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Unaffected patients with a homozygous absence of the *SMN1* gene

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In this report, we present three families in which we identified asymptomatic carriers of a homozygous absence of the *SMN1* gene. In the first family, the bialleleic deletion was found in three of four siblings: two affected brothers (SMA type 3a and 3b) and a 25-years-old asymptomatic sister. All of them have four *SMN2* copies. In the second family, four of six siblings are affected (three suffer from SMA2 and one from SMA3a), each with three *SMN2* copies. The clinically asymptomatic 47-year-old father has the biallelic deletion and four *SMN2* copies. In the third family, the biallelic *SMN1* absence was found in a girl affected with SMA1 and in her healthy 53-years-old father who had five *SMN2* copies. Our findings as well as those of other authors show that an increased number of *SMN2* copies in healthy carriers of the biallelic *SMN1* deletion is an important SMA phenotype modifier, but probably not the only one.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by loss of lower motor neurons.[1] The estimated incidence of SMA is 1 per 7000–10 000 live births, corresponding to the prevalence of a mutated gene in 1 per 42–50 persons. Recent studies suggest a higher prevalence of the carrier state amounting to 1 per 34 persons.[2,3] The disease is characterized by significant phenotype variability. According to the International SMA Consortium, three clinical forms are distinguished depending on the age at onset and motor development milestones, designated as SMA1 (onset occurs within 6 months after birth and affected children are never able to sit unaided), SMA2 (onset occurs within 18 months and children are able to sit but they never walk) and SMA3 (weakness develops in patients who were previously able to walk unassisted).[4] Type 3 SMA is further subdivided into two subgroups: SMA3a (onset below 3 years of age) and SMA3b (onset at the age ≥ 3 years). This distinction is related to the observation that children presenting with symptoms below 3 years of age lose ability to walk sooner than the children with symptoms over 3 years of age (about 30% in 3a *versus* 3% in 3b in the first 10 years after the onset of symptoms).[5] Cases presenting with the first symptoms of the disease at the age of 20–30 years are classified as SMA4, or proximal adult type SMA.[6,7]

All these forms of SMA result from low levels of the survival motor neuron (SMN) protein, which is insufficient to protect motor neurons.[8] The SMN protein plays a major role in the metabolism of RNA.[9] In addition, it probably

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has a specific function in the nervous system related to the neuromuscular junction maturation.[10,11] The SMN protein is coded by two genes, *SMN1* and *SMN2*. In SMA patients, the *SMN1* gene is mutated (a biallelic deletion occurs in more than 95% of patients) and the SMN protein is produced in reduced amounts by the *SMN2* gene.[12] The *SMN2* gene may have several copies. In approximately 80% of the general population, one or two copies of the *SMN2* gene are found.[13–15] In SMA patients, the diversity in number of the *SMN2* copies is much larger, ranging from 1 to 6 copies. The higher the number of *SMN2* copies, the more benign is the SMA phenotype. Patients with the severe form of the disease usually have 1–2 copies, while patients with the intermediate form have 2–3 copies, and the patients with the mild or adult form have 3–4 or even 5–6 copies.[3,16] Thus the number of the *SMN2* copies is undoubtedly the major SMA phenotype modifier. Some reports suggest that the presence of five *SMN2* copies compensates the loss of both *SMN1* gene alleles and may be responsible for the exceptionally rare asymptomatic biallelic deletion of the *SMN1* gene.[17] However fewer than five copies of *SMN2* found in some asymptomatic carriers of a biallelic deletion of the exon 7 of the *SMN1* gene suggest that other factors also play a role in the determination of the SMA phenotype.[18]

In this report, we present three asymptomatic carriers of biallelic absence of the *SMN1* gene detected in three families affected with SMA. Two subjects had four *SMN2* copies, which is the average number seen in the mild form of SMA, and one subject had five *SMN2* copies.

Patients and methods
Molecular testing for a biallelic deletion in the *SMN1* gene was performed in 490 clinically healthy members of families affected with SMA, including 386 parents (213 mothers and 173 fathers), 63 siblings (29 brothers and 34 sisters), 9 children (3 sons and 6 daughters) and 32 cousins, and also in 300 healthy controls (persons with a negative family history of neuromuscular disorders).

