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Mildly affected patients with spinal muscular atrophy are partially protected by an increased *SMN2* copy number

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Abstract Spinal muscular atrophy (SMA) is a recessive neuromuscular disorder caused by loss of the *SMN1* gene. The clinical distinction between SMA type I to IV reflects different age of onset and disease severity. *SMN2*, a nearly identical copy gene of *SMN1*, produces only 10% of full-length *SMN* RNA/protein and is an excellent target for a potential therapy. Several clinical trials with drugs that increase the *SMN2* expression such as valproic acid and phenylbutyrate are in progress. Solid natural history data for SMA are crucial to enable a correlation between genotype and phenotype as well as the outcome of therapy. We provide genotypic and phenotypic data from 115 SMA patients with type IIIa (age of onset <3 years), type IIIb (age of onset >3 years) and rare type IV (onset >30 years). While 62% of type IIIa patients carry two or three *SMN2* copies, 65% of type IIIb patients carry four or five *SMN2* copies. Three type IV SMA patients had four and

one had six *SMN2* copies. Our data support the disease-modifying role of *SMN2* leading to later onset and a better prognosis. A statistically significant correlation for ≥ 4 *SMN2* copies with SMA type IIIb or a milder phenotype suggests that *SMN2* copy number can be used as a clinical prognostic indicator in SMA patients. The additional case of a foetus with homozygous *SMN1* deletion and postnatal measurement of five *SMN2* copies illustrates the role of genotypic information in making informed decisions on the management and therapy of such patients.

Keywords Spinal muscular atrophy (SMA) · Survival motor neuron (SMN) · Quantification of *SMN2* · Natural history

Database: *SMN1*—OMIM: 600354; GeneBank: U18423,
SMN2—OMIM: 601627; GeneBank: NM_022875

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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterised by the degeneration of α -motor neurons in the anterior horns of the spinal cord, leading to progressive symmetrical muscle weakness and atrophy. The incidence of SMA is 1 in 6,000–10,000 newborns (Pearn 1978) and the carrier frequency is 1 in 35 among Europeans (Cusin et al. 2003; Feldkotter et al. 2002). SMA is classified into four types (type I–IV) according to the age of onset and disease severity (Munsat and Davies 1992; Zerres and Rudnik-Schoneborn 1995). Type I SMA patients (Werdnig-Hoffmann disease, OMIM #253300) show onset within 6 months after birth and usually die before 2 years of age. They are never able to sit or walk. Type II SMA patients (intermediate form, OMIM #253550) have an onset after 6 months of age. They are able to sit but cannot stand. Type III SMA patients (Kugelberg-Welander disease, OMIM #253400) show their first symptoms after 18 months of age. They are able to sit and walk but often become wheel-chair bound in the course of disease progression. Patients with an age of onset before 3 years are subclassified as IIIa, those with an age of onset after

3 years as IIIb (Zerres and Rudnik-Schoneborn 1995). Type IV SMA patients (adult form of SMA, OMIM #271150) are characterised by an age of onset > 30 years and only very mild signs of muscle weakness.

A correlation of phenotype with genotype is important not only for individual genetic counselling but also for therapeutic trials. In 97% of type I–III SMA patients, homozygous mutations (deletions, gene conversions or point mutations) of the survival motor neuron gene (*SMN1*; OMIM 600354) were detected (Lefebvre et al. 1995; Wirth 2000). So far, homozygous absence of *SMN1* was found only in very few patients with type IV SMA (age of onset > 30 years) (Brahe et al. 1995; Clermont et al. 1995) while most of these individuals do not carry any detectable deletions in this gene (Zerres et al. 1995).

An increased number of *SMN2* copies is the result of gene conversion of *SMN1* into *SMN2* (Burghes 1997; Hahnen et al. 1996; van der Steege et al. 1996; Wirth et al. 1997). The severity of SMA has been proven to be influenced by the number of *SMN2* (OMIM 601627) copies: about 70% of type I SMA patients carry two *SMN2* copies, 82% of type II SMA patients have three *SMN2* copies, whereas type III patients have with very few exceptions a minimum of three to four *SMN2* copies (Feldkotter et al. 2002; Mailman et al. 2002; Ogino et al. 2003). *SMN1* produces almost 100% of full-length (FL) mRNA whereas *SMN2* primarily produces transcripts lacking exon 7 (Gennarelli et al. 1995; Helmken et al. 2003; Lefebvre et al. 1995). Only about 10% of *SMN2* transcripts are correctly spliced and encode a protein identical to *SMN1*.

