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Phenotype modifiers of spinal muscular atrophy: the number of *SMN2* gene copies, deletion in the *NAIP* gene and probably gender influence the course of the disease

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Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by mutations of the *SMN1* gene. It is characterized by significant phenotype variability. In this study, we analyzed possible phenotype modifiers of the disease — the size of the deletion in the SMA region, the number of *SMN2* gene copies, as well as the effect of gender. Among the factors analyzed, two seem to influence the SMA phenotype: the number of *SMN2* gene copies and a deletion in the *NAIP* gene. A higher number of *SMN2* copies makes the clinical symptoms more benign, and the *NAIP* gene deletion is associated with a more severe phenotype. The influence of gender remains unclear. In a group of 1039 patients, 55% of whom were male, the greatest disproportion was in the SMA1 (F/M=0.78) and SMA3b (F/M=0.45) forms. In SMA1 a deletion in the *NAIP* gene was seen twice as frequently in girls compared to boys. In three patients, we observed genotypes atypical for the chronic forms of SMA: two patients with SMA3a and 3b had a deletion of the *NAIP* gene, and a third patient with SMA2 had one copy of the *SMN2* gene.

Keywords: SMA modifiers, *SMN2* gene copy number, *NAIP* deletion, gender influence

INTRODUCTION

Infantile and juvenile spinal muscular atrophy (SMA) is one of the most frequent autosomal recessive genetic disorders. The incidence of SMA is estimated at 1 per 5000 to 10000 births (Thieme *et al*., 1993; 1994). The disorder leads to a loss of lower motor neurons, resulting in muscle wasting and atrophy (Dubowitz, 1995). However, the age at the onset of the disease and the severity of clinical symptoms are highly variable. According to the International SMA Consortium, three clinical forms are distinguished: the severe form or Werdnig-Hoffmann disease (SMA1, children are never able to sit unaided), the intermediate form (SMA2, children sit but never walk) and the mild form or SMA3, in which weakness develops in patients who were previously able to walk unassisted (Munsat & Davies, 1992). There are two subgroups in the third form — SMA3a (clinical symptoms before 3 years of age) and 3b (onset between 3 and 20 years of age). This distinction is related to the observation that children in whom the disease manifests at the age of more than 3 years maintain their ability to walk unaided for a longer time (Zerres *et al*., 1997). The probability of maintaining the ability to walk unassisted after 10, 20 and 40 years of the disease is 73%, 44% and 34%, respectively, in SMA 3a, and 97%, 89% and

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Abbreviations: NAIP, neuronal apoptosis inhibitor protein; SMA, spinal muscular atrophy; SMN1, survival of motor neuron 1; SMN2, survival of motor neuron 2.

67% in SMA 3b. Apart from these three basic clinical forms, two other forms have been described in the literature: an inborn form or SMA0 in which symptoms are already present in the foetus, and an adult form or SMA4, with variously defined age of onset (from 20 to 30 years) (Brahe *et al*., 1995, Zerres *et al*., 1995, Macleod *et al*., 1999; Dubowitz *et al*., 1999).

