SMN2 and NAIP gene dosages in Vietnamese patients with spinal muscular atrophy

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SMN2 and *NAIP* gene dosages of Vietnamese patients with spinal muscular atrophy

Running title: *SMN2* and *NAIP* gene dosages in SMA

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Abstract

Background: The *SMN1* gene is now recognized as a spinal muscular atrophy (SMA)-causing gene, while *SMN2* and *NAIP* have been characterized as a modifying factor of the clinical severity of SMA. Gene dosage of *SMN2* is associated with clinical severity of SMA. However, the relationship between gene dosage of *NAIP* and clinical severity of SMA remains to be clarified, although complete deletion of *NAIP* is frequent in type I patients.

Methods: To evaluate the contribution of the *SMN2* and *NAIP* gene dosages to SMA, we measured using quantitative real-time PCR the copy numbers of *SMN2* and *NAIP* in 34 Vietnamese SMA patients lacking *SMN1* (13 type I, 11 type II and 10 type III patients).

Results: The *SMN2* copy number in type I patients was significantly lower than that in type II-III patients, which was compatible with the previous reports. On the other hand, 25 out of 34 patients showed only 0 or 1 copy of *NAIP*, while 50 out of 52 controls showed 2 or more copies. For *NAIP* (+) genotype, 6 out of 13 type I patients, 8 out of 11 type II patients and 6 out of 10 type III patients carried one *NAIP* copy.

Conclusions: The *SMN2* copy number was related to the clinical severity of SMA among Vietnamese patients. However, 1 *NAIP* copy, i.e., heterozygous *NAIP* deletion was common in Vietnamese SMA, regardless of their clinical phenotypes.

Key words

Spinal muscular atrophy, *SMN1*, *SMN2*, *NAIP*, gene dosage.

Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder characterized by degeneration of anterior horn cells (motor neurons) in the spinal cord, resulting in weakness of the proximal limb and trunk muscles. According to the disease severity, childhood-onset SMA is classified into three subtypes: type I (severe form, unable to sit unsupported), type II (intermediate form, unable to stand or walk unsupported) and type III (mild form, able to stand or walk unsupported).[1]

All three clinical subtypes were mapped to chromosome 5q11.2-13.3 using linkage analysis,[2-4] and the *SMN1*, *SMN2* and *NAIP* genes were subsequently identified in this SMA-related region.[5,6] *SMN1* and *SMN2* are almost identical genes that encode the same protein.[5]

To date, the SMN protein, the product of *SMN1*, has been reported to be involved in general cellular processes, such as pre-mRNA splicing, transcription, nucleocytoplasmic transportation, apoptosis and ribosomal metabolism.[19] For example, a mutation identified in a type I SMA patient, namely a glutamate-to-lysine substitution at amino acid 134 (E134K) in the central part of SMN (so-called Tudor domain), was found to prevent SMN from binding to Sm proteins.[20,21] These SMN-Sm interactions are a prerequisite for the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) prior to their entry into the nucleus, where pre-mRNA splicing takes place.[22]

SMN1 is homozygously deleted in 90% of SMA patients,[5,7,8] and deleteriously mutated in the remaining 10% of SMA patients.[9] Therefore, *SMN1* is now recognized as

an SMA-causing gene. In contrast, the *SMN2* gene has been characterized as a modifying factor of the clinical severity of SMA. The *SMN2* copy number has been reported to be higher in type II-III patients than in type I patients, suggesting that *SMN2* compensates for the loss of *SMN1* to some degree.[10-18]

Some functions of the NAIP protein, the product of *NAIP*, have been reported. For example, Götz et al.[27] showed that overexpression of NAIP protein impairs neurite outgrowth of neuronal cells, indicating that it may have an effect on the differentiation and survival of motor neurons, while Perrelet et al.[28] found that NAIP protein could prevent motor neuron degeneration after sciatic nerve axotomy in rats. These findings suggest that *NAIP* mutation is linked to motor neuron degeneration.