Methods
Genomic DNA was collected from peripheral blood of all family members. DNA was isolated from blood samples by the salting-out procedure.[19]

Homozygous deletion of exons 7 and 8 of *SMN1* was detected using the restriction enzyme analysis, as described elsewhere.[20]

For *SMN1* and *SMN2* dosage analysis, the method described by Anhuf *et al* was employed with modification.[14,21,22] Photometric measuring of DNA was performed on Gene Quant II (Pharmacia) spectrophotometer. DNAs were diluted to a concentration of 10 ng/μl. The same DNA samples of three normal individuals were used as a standard in all amplification reactions for *SMN1* and

SMN2. We used MGB probes to distinguish between *SMN1* and *SMN2*. The MGB probes were specific for the *SMN1* and the *SMN2* at position 6 in exon 7. The *SMN1* probe contained a VIC reporter dye connected to the 5′ end; the *SMN2* probe contained a FAM reporter dye. Primers were identical for *SMN1* and *SMN2*. As a reference gene (one copy, external control), we used an *RN-ase P* gene (TaqMan RN-ase P Control Reagents System Kit, Applied Biosystems). The RQ-PCR was performed on 96-well MicroAmp optical plates in a total volume of 25 μl that contained 50 ng of DNA, 300 nM of each primer, 100 nM of *SMN1* and *SMN2* probes and 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystem). PCR was performed in the 7500 ABI Prism Sequence Detection System producing typical TaqMan reaction: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Every sample was analyzed for *SMN1* and *SMN2* genes and independently for the *RN-ase P* gene in triplets. Each reaction was duplicated and mean results were calculated. Date evaluation was carried out using Relative Quantification (ddCt) Study 7500 System SDS Software.

EMG was performed in six patients, in four of them it was analyzed quantitatively. EMG activity was estimated at rest, and at maximal voluntary contraction, at least 20 motor unit potentials (MUP) were collected in each muscle for quantitative analysis. Two patients were examined with Keypoint Medtronic and two with Counterpoint Medtronic systems. Collected data were compared with our laboratory's reference values. In the upper extremity the biceps brachii muscle was tested in three subjects, and the deltoid in one (family 2, I.1); in the lower extremity the rectus femoris muscle was tested.

The SMN protein levels in fibroblast cells were determined in eight members of families 1 and 2 and in seven healthy volunteers with no family history of the disease. The western blot analysis was performed with monoclonal anti-SMN antibodies (BD Biosciences; diluted 1:1000), and monoclonal anti-β-tubulin antibodies (Sigma-Aldrich Inc.; diluted 1:3000), as described previously.[8] At least two independent cell lysates from cultured fibroblasts were analyzed, each in duplicate. The mean SMN level for the control group was assigned as 100%, and the final results for tested individuals were expressed as the mean percentage values. The mean value for the affected and unaffected patients was estimated and statistical analysis (Student's *t*-test) was performed.

Results
A biallelic deletion in the *SMN1* gene was found in three clinically healthy members of families affected with SMA. We did not find a biallelic deletion in the *SMN1* gene in any of 300 subjects from the control group.

Detailed characteristics of asymptomatic patients with biallelic deletion and their families is presented below.

Family 1 (no. 528)

There are four siblings in family 1 (Figure 1a; Table 1). Two brothers (II4 and II5) are affected with SMA 3b and 3a, respectively. The diagnosis was confirmed by molecular
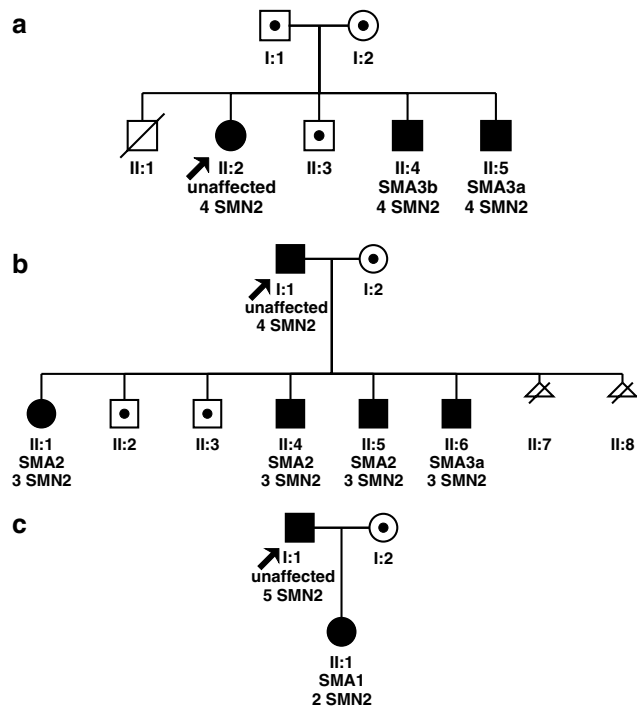

Figure 1 Pedigrees of spinal muscular atrophy (SMA) families with biallelic absence of the *SMN1* gene in unaffected subjects. (**a**) family 1, (**b**) family 2, (**c**) family 3; circles represent females, squares represent males, black symbols represent biallelic *SMN1* absence, dots represent heterozygous SMA carriers, arrows represent asymptomatic carriers of biallelic *SMN1* absence.