All the mentioned forms of spinal muscular atrophy are associated with mutations of the *SMN1* gene, located in the SMA region (Lefebvre *et al*., 1995). This region is located on the long arm of chromosome 5 (5q12.3) and is composed of two similar segments – the centromeric and the telomeric one. The *SMN1* gene is found in the telomeric segment, and its equivalent, the *SMN2* gene, in the centromeric segment. In addition, there are several other genes in this region: *NAIP*, *H4F5* and *p44*, and many repeated sequences and pseudogenes, making it genetically unstable (Roy *et al*., 1995; Bürglen *et al*., 1997; Scharf *et al*., 1998). Over 95% of the mutations responsible for SMA are biallelic absence of exon 7 of the *SMN1* gene (Lefebvre *et al*., 1995). Such a high homogeneity of the molecular basis is inconsistent with the phenotype variability of the disease and stimulated us to search for potential phenotype modifiers. Initially, the variability was thought to be linked to the size of the deletion in the SMA region (Lefebvre *et al*., 1995). It seemed that large deletions including neighboring genes such as *NAIP* might cause the severe form of the disease. This explanation was deemed probable as the *NAIP* gene belongs to a group of apoptosis inhibitors, so its damage might be involved in the loss of spinal motor neurons. Later on, phenotype variability began to be linked to the variable number and quality of *SMN* genes (Burghes, 1997). *SMN1* and *SMN2* genes are bigeminally similar. Their sequences differ by five nucleotides, of which only the change in exon 7 is functionally significant. The *SMN* genes may undergo conversions, leading to formation of hybrid genes. Burghes (1997) proposed that the severe form of the disease might be caused by true deletions in the SMA region, and the mild form — by conversion of *SMN* forms. Since the *SMN1* and *NAIP* deletions and the 1 or 2 *SMN2* copies (an average number present in the population) are mostly seen in the severe form, this is an evidence of a true deletion including the entire telomeric section of the SMA region, with the centromeric part remaining intact. In turn, the 3 or 4 *SMN2* copies present in patients with the mild form of the disease probably result from the conversion of *SMN1* into *SMN2*. In line with this, studies regarding phenotype modifiers concentrated solely on the number of *SMN2* copies. A strong correlation was shown between the number of *SMN2* copies and the disease phenotype. Patients with the severe form usually have 1 or 2 copies, those with the intermediate form have 2 or 3 copies, and those with the mild form have 3–4 or even 5 or 6 copies (Feldkötter *et al*., 2002; Mailman *et al*., 2002; Yamashita *et al*., 2004). However, the influence of the number of *SMN2* copies is not implicit, e.g. three *SMN2* copies have been observed in both SMA1 and SMA3. Probably the *SMN2* copies are not functionally equivalent and produce various amounts of a fully functional SMN protein (Harada *et al*., 2004).

Apart from the evident influence of the differences in the SMA region on the phenotype, the effect of gender on the disease phenotype has also been noted in the literature. It has been observed that women compared to men are half as often affected with the mild form and the protective effect of estrogens has been suggested (Hausmanowa-Petrusewicz *et al*., 1984). In addition, cases of asymptomatic biallelic mutation of the *SMN1* gene occur more frequently in women than in men (Helmken *et al*., 2003).

Despite many studies on the phenotype variability, to date no simultaneous analysis of all putative phenotype modifiers mentioned above has been carried out. Indeed, any effect of the size of the deletion in the SMA region has been negated. The effect of gender also remains unclear. Therefore, we decided to carry out a simultaneous analysis of the size of deletion of the SMA region, the number of *SMN2* copies and the effect of gender on the SMA phenotype.

PATIENTS AND METHODS

Gender analysis was performed in a group of 1039 patients diagnosed in our centers, which included 204 cases with SMA1 (114 males (M), 90 females (F)), 330 — with SMA2 (171M, 159F), 298 — with SMA3a (142M, 156F), 206 — with SMA3b (142M, 64F) and one female with SMA4 (disease onset at the age of 34 years). Extended molecular studies were carried out in a group of 240 patients, including 86 patients with SMA1 (38F, 48M), 68 with SMA2 (26F, 42M), 48 with SMA3a (21F, 27M), 37 with SMA3b (13F, 24M) and 1 patient (F) with SMA4. Molecular studies included analysis of the size of the deletion in the SMA region (presence of a deletion of exons 7 and 8 of the *SMN1* gene and exons 5 and 6 of the *NAIP* gene) and the number of *SMN2* copies.

Genomic DNA was collected from the peripheral blood of SMA patients. DNA was isolated from blood samples by salting-out procedure (Miller *et al*., 1988). Homozygous absence of exons 7 and 8 of *SMN1* was detected using restriction analysis, as described elsewhere (Scheffer *et al*., 2001). Deletion

of the *NAIP* gene was analyzed using exon 5 and 13 primers as previously described (Roy *et al*., 1995). For *SMN1* and *SMN2* dosage analysis, the method described by Anhuf *et al*. (2003) was employed with some modification (Jędrzejowska *et al*., 2008).

Statistical analysis was performed using multifactorial logistic regression analysis with step-wise strategy of factor selection, log-linear models, and Fisher exact statistics (Armitage, 2002). Correlations between variables were measured using Spearman coefficients.

RESULTS

Size of the deletion in the SMA region

In the study group of 240 patients, a large deletion including the *NAIP* gene was found in 48 patients, an isolated loss of exon 7 of the *SMN1* gene in 38 patients, and in the remaining 154 patients a deletion of exons 7 and 8 of *SMN1* was found, with the *NAIP* gene intact (Table 1).