Homozygous *NAIP* deletion has been more frequently observed in type I patients than in type II-III patients.[6,23,24] However, Taylor et al.[14] found no differences in the age of onset and length of survival between type I patients lacking or retaining the *NAIP* gene, and concluded that the *NAIP* gene may not have an effect on the clinical severity.

However, despite the above-mentioned observations, the contribution of *NAIP* to the pathogenesis of SMA remains unclear, since its copy numbers have not been measured. To evaluate the contributions of the *SMN2* and *NAIP* genes to SMA among the Vietnamese population, we measured the copy numbers of these genes in 34 Vietnamese SMA patients lacking the *SMN1* gene and 52 healthy Vietnamese using a quantitative real-time PCR (QRT-PCR) method.

Patients and methods

Patients

A total of 34 Vietnamese SMA patients were enrolled in the study (Table 1). All of these patients fulfilled the diagnostic criteria of the International SMA Consortium,[1] and showed *SMN1* deletion according to the method of van der Steege.[25] In addition, 52 healthy Vietnamese adults without any neuromuscular symptoms volunteered to participate as controls. The study was approved by the Institutional Review Board of the Department of Endocrinology, Metabolism and Genetics, National Institute of Pediatrics, Hanoi, Vietnam. Informed consent was obtained from all subjects.

→ **Table 1**

QRT-PCR of the *SMN1, SMN2* and *NAIP* copy numbers

Genomic DNA was extracted from peripheral blood following a standard phenol-chloroform method.[29] For determination of the *SMN1*, *SMN2* and *NAIP* gene copy numbers, we established a new calibrator-normalized relative quantification method using real-time PCR and a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). *SMN1* exon 7 was amplified with a primer set of telSMNex7forw (5'-TTT ATT TTC CTT ACA GGG TTT *C*-3') [13] and telSMNint7rev (5'-GTG AAA GTA TGT TTC TTC CAC GTA-3').[13] *SMN2* exon 7 was amplified with a primer set of cenSMNex7forw (5'-TTT ATT TTC CTT ACA GGG TTT TA-3') [13] and cenSMNint7rev (5'-GTG AAA GTA TGT TTC TTC CAC GCA-3').[13] *NAIP* exon 5 was amplified with a primer set of 1863-Mod (5'-CTC TCA GCC TGC TCT TCA G-3') [6] and 1864-Mod (5'-AAA GCC TCT GAC GAG AGG ATC-3').[6] The *CFTR* gene was used as a reference for the relative quantification of *SMN1*, *SMN2*, and *NAIP*. *CFTR* was

amplified with a primer set of CF621F (5'-AGT CAC CAA AGC AGT ACA GC-3') [10] and CF621R (5'-GGG CCT GTG CAA GGA ATG TTA-3').[10]

The PCR amplifications of *SMN1*, *SMN2*, *NAIP* and *CFTR* were performed in total reaction volumes of 20 µl, containing 5 ng of genomic DNA, 10 pmol of each primer, 4 mM $MgCl_2$ and 2 µl of FastStart DNA Kit SYBR Green I (Roche Diagnostics). The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58-62°C for 10 s and 72°C for 25 s. The appropriate annealing temperatures were as follows: 58°C for *SMN1*, 62°C for *SMN2*, 60°C for *NAIP* and 60°C for *CFTR*. A fluorescence detection step was carried out at the end of the elongation step in each cycle. The analysis was performed using the second derivative maximum method of the LightCycler software (Roche Diagnostics).

Statistical analysis

The copy numbers of the *SMN1*, *SMN2* and *NAIP* genes in the controls, clinical subtypes and *NAIP* genotypes of SMA patients were compared by t-tests. A *p*-value of less than 0.05 was considered to indicate a significant difference.

