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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

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 TFIIF, has been cloned from the SMA critical region (3,6). TFIIF 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 *SMN2* 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	<i>SMN1</i>		<i>NAIP</i>	<i>p44t</i>
					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.

<i>SMN1</i>		<i>NAIP</i>	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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