The deletion in the *NAIP* gene correlated with the severity of clinical symptoms (Fisher's exact test, $P<0.0001$). The *NAIP* gene was absent in the majority (73%) of patients with the severe form. In the mild form (3a and 3b), only two such deletions were found (described in the following section). On the other hand, an isolated absence of exon 7 was seen with a similar frequency in all forms of SMA.

Number of copies of the *SMN2* gene

The presence of one copy of the *SMN2* gene was shown in 4 patients (1.67%) in the studied group, of two copies in 50 patients (20.83%), three copies in 131 patients (54.58%), four copies in 53 patients (22.08%) and five copies of the gene in two patients (0.83%) (Tables 1 and 2).

The number of *SMN2* gene copies correlated with the phenotype of the disease. The greater the number of *SMN2* copies, the more benign was the phenotype (Spearman's correlation coefficient $r=0.74$, $P<0.05$). In the severe form, one or two *SMN2* copies (59% patients) were most frequently seen, but three copies of the gene (41%) were also quite frequent. In SMA2, 84% of patients had three copies of the gene. In SMA3a, three or four *SMN2* copies were seen (in 66% and 33% patients, respectively). In SMA 3b, four *SMN2* copies (78%) were most frequently seen. In one patient with SMA2 (the case is described below), the presence of one *SMN2* copy was demonstrated.

Gender of patients

Overall, male patients with SMA outnumbered female patients (470F *vs*. 569M). This was particularly true for the benign form with the onset at the age of over 3 years (64F *vs*. 142M, $P<0.0001$) as well as the severe form – SMA1 (90F *vs*. 114M) ($P=0.0042$, compared to the proportion in the gener-

Table 1. Number of *SMN2* gene copies and the size of deletion in SMA region in particular types of SMA by gender.

The disease phenotype correlated with the number of *SMN2* copies and the deletion of the *NAIP* gene. When gender proportions were taken into account in particular forms, we found a higher percentage of girls with the severe form, two *SMN2* copies and a deletion including the *NAIP g*ene. M, male; F, female.

Type of SMA	Size of deletion	Number of *SMN2* gene copies					Overall	
		1	2	3	4	5		
SMA1	NAIP	2 (2M)	27 (18F, 9M)	6 (3F, 3M)	0	0	35 (21F, 14M)	86 (38F, 48M)
	Exons 7 and 8	1 (1F)	18 (8F, 9M)	24 (7F, 17M)	0	0	42 (16F, 26M)	
	Exon 7	0	3 (3M)	6 (1F, 5M)	0	0	9 (1F, 8M)	
SMA2	NAIP	0	2 (2M)	9 (2F, 7M)	0	0	11 (2F, 9M)	68 (26F, 42M)
	Exons 7 and 8	1 (1M)	0	38 (15F, 23M)	4 (2F, 2M)	0	43 (17F,26M)	
	Exon 7	0	1 (1F)	10 (4F, 6M)	3 (2F, 1M)	0	14 (7F, 7M)	
SMA3a	NAIP	0	0	1 (1M)	0	0	1 (1M)	48 (21F, 27M)
	Exons 7 and 8	0	0	24 (13F, 11M)	13 (4F, 9M)	0	37 (17F, 20M)	
	Exon 7	0	0	7 (4F, 3M)	3 (3M)	0	10 (4F, 6M)	
SMA3b	NAIP	0	0	0	1 (1M)	0	1 (1M)	37 (13F, 24M)
	Exons 7 and 8	0	0	6 (3F, 3M)	23 (7F, 16M)	2 (2F)	31(12F, 19M)	
	Exon 7	0	0	0	5 (1F, 4M)	0	5 (1F, 4M)	
SMA4	Exon 7 and 8	0	0	0	1 (1F)	0	1 (1F)	1 (1F)
Overall		4 (1F, 3M)	50 (27F, 23M)	131 (52F, 79M)	53 (17F, 36M)	2 (2F)	240 (99F, 141M)	

al population at the age of 0–14 years). The ratio of females to males in the whole study group (1039 patients) was F/M=0.82, and for SMA1, SMA2, SMA3a and SMA3b the F/M ratio was 0.78, 0.92, 1.1 and 0.45, respectively.

