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Molecular Genetics of Spinal Muscular Atrophy: Contribution of the *NAIP* Gene to Clinical Severity

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Spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders characterized by degeneration of anterior horn cells in the spinal cord, and leads to progressive muscular weakness and atrophy. At least three SMA-related genes have been identified: *SMN1*, *NAIP* and *p44t*. We analyzed these genes in 32 SMA patients and found that the *SMN1* gene was deleted in 30 of 32 patients (94 %), irrespective of clinical type. The *NAIP* gene was deleted in 6 patients and its deletion rate was higher in type I patients than that in type Ⅱ or Ⅲ. Further, in type I patients lacking the *NAIP* gene, deterioration in their respiratory function is more rapid than in those type I patients retaining the *NAIP* gene. Since complete *p44t* deletion was observed in only 3 patients, the correlation between the *p44t* deletion and severity of SMA remained ambiguous. We concluded that the *NAIP* deletion was closely related to the clinical severity of SMA and was a predictive marker of SMA prognosis, while the *SMN1* deletion did not correlate with clinical severity.

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders (1 in 10,000 newborns (8)) resulting from the degeneration of anterior horn cells of the spinal cord, and leads to progressive muscular weakness and atrophy. SMA is clinically divided into three subtypes depending on age at onset and clinical course (19). Type I SMA (Werdnig-Hoffmann disease) is the severest form and is recognized within the first 6 months of age; type I patients are never able to sit without aid. Type Ⅱ SMA is an intermediate form whose onset is before 18 months of age; type Ⅱ patients are never able to stand or walk without aid. Type Ⅲ SMA (Kugelberg-Welander disease) is the mildest form and manifest after 18 months; type Ⅲ patients become ambulant but they may have difficulty with gait. Genetic linkage studies have mapped responsible genes for all three clinical types of SMA to chromosome 5q13 (2,16,17), with at least three SMA-related genes having so far been identified in the SMA critical region; the survival motor neuron (*SMN*) gene (13), the neuronal apoptosis inhibitory protein (*NAIP*) gene (22) and the *p44t* gene (3,6).

The *SMN* gene is present in two highly homologous copies within the SMA region, the *SMN1* (telomeric *SMN*) and *SMN2* (centromeric *SMN*) genes (13). Both copies generate 1.7

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kb mRNAs which code identical amino acid sequences. In control subjects, the *SMN1* gene is almost always present whereas the *SMN2* gene is absent in about 4%, without any pathological consequence (13). Single nucleotide changes in exons 7 and 8 allow the *SMN1* and *SMN2* genes to be distinguished. The *SMN1* gene is lacking or interrupted in more than 90% of SMA patients (13,27,29) and deleteriously mutated in the remaining (1,4,13,24), providing strong evidence that this gene is an SMA-determining gene.

The *NAIP* gene lies in the region adjacent to the *SMN1* gene and is present in the SMA region with multiple pseudogenes, which apparently arise independently of the inverted duplication (22). The centromeric truncated *NAIP* gene, *NAIP* ϕ, lacks the two coding exons, exons 5 and 6 (22). Approximately 2 to 5 copies of intact or truncated *NAIP* genes have been identified in the general population (22). The functional role of the *NAIP* gene in the pathogenesis of SMA has not been elucidated. Although some reports have demonstrated a correlation between deletion of the *NAIP* gene and severity of SMA (22,27,29), the *NAIP* deletion has been found in controls with no phenotypic evidence of SMA (22).

The third gene encoding p44 protein, which is a subunit of the basal transcription factor TFIIH, has been cloned from the SMA critical region (3,6). TFIIH is implicated in basal transcription, DNA repair and cell cycle control. Like the *SMN* and *NAIP* genes, the *p44* gene also exists in at least two copies within the SMA region, the telomeric (*p44t*) and centromeric (*p44c*) copies. Little has been reported on involvement of the *p44t* gene in SMA.

In the present study, to elucidate the correlation between genotype and clinical severity in SMA patients, we analyzed the molecular genetic features of 32 Japanese patients with SMA, from 30 unrelated families.

PATIENTS AND METHODS

Patients.

After obtaining informed consent we analyzed the molecular genetic features of the 32 Japanese patients with SMA from 30 unrelated families (Table Ⅰ). All patients fulfilled the diagnostic criteria of SMA as defined by the International SMA Consortium (19). Ten patients had type I, 18 patients type Ⅱ and 4 patients type Ⅲ SMA. The 18 patients with type Ⅱ SMA included two pairs of affected siblings.