testing (del. exon 7 and 8 of the *SMN1* gene). Both showed neurogenic changes in upper and lower extremity muscles. Two other siblings are clinically healthy, they did not show any symptoms on neurological examination. EMG revealed chronic neurogenic changes in the lower extremity muscles in both of them (sister II-2: m. biceps: N; m. rectus femoris: amp. 2995; area 6542; 63% of outliners; brother II-3: m. biceps: N; m. vastus: 1228/1856; 18% of outliners). Molecular testing showed the biallelic deletion in a healthy sister (II-2) and a heterozygous deletion carrier state in a healthy brother (II-3). Both parents are deletion carriers (one *SMN1* copy). A fifth child (II-1) born at 7 months of gestation died soon after birth.

Family 2 (no. 899)

There are six siblings in this family (Figure 1b; Table 1), including three affected with SMA2, the youngest boy affected with SMA3a and two healthy ones. The parents are clinically healthy. Prenatal testing was carried out twice (both results were abnormal). Molecular testing showed a biallelic deletion of exons 7 and 8 of the *SMN1* in the four affected siblings and in the clinically healthy father. EMG performed in the asymptomatic father (II–1) revealed chronic neurogenic changes in the lower extremity muscles (m. deltoid: N; m. rectus femoris amp.1680; area 3341). The mother and the healthy siblings are heterozygous deletion carriers.

Family 3 (no. 676)

In this family (Figure 1c; Table 1), a biallelic *SMN1* gene deletion was found in a child with an acute form of SMA and in her asymptomatic father. The family was clinically

Table 1 Clinical and molecular genetic findings in families 1, 2, 3

Family	Identifications in pedigrees	Phenotype	Age at examination	Age at onset	EMG	SMN1 copies no.	SMN2 copies no.	SMN protein in fibroblasts
1	I.1	Unaffected	47 years	—	Not examined	1	3	Not examined
	I.2	Unaffected	48 years	—	Not examined	1	3	Not examined
	II.2	**Unaffected**	25 years	—	Neurogenic	**del**	**4**	100.3%
	II.3	Unaffected	24 years	—	Neurogenic	1	3	79.8%
	II.4	SMA3b	20 years	5 years	Neurogenic	del	4	94.2%
	II.5	SMA3a	19 years	12 month	Neurogenic	del	4	82.9%
2	I.1	**Unaffected**	47 years	—	Neurogenic	**del**	**4**	103.5%
	I.2	Unaffected	47 years	—	Not examined	1	2	Not examined
	II.1	SMA2	23 years	9 month	Neurogenic	del	3	79.7%
	II.2	Unaffected	22 years	—	Not examined	1	3	Not examined
	II.3	Unaffected	21 years	—	Not examined	1	3	Not examined
	II.4	SMA2	14 years	9 month	Not examined	del	3	71.3%
	II.5	SMA2	12 years	6 month	Not examined	del	3	79.7%
	II.6	SMA3a	10 years	18 month	Not examined	del	3	96.0%
3	I.1	**Unaffected**	53 years	—	Not examined	**del**	**5**	Not examined
	I.2	Unaffected	22 years	—	Not examined	1	1	Not examined
	II.1	SMA1	9 month	3 month	Not examined	del	2	Not examined

Bold indicates asymptomatic cases and their results presented in the article.

examined only once and currently they are lost to follow-up and the affected child has died.

The SMN protein analysis showed no statistically significant differences between the mean value for the affected (84.0 ± 9.5) and unaffected patients (94.5 ± 12.9) ($P = 0.199$) from SMA families.

Discussion

An asymptomatic biallelic deletion in the *SMN1* gene is exceptionally rare. We found this deletion only in three cases among 490 clinically healthy relatives of SMA patients (including 437 obligatory deletion carriers, that is parents and offspring as well as siblings at a 2/3 chance of being heterozygous carriers) but in none of control group. Prior *et al* found the biallelic deletion in two cases among 408 deletion carriers.[17] In a group of 214 parents and 66 siblings, Wang *et al* found a definite biallelic deletion in two clinically healthy subjects.[23] Thus, the prevalence of an asymptomatic biallelic deletion carrier state may be estimated at approximately 0.5–0.7% of first-degree relatives of SMA patients. This is a low rate but highlights the need for genetic testing in members of families affected with SMA and bears importance to genetic counseling, since the presence of such a variant in one of the parents means that the risk of having an affected offspring is not 25 but 50% (as in the family 2 of this report).