Table 2. Number of *SMN2* gene copies in the study group

Analysis Variable : st_*SMN2*						
SMN2 copies	N obs.	Mean	Std. Dev.*	Median	Minimum	Maximum
1	4	1.0058	0.1295	0.9960	0.8600	1.1710
2	50	2.0468	0.1284	2.0590	1.7240	2.2530
3	131	2.9949	0.1569	3.0150	2.7020	3.2560
4	53	3.9425	0.1307	3.9590	3.6340	4.1660
5	2	4.9180	0.1782	4.9180	4.7920	5.0440

*Std. Dev., Standard deviation.

Correlation analysis between the size of the deletion and the number of *SMN2* copies in various types for each gender separately did not show significant differences between male and female sex (in log-linear models). However, in the group with SMA1 there were more girls with a deletion in the *NAIP* gene (21/38, 55%) compared to boys (14/48, 29%). Also, more girls had two *SMN2* copies (26/38, 68%) compared to boys (21/48, 43%). Of particular note was the greater number of girls in the group with the *NAIP* deletion and two *SMN2* copies (47.3% girls *vs*. 18.7% boys with SMA1). In the remaining groups, particular sizes of the deletion and the number of *SMN2* copies occurred with similar frequency both in men and women, giving rise to a similar phenotype.

Multifactorial logistic regression analysis with step-wise factor selection (number of *SMN2* copies, presence of a deletion of exons 7 and 8, isolated deletion of exon 7, deletion of exon 7 and 8 *SMN1* and *NAIP*, gender of patients) carried out in the group of 240 patients showed an independent significant effect of two factors on the SMA phenotype — *NAIP* gene deletion ($P=0.0022$) and the number of *SMN2* copies ($P<0.0001$).

Cases with a genotype atypical for chronic form

In the studied cohort, we identified several patients who differed from the usually observed correlation, including two patients with the mild form and a deletion of the *NAIP* gene and one patient with SMA2 and one copy of the *SMN2* gene. A short description of these cases is presented below:

1. A boy born in 2000, SMA3a. He was born following a first, uneventful pregnancy. Initial symptoms occurred in the second year of life. These included difficulties in walking that were seen already at the outset of that developmental stage and frequent falls. The patient never became able to run. When examined at 5 years of age, he sat and walked unaided, his gait was rolling, and he stood up from squatting with assistance. Molecular studies showed the presence of a large deletion, also including the *NAIP* gene, and three *SMN2* copies. The level of SMN protein in skin fibroblasts measured by the Western blotting was 97.7% of normal.

2. A man born in 1952, SMA3b, first symptoms at around 14 years of age, including difficulty in climbing stairs, walking, and hand tremor. When last examined at the age of 48 years, the patient sat and walked unaided (albeit with slightly rolling gait), could stand up from the sitting position with assistance, but was unable to stand up from squatting. Genetic studies showed deletion of *SMN1* and *NAIP* genes and four *SMN2* copies.

3. A boy born in 1999, SMA2/3a. He was born following a second pregnancy which was complicated by frequent uterine contractions. At birth he presented flaccidity. At 6 months of age, the boy sat when put in position and crawled. He never sat unaided, did not stand up from squatting, but stood with assistance when positioned to standing. He started walking with assistance at around 15 months. He could never climb stairs or run. Genetic examination showed a deletion of exons 7 and 8 of the *SMN1* gene, while the *NAIP* gene was remained intact and there was one *SMN2* copy.

DISCUSSION

Our study is the first reported evaluation of the patients' gender impact on the SMA phenotype and analysis of its correlation with two critical molecular factors: the copy number of the *SMN2* gene and the deletion status of the *NAIP* gene.

Of the several factors considered as SMA phenotype modifiers, two seem to have an influence — the number of *SMN2* gene copies and the deletion of the *NAIP* gene. The effect of the number of *SMN2* gene copies seems to be quite evident. Similar to previous studies, the correlation between the number of *SMN2* gene copies and the phenotype was robust. Of note, the low percentage of patients with only one copy of the *SMN2* gene (4/240) is consistent with the results of previous studies (9/375 in a study by Feldkötter, and 7/142 in a study by Mailman) (Feldkötter *et al*., 2002; Mailman *et al*., 2002). The loss of both *SMN1* alleles in the presence of only one *SMN2* copy may thus be a lethal characteristic.

