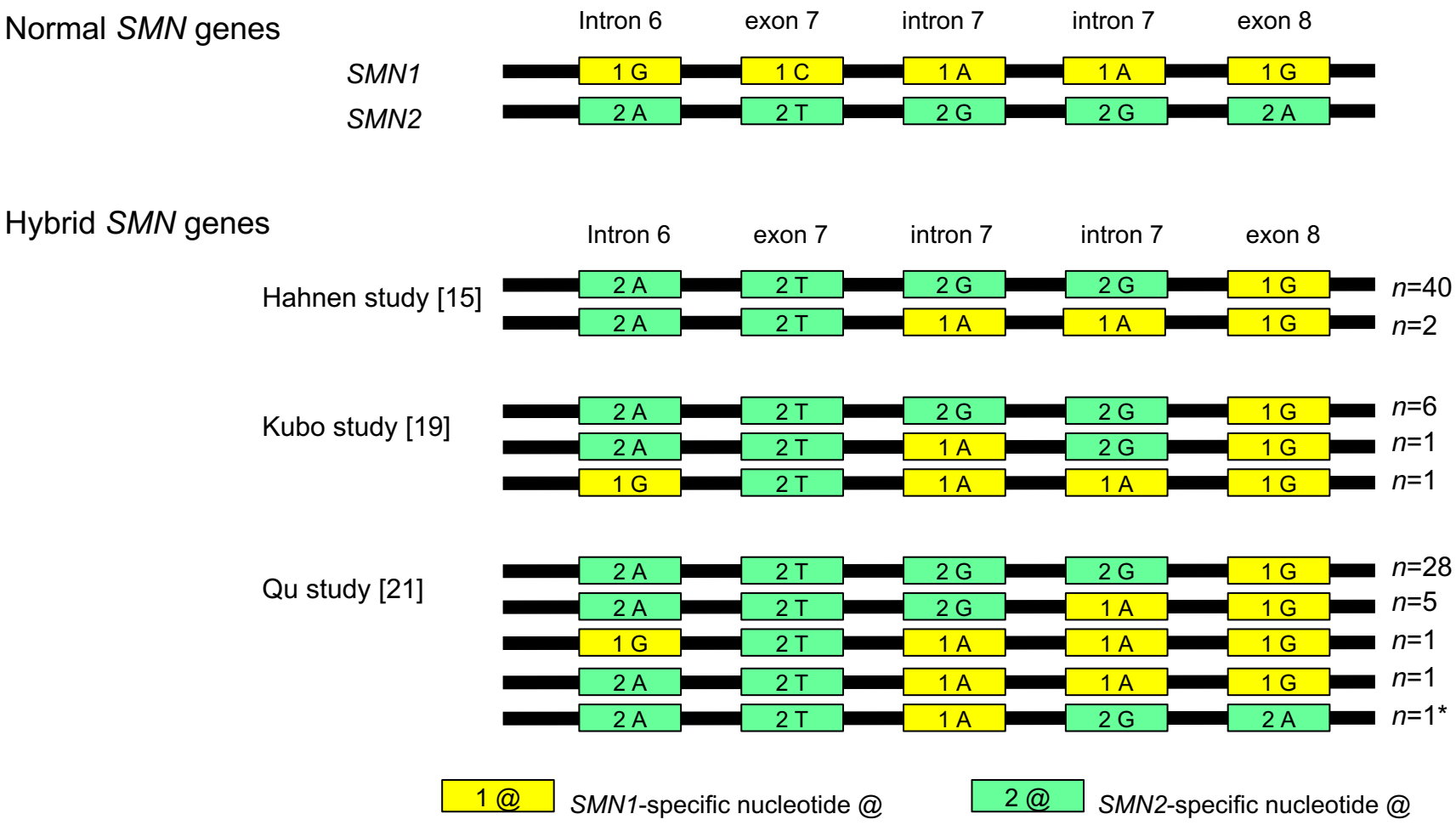


(Fisher's exact probability test $p<0.01$)

Fig. 5


A. Hybrid *SMN* gene (+)
SMN2 copy number (Mean)
0
1
2
3
4
I
II
III
Subtype
B. Hybrid *SMN* gene (-)
SMN2 copy number (Mean)
0
1
2
3
4
I
II
III
Subtype

Supplementary Fig. 1



* This case carried *SMN2* exon 8 in the study of Qu et al.

Schematic representation of the normal and hybrid *SMN* genes identified in different studies. Normal *SMN1* and *SMN2* genes differ in five nucleotides. About six different types of hybrid *SMN* genes have been reported in three different studies.

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		EUR/USD	≧10,000	≧10,000	≧10,000	≧5,000	≧5,000	≧10,000	≧10,000	≧10,000	≧500
ETEN	Y/N										
HN	Y/N										
YOSW	Y/N										
PSL	Y/N										
TT	Y/N										
TC	Y/N										
MY	Y/N										
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		JPY	≧1,000,000	≧1,000,000	≧1,000,000	≧500,000	≧500,000	≧1,000,000	≧1,000,000	≧1,000,000	≧50,000
		EUR/USD	≧10,000	≧10,000	≧10,000	≧5,000	≧5,000	≧10,000	≧10,000	≧10,000	≧500
HA	Y/N										
YT	Y/N										
TS	Y/N										
MS	Y/N										
	Y/N										
	Y/N										
	Y/N										
	Y/N										
	Y/N										

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