Since each SMA patient retains at least one *SMN2* copy and since the number correlates with the phenotype, *SMN2* is considered as an interesting gene target for therapy (Wirth 2002).

A large number of various drugs and molecules have been reported to increase the *SMN2* protein level in vitro via transcriptional activation and/or by restoring correct *SMN2* pre-mRNA splicing. This makes SMA one of the few genetic disorders in humans, in which activation of a copy gene opens a therapeutic approach. The most promising drugs to date are the histone deacetylase inhibitors valproic acid (VPA) (Brichta et al. 2004, 2003; Sumner et al. 2003) and phenylbutyrate (Andreassi et al. 2004; Brahe et al. 2005). Both compounds are currently investigated in phase II and III clinical trials in Europe and US (<http://www.fsma.org> and www.sma-initiative.de).

In some families, different SMA phenotypes of siblings who carry identical homozygous absence of *SMN1* and identical *SMN2* copy numbers have been reported (Brahe et al. 1993; Cobben et al. 1995; DiDonato et al. 1997; Hahnen et al. 1995; Prior et al. 2004; Wang et al. 1996). A so far unknown modifying factor has been shown to up-regulate the *SMN* protein level and to protect some individuals from developing SMA (Helmken et al. 2003). In conclusion, there are two major factors that influence the severity of SMA: the number of *SMN2* copies and the SMA modifying gene(s).

In this study, we analysed the phenotype–genotype correlation especially in SMA patients affected by milder forms of the disease. In none of the molecular genetic studies carried out so far, SMA patients were subdivided into type IIIa and IIIb according to their age of onset, before or after 3 years, respectively. However, there is a marked difference in the preservation of ambulatory capacity between type IIIa and IIIb. Whereas only 44% of the type IIIa individuals are still able to walk by the age of 20 years, 90% of type IIIb patients of the same age have retained this ability (Zerres et al. 1995). The aim of this study was to provide solid data concerning the correlation between the number of *SMN2* copies and the age of onset especially in mildly affected SMA patients as an important parameter for future clinical trials.

Materials and methods

One hundred and fifteen patients with SMA IIIa, IIIb or IV and confirmed homozygous absence of *SMN1* were analysed for their *SMN2* copy numbers by real-time quantitative PCR using a SYBR Green assay on a LightCycler® machine as previously described (Feldkotter et al. 2002). Direct screening of SMA patients for homozygous absence of *SMN1* was carried out by PCR followed by restriction digestion with *HinfI* and agarose gel electrophoresis to distinguish *SMN1* from *SMN2* as described elsewhere (Wirth et al. 1999). Haplotype analysis with the multicopy polymorphic marker C212 localised at the 5' end of the *SMN* genes was performed as described (Wirth et al. 1995). Blood samples had been obtained after informed and written consent from each participant or the legal guardian according to a protocol reviewed and approved by the local Ethics Committee. Phenotypic data had largely been collected together with the original blood samples and were completed by individual telephone interviews of the physicians in charge where necessary.

Results and discussion

Previous studies by others and us have analysed type III SMA patients only as a whole group based on the main motor ability: to walk without support. Here, we analysed 111 patients with homozygous absence of *SMN1* and type III SMA. Sixty of these patients showed disease onset before 3 years of age and were defined as type IIIa. The remaining 51 patients are characterised by an onset after 3 years of age and consequently were classified as type IIIb (Table 1).

Of note, 4 additional patients with adult (type IV) SMA and homozygous absence of *SMN1* were identified amongst a total of 21 patients with an SMA type IV phenotype tested in our routine molecular genetic laboratory. The highest age of onset observed among these patients was 40 years (Table 2). Remarkably, all four patients showed a homogeneous clinical picture with

Table 1 Quantitative analysis of *SMN2* copy numbers in 115 patients with type IIIa, IIIb and IV SMA