One of our most important observations is that the presence of a deletion in the *NAIP* gene worsens the prognosis independently of the number of *SMN2* copies. Although the deletion of *NAIP* probably only indicates the mechanism of loss of *SMN1*, it is an independent prognostic factor. As we observed three copies of the *SMN2* gene in all phenotypes (41.86% in SMA1; 83.82% in SMA2; 66.43% in SMA3a, and 16.22% in SMA3b), the *NAIP* gene deletion and three *SMN2* copies was found only in SMA1 and 2 (with one exception).

The issue of the influence of gender has been briefly mentioned in the literature on spinal muscular atrophy but has never been fully explained. The phenomenon of half as many girls over the age of 8 years being affected by SMA compared to boys at the same age has been observed for many years (Hausmanowa-Petrusewicz *et al*., 1984). The reverse gender proportions occur among asymptomatic carriers of biallelic deletions, where women predominate (14 *vs*. 9 in our data, and 13 *vs*. 5 when only siblings were taken into account) (Cobben *et al*., 1995; Hahnen *et al*., 1995; Capon *et al*., 1996; Wang *et al*., 1996; Bussaglia *et al*., 1997; Helmken *et al*., 2003; Prior *et al*., 2004; Cuscó *et al*., 2006; Jędrzejowska *et al*., 2008). In our study group, the predominance of the male gender was noticeable, being the most strongly represented in the mild form 3b, which is in line with previous observations. However, molecular studies of the SMA region in this group did not show any significant differences depending on gender, indicating the presence of an independent phenotype modifier. Thus, it seems that the lower frequency of girls affected with the mild form is not associated with differences in the molecular basis of the SMA region and requires further investigation.

We also observed a great disproportion between females and males in the severe SMA1 form. However, the differences in this group also included the molecular basis of the disease. The more severe genotype (*NAIP* gene deletion and the presence of two *SMN2* copies) was observed three times as often in girls (47.3% (18/38) in F *vs*. 18.7% (9/48) in M). In SMA1, Novelli *et al.* (1997) found a higher percentage of girls with the *NAIP* gene deletion (75.6%) compared to boys (52.5%). In his opinion the presence of the *NAIP* gene might be essential to initiate a protective factor dependent on gender. Lack of the *NAIP* gene would inhibit this factor and thus increase the percentage of girls with the severe form in the group with a deletion of the *NAIP* gene. If this hypothesis is true, we should expect a lower percentage of females with *NAIP* in the chronic forms, but this was not seen in the group studied by Novelli. In our group, very few patients with the chronic form had the *NAIP* gene deletion, but a large majority of them were boys (11 *vs* 2). Thus, our data would seem to be concordant with the hypothesis put forward by Novelli.

In our study group, we identified three cases of the chronic form with an atypical genotype: two patients with the mild form of SMA and a deletion including *NAIP* gene and one boy with a borderline form 2/3a and one *SMN1* copy. A deletion in the mild form of the disease is rare, although it has already been reported in the literature (Yamashita *et al*., 2004). If we assume that the *NAIP* gene deletion only indicates the genetic mechanism of the absence of the *SMN1* gene, i.e. a true deletion, then the question arises how the presence of 3 or 4 *SMN2* copies could be explained. One can speculate that a deletion involved an allele that initially underwent a conversion of the *SMN1* genes into *SMN2*. Taking into account the instability of the SMA region, such a sequence of events seems feasible. We found the presence of only one copy of the *SMN2* gene in a patient with the intermediate form more surprising. In a large study of the correlation between the number of *SMN2* gene copies and the phenotype, Feldkötter found that the presence of one copy of this gene was unconditionally associated with the severe form. In addition, the maximum lifespan of such patients was 11 months (Feldkötter *et al*., 2002). Also in the study by Mailman *et al*. (2002), the presence of one copy was closely linked with the severe form. Only Burghes (1997) observed several patients (seven) with the intermediate and mild form and only one *SMN2* copy.

Our study indicates that despite progress in the understanding of the phenotype-genotype correlations in spinal muscular atrophy, there is a number of issues that still need to be clarified. The effect of gender that seems to be significant but still unclear is the most controversial. Our data show that many factors can change the phenotype and much more needs to be explained by examining the phenotype-genotype correlations. Nevertheless, when assessing the prognosis in spinal muscular atrophy, two characteristics should be borne in mind, namely the number of *SMN2* gene copies and the presence of a deletion in the *NAIP* gene.

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