Results

Establishment of a QRT-PCR assay for the *SMN1*, *SMN2* and *NAIP* copy numbers

The copy numbers of the *SMN1*, *SMN2* and *NAIP* genes were determined using a relative quantification method based on the calibrator-normalized ratios (CNRs), i.e., *CNR-SMN1*, *CNR-SMN2* and *CNR-NAIP* (Table 2). Since the relative quantification does not require fresh and intact DNA, the DNA samples sent from Vietnam to Japan can be analyzed. The gene dosages based on the CNRs were calculated by the following formula: 2× [amount of target gene amplicon (test sample) / amount of reference gene amplicon (test sample)] / [amount of target gene amplicon (calibrator sample) / amount of reference gene amplicon (calibrator sample)]. The phrase 'target gene' denotes *SMN1*, *SMN2* or *NAIP*, while 'reference gene' denotes *CFTR*. Furthermore, 'test sample' denotes genomic DNA from the participants in the study and 'calibrator sample' denotes a standard DNA sample from a healthy control with 2 *SMN1* copies, 2 *SMN2* copies and 2 *CFTR* copies.

→ Table 2

For *SMN1* and *SMN2* standards, we used 5 Japanese controls and 5 Japanese SMA patients whose copy numbers had already been determined by our previous competitive PCR method.[16] We did not have an appropriate *NAIP* standard, and therefore, we examined 52 Vietnamese controls by QRT-PCR assay and postulated that the majority in the controls carried two *NAIP* copies. The interassay and between-sample coefficients of variation (CVs) for samples from 5 unrelated individuals with two *SMN1* copies, two *SMN2* copies and two *NAIP* copies ranged from 2.24-10.9, 4.71-9.95 and 3.84-6.60, respectively.

SMN1 copy number in controls

A total of 2 *SMN1* copies were found in 47 of 52 controls (90.4%), while 3 *SMN1* copies were found in 5 of 52 controls (9.6%) (Table 3).

→ Table 3

SMN2 copy number in controls and patients

We found remarkable differences in the *SMN2* copy numbers between the SMA patients and controls. Specifically, 0 or 1 *SMN2* copy were found in 0 of 34 SMA patients (0%), compared to 24 of 52 controls (46%) (1 *SMN2* copy was found in 22 controls and 0 copies in 2 controls). In contrast, 3 or more *SMN2* copies were found in 19 of 34 SMA patients (56%), compared to only 2 of 52 controls (3.8%) (Fig. 2 and Table 3a-b). The average *SMN2* copy number was significantly higher in SMA patients than in controls (2.7 ± 0.6 vs. 1.5 ± 0.6, $p<0.001$). It should be noted that 0 *SMN2* copies were often found in controls, but never in SMA patients.

→ Fig. 1

→ Table 3

Next, the relationships between the *SMN2* copy number and clinical features were examined. Among the 34 SMA patients, 2 *SMN2* copies were found in 11 of 13 type I patients (85%), compared to only 2 of 11 type II patients (18.2%) and 2 of 10 type III patients (20%). In contrast, 3 *SMN2* copies were found in only 2 of 13 type I patients (15.4%), compared to 10 of 11 type II patients (90.9%) and 5 of 10 type III patients (50%). Moreover, 4 *SMN2* copies were only found among type III patients (30%), and not in any of the other types (Fig. 1 and Table 3b). The average *SMN2* copy numbers in

type II and type III patients were similar (2.9 ± 0.3 vs. 3.2 ± 0.7, p=0.23). We therefore classified the type II and III patients into one group, designated type II-III: the average *SMN2* copy number was significantly lower in type I patients than in type II-III patients (2.0 ± 0.3 vs. 3.0 ± 0.5, p<0.001).

NAIP copy number in controls and patients

We also found significant differences in the *NAIP* copy numbers between SMA patients and controls. Specifically, 0 or 1 *NAIP* copy were found in 25 of 34 SMA patients (73.5%), compared to only 2 of 52 controls (3.8%). In contrast, 2 or more *NAIP* copies were found in 9 of 34 SMA patients (26.5%), compared to 50 of 52 controls (96.2%) (Fig. 1 and Table 3a-b). The average *NAIP* copy number was significantly lower in SMA patients than in controls (1.1 ± 0.5 vs. 2.3 ± 0.6, p<0.001).