No. of <i>SMN2</i> copies	SMA IIIa				SMA IIIb				SMA IV			
	No. of patients	Percentage of patients	Sex (m/f)	Mean age by sex (m/f)	No. of patients	Percentage of patients	Sex (m/f)	Mean age by sex (m/f)	No. of patients	Percentage of patients	Sex (m/f)	Mean age by sex (m/f)
2	7	11.7	3/4	25.1/17.5	2	3.9	1/1	37.4/20.2	0	0	–	–
3	30	50.0	17/13	28.4/29.7	16	31.4	8/8	39.8/38.3	0	0	–	–
4	23	38.3	11/12	27.0/33.3	31	60.8	21/10	40.9/48.7	3	75	0/3	–/48.2
5	0	0	–	–	2	3.9	2/0	61.5/–	0	0	–	–
6	0	0	–	–	0	0	–	–	1	25	1/0	63.8/–
Total	60	100	31/29		51	100	32/19		4	100	1/3	

nearly stationary or very slowly progressive weakness and atrophy of hip flexor and knee extensor muscle starting in the fourth decade of life. Slowly progressive segmental anterior horn cell disease of the proximal lower extremities thus forms part of the clinical spectrum of chromosome 5q13-linked spinal muscular atrophy and should be considered in the differential diagnosis even in sporadic cases. However, in 17 out of these 21 patients presenting with an adult SMA phenotype, homozygous absence of *SMN1* was not detected. Quantitative genetic analysis revealed that these patients carry two *SMN1* copies, thus excluding a compound heterozygosity with a deletion of *SMN1* on one chromosome 5 and a subtle *SMN1* mutation on the other chromosome 5, a genotype found in about 3% of SMA patients (Wirth et al. 1999). These data confirm association of some adult SMA cases with mutations in *SMN1* (Brahe et al. 1995; Clermont et al. 1995; Zerres et al. 1995).

As demonstrated in Table 1, 50% of type IIIa, 31.4% of type IIIb and none of the type IV SMA patients, respectively, carry three *SMN2* copies. Conversely, only 38.3% of type IIIa, but 60.8% of type IIIb and 75% of type IV SMA patients, respectively, carry four *SMN2* copies (Fig. 1). Two additional patients affected by very mild type IIIb SMA (onset with 19 and 25 years, respectively) carry five *SMN2* copies (Table 2). These results suggest that the genotype of type IIIa SMA patients rather resembles the genotype of type II patients [82% with three *SMN2* copies, 7% with four *SMN2* copies according to Feldkotter et al. (2002)] than the one of type IIIb patients. These data provide statistical significance for a correlation of ≥ 4 *SMN2* copies with type IIIb SMA versus ≤ 3 *SMN2* copies with type IIIa SMA (in the Fisher's exact test, the two-tailed *P* value equals 0.008; odds ratio = 2.87; 95% CI 1.326–6.209). Moreover, our analysis suggests a strong protective effect of four or more *SMN2* copies in SMA patients, which goes along with an age of onset after 3 years up to as late as 40 years. This is the first genotypic data set of statistical significance supporting the empirical subdivision of SMA type III patients into phenotypes IIIa and IIIb.

An even higher number of ≥ 5 *SMN2* copies, an event found only in very few SMA patients (Table 2), clearly indicates a very mild course of SMA. Importantly, even in the presence of six *SMN2* copies (estimated amount of

FL-*SMN* RNA: 60%), mild SMA symptoms may still occur. In contrast, SMA carriers with one *SMN1* and one *SMN2* who should also produce about 60% of FL-*SMN* are asymptomatic. The idea that $\Delta 7$ -*SMN2* might have some negative impact on the disease phenotype is in line with data suggesting a pro-apoptotic effect of the $\Delta 7$ -*SMN2* protein (Kerr et al. 2000; Vyas et al. 2002). However, the fact that eight *SMN2* copies in mice and humans completely rescue the phenotype (Monani et al. 2000; Vitali et al. 1999) and recent data on phenotypic improvements in SMA via increased $\Delta 7$ -*SMN2* protein levels in double transgenic SMA-like mice with the genotype *Smn*-/-;*SMN2*+; $\Delta 7$ *SMN2* strongly argue against a negative effect of the $\Delta 7$ -*SMN2* protein (Le et al. 2005).

Despite the crucial influence of the *SMN2* copy number on the phenotype in mild SMA, this does not fully explain variable expressivity. In each SMA category, there is still a substantial overlap of *SMN2* copy numbers, suggesting that there are other modifying factors such as size of the deletion and molecular rearrangements resulting in incomplete *SMN2* genes. The observation of rare phenotypically discordant SMA families, in which some of the siblings with homozygous mutation of *SMN1* do not show any symptoms, also points towards additional SMA modifiers (Brahe et al. 1993; Cobben et al. 1995; Hahnen et al. 1995; Helmken et al. 2003; Wang et al. 1996).