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

Genomic DNA was extracted from 3 ml of whole blood using DNA extraction kits, Genomix ® (Talent, Trieste, Italy) and SepaGene® (Sanko Junyaku Co., Ltd, Tokyo, Japan). Polymerase chain reaction (PCR) amplification was performed according to the method of van der Steege *et al.* (26). The oligonucleotide primers for exon 7 of the *SMN1* and *SMN2* genes were R111 (13) and X7-Dra (26), and those for exon 8 of the *SMN1* and *SMN2* genes were 541C950 (13) and 541C1120 (13). The restriction enzymes *Dra I* and *Dde I* cleaved the PCR-amplified fragments from *SMN 2* exons 7 and 8, respectively. To discriminate between *SMN1* and *SMN2* gene products, the PCR products were digested with *Dra I* for exon 7 and *Dde I* for exon 8, and the digested products were electrophoresed in 3 % agarose gels and visualized by ethidium bromide staining.

PCR amplification of *NAIP* gene exon 5.

PCR amplification of the *NAIP*-specific sequence, exon 5, was performed according to the method of Roy *et al.* (22). Here we adopted “exon 5” as a widely accepted exon number, although this exon has been noted as “exon 4” by Chen *et al*. (7). PCR products were electrophoresed in 3 % agarose gels and visualized by ethidium bromide staining.

Detection of telomeric *p44* gene deletion by PCR and enzyme-digestion analysis.

PCR amplification of the *p44t* and *p44c* genes was performed according to the method of Bürglen *et al.* (3). The oligonucleotide primers used in this study were 44SP (3) and 44D3 (3). Deletion or interruption of the telomeric copy at codon 151 abolishes the *Alu I* restriction site in the *p44* gene. To discriminate between *p44t* and *p44c* gene products, PCR product was digested with *Alu I*, and the digested products were electrophoresed in 3 % agarose gels and visualized by ethidium bromide staining.

Statistics.

The frequencies of *NAIP* deletion in SMA subtypes were compared by use of a chi-square test on Fisher's exact probability test. A value of p less than 0.05 was considered to indicate a significant difference.

Table Ⅰ. Clinical features and genotypes of 32 patients.

Case	Type	Onset Age	Sex	Outcome	*SMN1*		*NAIP*	*p44t*
					exon 7	exon 8	exon 5	
1	Ⅰ	0 m	M		del	del	non-del	non-del
2	Ⅰ	1 m	F	Died at 2 m	del	del	del	non-del
3	Ⅰ	2 m	M	Died at 4 m	del	del	del	non-del
4	Ⅰ	3 m	M	ARC at 5 m	del	del	del	del
5	Ⅰ	4 m	F	Died at 6 m	del	del	non-del	non-del
6	Ⅰ	4 m	M	ARC at 6 m	del	del	del	non-del
7	Ⅰ	4 m	M		del	del	non-del	non-del
8	Ⅰ	4 m	F	ARC at 26 m	del	del	non-del	non-del
9	Ⅰ	5 m	F	ARC at 32 m	non-del	non-del	non-del	non-del
10	Ⅰ	5 m	M	ARC at 13 m	del	del	non-del	non-del
11	Ⅱ	4 m	F		del	non-del	non-del	non-del
12	Ⅱ	4 m	F		del	non-del	non-del	non-del
13	Ⅱ	5 m	F		del	non-del	non-del	non-del
14	Ⅱ	6 m	M		del	del	non-del	non-del
15	Ⅱ	9 m	F		del	del	non-del	non-del
16	Ⅱ	9 m	M		del	del	non-del	non-del
17	Ⅱ	10 m	M		del	del	non-del	non-del
18	Ⅱ	10 m	M		del	del	non-del	non-del
19	Ⅱ	10 m	M		del	del	non-del	non-del
20	Ⅱ	10 m	M		del	del	non-del	non-del
21	Ⅱ	12 m	F	Died at 21 y	del	del	del	del
22	Ⅱ	12 m	F		del	del	del	del
23	Ⅱ	12 m	M		del	del	non-del	non-del
24	Ⅱ	14 m	F		del	del	non-del	non-del
25	Ⅱ	15 m	M		non-del	non-del	non-del	non-del
26	Ⅱ	18 m	M		del	del	non-del	non-del
27	Ⅱ	18 m	M		del	del	non-del	non-del
28	Ⅱ	18 m	F		del	del	non-del	non-del
29	Ⅲ	19 m	M		del	non-del	non-del	non-del
30	Ⅲ	3 y	F		del	del	non-del	non-del
31	Ⅲ	10 y	F		del	del	non-del	non-del
32	Ⅲ	19 y	M		del	del	non-del	non-del

del; deleted, non-del; not deleted,
ARC; artificial respiratory care
Case 19 and 24, and case 21 and 22 were siblings in two different families.

RESULTS

Genetic analyses of the 32 Japanese SMA patients enrolled in this study are summarized in Tables Ⅰ and Ⅱ. As shown in Table Ⅱ, 30 of the 32 SMA patients (94%), independent of clinical subtypes, were homozygous for deletion of *SMN1* exon 7. Twenty-six patients lacked *SMN1* exons 7 and 8. Four patients (3 type II and 1 type III) lacked *SMN1* exon 7 but retained *SMN1* exon 8 (20). We did not find any correlation between the *SMN1* deletion and clinical severity of SMA.