Among the 34 SMA patients, 0 *NAIP* copies were found in 4 of 13 type I patients (30.8%), compared to 0 of 11 type II patients (0%) and only 1 of 10 type III patients (10%). Furthermore, 1 *NAIP* copy was found in 6 of 13 type I patients (46.1%), 8 of 11 type II patients (72.7%) and 6 of 10 type III patients (60%) (Fig. 1 and Table 3b). 20 out of 29 patients with *NAIP* (+) genotypes carried only one *NAIP* copy, regardless of clinical severity (6 type I, 8 type II and 6 type III patients). Among the 29 patients with *NAIP* (+) genotypes, the average *NAIP* copy numbers did not differ significantly among type I, type II and type III patients (1.2 ± 0.4, 1.2 ± 0.4 and 1.3 ± 0.4, respectively).

Combinations of *SMN1, SMN2* and *NAIP* genotypes based on each copy number

The genotypes of the *SMN1 SMN2* and *NAIP* copy numbers were combined. A total of 12 different patterns were observed among the 52 controls (Table 3a), with the

following two main patterns: 2*SMN1*-2*SMN2*-2*NAIP* (49%) and 2*SMN1*-1*SMN2*-2*NAIP* (25%).

Among the 34 SMA patients, 8 different patterns were observed (Table 3b). The main genotypes in type I patients were 0*SMN1*-2*SMN2*-1*NAIP* (5 of 13 type I patients, 39%) and 0*SMN1*-2*SMN2*-0*NAIP* (4 of 13 type I patients, 31%). Both type II and type III patients frequently showed the genotype of 0*SMN1*-3*SMN2*-1*NAIP* (6 of 11 type II patients (54.5%); 3 of 10 type III patients (30%)).

Discussion

A correlation between the *SMN2* copy number and the clinical phenotype of SMA has previously been reported.[13] This finding was also observed in the present study. Specifically, type II-III SMA patients have higher *SMN2* copy numbers than type I SMA patients and controls. These findings can be explained by a gene conversion event, in which conversion of *SMN1* to *SMN2* causes an 'apparent' deletion of the *SMN1* gene, resulting in an increase in the *SMN2* copy number. The expression of the *SMN2* gene then compensates for the loss of the *SMN1* gene, leading to a mild phenotype of SMA.

We failed to find 0 or 1 *SMN2* copy in our 34 Vietnamese SMA patients lacking the *SMN1* gene. This situation arises partly because absence of both the *SMN1* and *SMN2* genes is believed to be lethal in utero, since *Smn* knockout mice show embryonic lethality,[26] and partly because patients with 0 *SMN1* and 1 *SMN2* copy are not expected to live beyond 1 month of age.[27]

Our present report is the first to document the results of a copy number analysis of the *NAIP* gene in SMA patients. The majority of SMA patients lacking *SMN1* carried one *NAIP* copy, irrespective of the clinical severity, indicating the presence of the gene on one chromosome and its absence on the other. This presence and absence of *NAIP* possibly reflects the extent of deletion, in which a large deletion involves both *SMN1* and *NAIP* and a small deletion only involves *SMN1*. This idea is highly plausible since *SMN1* and *NAIP* are adjacent to each other.

A small deletion, i.e. *NAIP* retention, may be associated with gene conversion of *SMN1* to *SMN2*, which would lead to apparent deletion of *SMN1*. However, we also revealed that gene conversion can occur on chromosomes with a large deletion, i.e. *NAIP* deletion. In the present study, one of our type III patients lacking *SMN1* showed 3

copies of *SMN2* and 0 copies of *NAIP* (Table 1), and we previously reported another type III SMA patient who showed 0 *SMN1* copies, 4 *SMN2* copies and 0 *NAIP* copies.[18] In these cases, gene conversion of *SMN1* to *SMN2* and deletion of *NAIP* seem to have occurred together on one chromosome.