To date, according to our knowledge, an asymptomatic biallelic deletion in the *SMN1* gene has been reported in 23 subjects from 19 families.[17,18,23–28] In 13 families, siblings of asymptomatic carriers were affected, while in 5 families children of such carriers and in one family, a brother and a son of a carrier were affected. In this report, we present the following three cases of an asymptomatic biallelic deletion in the *SMN1* gene.

Naturally we cannot exclude the possibility of a future occurrence of symptoms in these patients, particularly in view of their abnormal EMG results suggestive of an ongoing pathologic process. EMG data on the patients with asymptomatic biallelic deletion is scarce and electrophysiological methods employed differ between reports. Prior *et al* and Cobben *et al* described three patients with normal electrophysiological parameters.[17,24] Yet, one of the subjects was 6 months of age at the time of EMG, and one tested at 35 years of age had normal motor unit number estimate, but data on concentric needle EMG was not given. Mild neurogenic changes with increased MUP amplitude but no denervation were detected in 16- and 22-years old asymptomatic females.[27,28] The findings are not easy to interpret in view of the fact that mild neurogenic changes were also found in a healthy brother who is a heterozygous deletion carrier, with only one copy of *SMN1* (subject II.3 in family 1). Mild neurogenic changes were previously reported in some parents of the children affected with SMA, who are nearly obligatory deletion

carriers.[29] The presence of neurogenic changes in un-affected heterozygous deletion carriers may suggest a subclinical defect resulting from SMN protein deficiency, detected with EMG. Yet, comparison of the severity of denervation/reinnervation process between heterozygous deletion carriers is not possible from the published data, as EMG was reported in a qualitative way.[24,28] The lack of symptoms in subjects with the biallelic deletion in the *SMN1* gene may be related to two factors, that is the increased number of *SMN2* gene copies or the effect of other unknown phenotype modifiers. The presence of five *SMN2* gene copies may explain the lack of clinical symptoms in the patient in family 3. Prior *et al* found five *SMN2* gene copies in three asymptomatic carriers.[17] However, the effect of the number of *SMN2* gene copies is not absolute and the presence of five copies does not always compensate for the loss of the *SMN1* gene. Wirth *et al* described three patients affected with a mild SMA form who had five or even six *SMN2* copies.[16] In addition, an increased number of *SMN2* gene copies cannot explain the lack of clinical symptoms in cases in families 1 and 2. We found four copies of the *SMN2* gene in these patients that is typical for SMA3. Molecular testing showed 2–3 *SMN2* copies in asymptomatic carriers also in the families described by Helmken *et al*.[18] In these families, the number of *SMN2* gene copies was identical in affected subjects and their asymptomatic siblings. Thus, it seems that apart from *SMN2* copies some other phenotype modifiers are also effective in SMA.

It is possible that gender may be one of the phenotype modifying factors. Twice lower incidence of SMA among girls above 8 years of age compared to age-matched boys was already reported long time ago.[30] In contrast, biallelic deletion in unaffected subjects described earlier was more prevalent among females (14 females *versus* 9 males).[17,18,23–28] This is even more pronounced if only siblings (sharing identical genotype of SMA locus) are taken into account. Among 14 families, females were asymptomatic carriers in 9, while males in only 4 families, and one family included both a male and a female asymptomatic carrier (together 13 females *versus* 5 males). This gender proportion may be purely coincidental, but it may also suggest the presence of a gender-related protective factor.

Determination of the SMN protein level in family no. 528 showed nearly identical values in the affected brothers (83% and 94%) and their asymptomatic sister who was a carrier of the biallelic deletion (100%). In family no. 899, the SMN protein level ranged from 71 to 96% in the affected siblings and was 103% in the asymptomatic father. These differences between affected and unaffected biallelic deletion carriers were not statistically significant ($P = 0.199$). It is in agreement with previous papers. The SMN protein levels have been shown to be not helpful to differentiate milder forms and controls, even in limphoblastoid cell lines.[8,31,32] However Helmken *et al* showed

that the protein level was higher in asymptomatic deletion carriers but only in lymphocytes, whereas fibroblasts showed identical protein expression in affected and unaffected siblings.[18] In contrast, the total amount of the SMN transcript was identical in phenotypically discordant siblings. Thus, it seems that the SMN protein level may be tissue specific and phenotypic variability can be caused by changes at the translational level or through some completely different pathway.

The disease phenotype is undoubtedly influenced by the number of *SMN2* copies. However, molecular DNA changes in the SMA region seem to be identical in both affected and asymptomatic carriers of the biallelic deletion, which was shown both in our cases and other similar subjects described earlier.[17,18,23–28] The varying phenotype may be related to some post-translational, epigenetic, perhaps tissue-specific changes affecting the SMN protein.

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