Table Ⅱ. Gene deletion pattern and clinical subtype.

SMN1		*NAIP*	Clinical Subtype		
exon 7	exon 8	exon 5	Ⅰ	Ⅱ	Ⅲ
del	del	del	4 (40%)	2 (11%)	0 (0%)
del	del	non-del	5 (50%)	12 (67%)	3 (75%)
del	non-del	non-del	0 (0%)	3 (17%)	1 (25%)
non-del	non-del	non-del	1 (10%)	1 (5%)	0 (0%)
			10 (100%)	18 (100%)	4 (100%)

del; deleted, non-del; not deleted

NAIP exon 5 was deleted in 6 of 32 SMA patients (19%, 4 type I and 2 type Ⅱ, Tables Ⅰ and Ⅱ). As shown in Table Ⅱ, in all patients with a deletion of *NAIP* exon 5, there was also a deletion of *SMN1* exons 7 and 8.

The 2 type Ⅱ patients with *NAIP* deletion were sisters in the same family. When the patients in the same family are counted as one patient, frequency of *NAIP* deletions was significantly higher in type I patients than in type Ⅱ or Ⅲ patients ($p=0.03$). Five type I patients, the severest phenotype, died or required artificial respiratory care before the age of 6 months. Of these five patients, 4 lacked the *NAIP* gene.

The *p44t* gene was deleted in only 3 of the 32 SMA patients (9%, 1 type I and 2 type Ⅱ, Table Ⅰ). Because of the small number of the patients lacking *p44t*, we could not elucidate any correlation between the deletion of *p44t* and the clinical severity of SMA. In 2 of the 32 patients (6%), we did not detect any deletion of these 3 genes (Table Ⅰ).

DISCUSSION

In the present study, the *NAIP* deletion rate was higher in type I patients than that in type Ⅱ or Ⅲ patients, while the *SMN1* gene was deleted in most patients irrespective of clinical subtypes. This is compatible with reports from various countries including Japan (9,11,22,23,27).

However, Taylor *et al.* placed the focus on type I patients and reported that there was no difference in age of onset and length of survival in type I patients lacking or retaining the *NAIP* gene (25). In our study, all four type I patients lacking the *NAIP* gene had the severest phenotype (died or required artificial respiratory care before 6 months of age), while only one of six patients retaining the *NAIP* gene (Case No. 5) showed the severest phenotype. Our findings indicated that, in type I patients lacking the *NAIP* gene, deterioration in their respiratory function is more rapid than those type I patients retaining the *NAIP* gene.

The role of the *NAIP* gene has not been elucidated in the pathogenesis of SMA. To date, several experiments have suggesting that the *NAIP* gene exerts effects on differentiation and survival of neuronal cells including motor neurons (10,14,18). Further, a close correlation between the regional distribution of NAIP in CNS and the neurodegenerative alterations in SMA has been reported (21,28). These findings could be of relevance concerning a potential

role of the *NAIP* gene as a modifying factor in the pathogenesis of SMA, but further studies will be necessary to clarify this functional role.

On the other hand, the *SMN2* copy number has been well established as a modifying factor of clinical severity, many researchers reporting that a higher number of *SMN2* copies are observed in type Ⅱ or Ⅲ SMA patients than in type I patients (5,15). Hsieh-Li *et al*. demonstrated in SMA model mice that there was a strong correlation between the copy number of the human *SMN2* transgene and phenotype (12). These reports concluded that the copy number of the *SMN2* gene influenced severity of SMA by way of compensation for the lack of the *SMN1* gene.

A question arises whether the *NAIP* deletion is linked to the decrease of the *SMN2* copy number. Campbell *et al.*, using pulsed-field gel electrophoresis, reported a simultaneous deletion of *SMN1* and *NAIP* genes in type I patients (5). They also reported the presence of the *NAIP* gene and an increase of *SMN2* copy number in type Ⅱ or Ⅲ patients, suggesting that type Ⅱ or Ⅲ patients with the *NAIP* gene retain a total copy number of the *SMN* genes by a mechanism of gene conversion of *SMN1* to *SMN2*. Thus, deletion of the *NAIP* gene is often accompanied by a decrease of the total copy number of *SMN* genes in type I SMA and the presence of the *NAIP* gene, by an increase of the *SMN2* copy number or retention of total copy number of the *SMN* genes, in type Ⅱ or Ⅲ patients.

In conclusion, we have shown that deletion of the *NAIP* gene is closely related to the clinical severity of SMA, while the deletion of *SMN1* does not correlate with clinical severity. Although the function of the *NAIP* gene remains to be clarified, we have indicated that the *NAIP* gene can be a significant predictive marker of SMA prognosis.

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