According to our calculations, *NAIP* deletion occurred on 30 of 68 chromosomes among the SMA patients (44%), but only 2 of 104 chromosomes among the healthy controls (1.5%). Therefore, *NAIP* deletion occurred much more frequently in SMA patients than in healthy controls, suggesting that *NAIP* deletion is associated with *SMN1*-deleted chromosomes in more than 40% of SMA patients.

However, among our patients with *NAIP* (+) genotypes, the average *NAIP* copy numbers did not differ significantly between type I and type II-III patients. These findings strongly suggest that the *NAIP* copy number does not change the clinical severity of SMA and that the *NAIP* gene itself does not appear to be related to SMA severity.

One might argue that, without considering the *SMN2* copy number, comparing the average *NAIP* copy number among the subtypes could not support the conclusion described above. However, the data in table 3b shows that the conclusion would not change even after considering the *SMN2* copy number.

In conclusion, the majority of SMA patients lacking *SMN1* carried one *NAIP* copy, indicating that heterozygous *NAIP* deletion is common in SMA patients, regardless of the clinical severity. Furthermore, our results show that the *SMN2* copy number, but not the *NAIP* copy number, is associated with the severity of SMA. However, in this study, we did not evaluate the qualitative changes (ex. point mutations) in *NAIP* affecting the protein activity, which remains to be studied.

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Figure Legends

Figure 1 Genotype frequencies based on the *SMN2* and *NAIP* copy numbers in each clinical subtype.

Table 1: The clinical information and gene dosage data of Vietnamese SMA patients lacking SMN1

Patient	Sex	Type	Age at onset	Age at death (DE) or follow up (FU)	Gene deletion test			Gene dosage analysis			
					SMN1		NAIP	SMN2		NAIP	
					exon 7	exon 8	exon 5	CNR-SMN2 values	SMN2-copy number	CNR-NAIP values	NAIP-copy number
1	M	I	1 m	2 m (DE)	del	del	non-del	1.94	2	1.07	1
2	F	I	2.5 m	5 m (DE)	del	del	non-del	2.29	2	1.16	1
3	F	I	2.5 m	4 m (DE)	del	del	del	1.91	2	0	0
4	M	I	3.5 m	5 m (FU)	del	del	non-del	1.82	2	1.14	1
5	F	I	1 m	2 m (DE)	del	del	non-del	1.94	2	1.65	2
6	F	I	5 m	8 m (DE)	del	del	del	2.02	2	0	0
7	F	I	2 m	5 m (DE)	del	del	non-del	1.7	2	0.84	1
8	F	I	1 m	4 m (DE)	del	del	non-del	2.06	2	1.76	2
9	M	I	2 m	8 m (DE)	del	del	non-del	2.69	3	1.81	2
10	M	I	1 m	4 m (DE)	del	del	del	1.69	2	0	0
11	M	I	6 m	7 m (DE)	del	del	del	1.81	2	0	0
12	F	I	2 m	4 m (DE)	del	del	non-del	1.81	2	0.81	1
13	M	I	3 m	5 m (DE)	del	del	non-del	2.76	3	0.97	1
14	F	II	6 m	20 m (FU)	del	del	non-del	2.9	3	0.93	1
15	F	II	11 m	4 y (FU)	del	del	non-del	3.24	3	0.96	1
16	F	II	10 m	30 m (FU)	del	del	non-del	3.18	3	1.04	1
17	M	II	12 m	4 y (FU)	del	del	non-del	2.9	3	1.74	2
18	M	II	10 m	24 m (FU)	del	del	non-del	2.44	2	0.85	1
19	M	II	14 m	4 y (FU)	del	del	non-del	3.02	3	1.07	1
20	M	II	12 m	4 y (FU)	del	del	non-del	3.04	3	1.98	2
21	M	II	11 m	4 y (FU)	del	del	non-del	3.09	3	1.97	2
22	M	II	14 m	8 y (FU)	del	del	non-del	2.01	2	0.92	1
23	F	II	10 m	3 y (FU)	del	non-del	non-del	3.11	3	0.81	1
24	F	II	6 m	25 m (FU)	del	del	non-del	2.98	3	0.78	1
25	M	III	2 y	4 y (FU)	del	del	non-del	2.31	2	1.96	2
26	F	III	6 y	11 y (FU)	del	del	non-del	2.33	2	0.91	1
27	F	III	8 y	18 y (FU)	del	del	non-del	2.96	3	1.86	2
28	F	III	4 y	8 y (FU)	del	del	non-del	3.38	3	0.96	1
29	M	III	5 y	10 y (FU)	del	del	non-del	4.26	4	1.29	1
30	F	III	2 y	4 y (FU)	del	del	non-del	2.95	3	1.05	1
31	M	III	2 y	4 y (FU)	del	del	del	3.12	3	0	0
32	M	III	3 y	8 y (FU)	del	non-del	non-del	4.39	4	1.79	2
33	M	III	5 y	8 y (FU)	del	del	non-del	2.7	3	0.94	1
34	F	III	2 y	8 y (FU)	del	del	non-del	3.62	4	0.76	1

del: deletion; non-del: non-deletion; CNR: calibrator-normalized ratio (as explained in the text)

Table 2. Mean Values ± SD and CVs of CNR-SMN1, CNR-SMN2 and CNR-NAIP values in 86 individuals, including 52 controls and 34 SMA patients

Genes	Copy number	Individual number	Min/Max	Mean ± SD	CV (%)
SMN1	2	47	1.51/2.29	1.94 ± 0.16	8.46
	3	5	2.69/2.94	2.82 ± 0.08	3.09
SMN2	0	2	0	0	0
	1	22	0.69/1.35	1.03 ± 0.17	16.7
	2	41	1.602.327	1.98 ± 0.19	9.87
	3	18	2.67/3.37	2.98 ± 0.19	6.54
	4	3	3.61/4.39	4.09± 0.42	10.15
NAIP	0	5	0	0	0
	1	22	0.76/1.29	0.96 ± 0.14	14.17
	2	51	1.61/2.44	1.99 ± 0.25	10.75
	3	4	2.79/3.32	3.04 ± 0.22	7.43
	4	4	3.72/4.32	4.02 ± 0.42	10.47

CNR: calibrator-normalized ratio (as explained in the text)
CV: coefficient of variation

Table 3: Combined genotypes in SMA patients and controls

a: Controls

	Genotype SMN1-SMN2-NAIP	Control number (%)
1	2 — 0 — 2	1 (2%)
2	2 — 0 — 4	1 (2%)
3	2 — 1 — 1	2 (3.5%)
4	2 — 1 — 2	13 (25%)
5	2 — 1 — 3	2 (3.5%)
6	2 — 1 — 4	1 (2%)
7	2 — 2 — 2	25 (49%)
8	2 — 2 — 3	1 (2%)
9	2 — 3 — 2	1 (2%)
10	3 — 1 — 2	2 (3.5%)
11	3 — 1 — 4	2 (3.5%)
12	3 — 3 — 3	1 (2%)
Total		52 (100%)

b: SMA patients

	Genotype SMN1-SMN2-NAIP	Patient number (%)	Patient number of each clinical subtype		
			Type I	Type II	Type III
1	0 — 2 — 0	4 (12 %)	4	0	0
2	0 — 2 — 1	8 (23%)	5	2	1
3	0 — 2 — 2	3 (9%)	2	0	1
4	0 — 3 — 0	1 (3%)	0	0	1
5	0 — 3 — 1	10 (29%)	1	6	3
6	0 — 3 — 2	5 (15%)	1	3	1
7	0 — 4 — 1	2 (6%)	0	0	2
8	0 — 4 — 2	1 (3%)	0	0	1
Total		34 (100%)	13	11	10

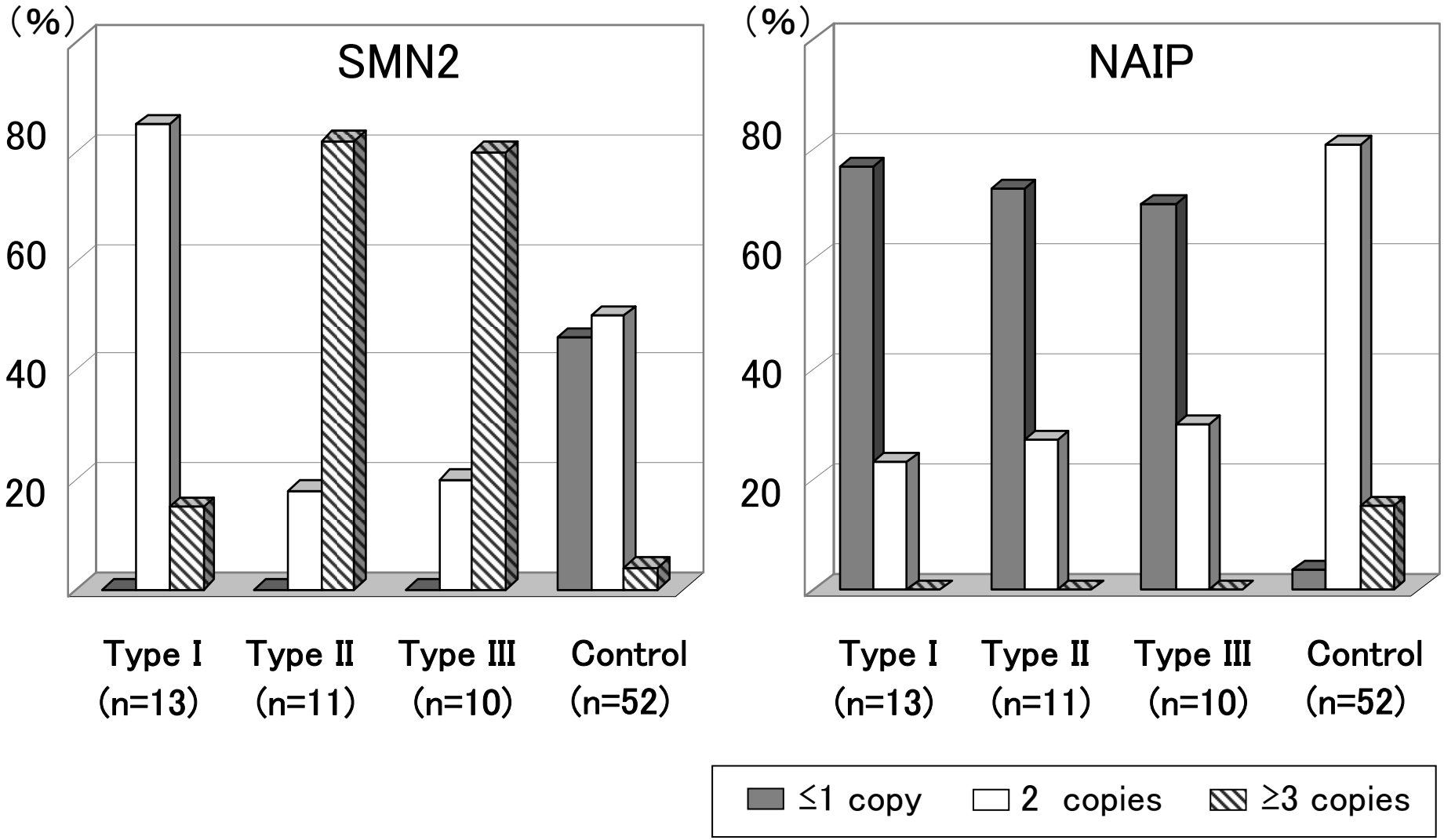

Figure 1　Genotype frequencies based on the *SMN2* and *NAIP* copy numbers in each clinical subtype