Long-term studies investigating the age of losing ambulatory capacities and the survival in SMA patients are important to further define genotype–phenotype correlations. Our previous study in type I SMA patients in whom the age of survival was correlated with the *SMN2* copy number revealed a significant difference between the type I SMA patients carrying three *SMN2* copies, who presented a mean age of survival of 36 months, and the majority with only two *SMN2* copies who showed a mean age of survival of only 11 months (Feldkotter et al. 2002). Thus, it can be assumed that patients with different numbers of *SMN2* copies can have a similar age of onset, but the individuals with an increased *SMN2* copy number may show slower disease progression and increased probability of staying ambulatory. Long-term observations in milder SMA phenotypes could further support this hypothesis and would be extremely helpful for clinical studies,

Table 2 Characteristics of type IV and type IIIb SMA patients with 4–6 and 5 *SMN2* copies, respectively

DNA	Sex	Age of onset	Actual age	Motor function (MRC semiquantitative scale)			Sit up	Stand up from a knee bend	Gait	<i>SMN2</i> copy number
				CN	UE	LE				
<i>Type IV</i>										
Pat. ID: 6162 (S.M.)	F	32	40	n	n	Hip flex.: 3/3 knee flex.: 4/4 knee ext.: 4/4 PR, AR: absent fasciculations and atrophy of m. quadriceps	n	Gower	Waddling, stairs difficult	4
Pat. ID: 6175 (R.K.L.)	M	35	64	n	n	Hip flex.: 2/2 hip abduct.: 5/5 hip adduct.: 4+/4+ knee flex.: 5/5 knee ext.: 2/2 PR, AR: absent atrophy of m. quadriceps	n	Unable to perform	Backkneeing (hyperextension of knees), several unprovoked falls, stairs difficult	6
Pat. ID: 6322 (K.A.)	F	40	55	n	n	Hip flex.: 2/2 hip abduct.: 4/4 hip adduct.: 4-/4- knee flex.: 4/4 knee ext.: 4/4+ PR, AR: reduced no visible atrophy or fasciculations	n	Butt first maneuver	mild waddling, stairs difficult	4
Pat. ID: 6236 (P.-A.G.)	F	34	49	n	n	Hip flex.: 2/2 hip abduct.: 4+/4+ hip adduct.: 4-/4- knee flex.: 4+/4+ knee ext.: 4/4 PR, AR: absent fasciculations and atrophy of m. quadriceps	n	Gower	Hyperextension of knees, several unprovoked falls, stairs with handrail	4
<i>Type IIIb</i>										
Pat. ID: 5873 (A.H.)	M	19	39			No data available			Waddling, stairs difficult	5
Pat. ID: 6241 (S.R.)	M	25	86	n	Abduct.: 2/2 rotat.: 3/3 triceps: 3/3 hands + fingers 4/4 fasciculation and atrophy of m. pectoralis, m. triceps and interossei	Hip flex.: 2/2 hip abduct.: 4+/4+ hip adduct.: 4-/4- knee flex.: 3/3 knee ext.: 2/2 PR, AR: absent fasciculation and atrophy of m. quadriceps	n	Gower	Hyperextension of knee joints	5

The numerical values given for the different types of movements indicate motor strength according to the MRC semiquantitative scale (Aids to the investigation of the peripheral nervous system. London: Her Majesty's Stationery Office; 1975): 0 = complete paralysis; 1 = palpable muscle contraction, but no visible movement of joint; 2 = movement only after elimination of gravity; 3 = full range movement against gravity but not against additional resistance; 4 = full range movement against resistance greater than gravity, but examiner can overcome the action; - = lower range of "4"; + = upper range of "4"; 5 = normal strength
CN cranial nerve status; *UE* upper extremities; *LE* lower extremities; *PR* patellar reflex, *AR* achilles tendon reflex; *m.* musculus; *flex.* flexion, *ext.* extension, *abduct.* abduction, *rotat.* rotation, *n* normal

particularly may help answering the question: at which time point a potential drug treatment should be started. We speculate that a person with four *SMN2* copies could afford to start a potential therapy later and may need lower drug doses in order to reach a certain SMN protein level sufficient to protect from developing the

disease as compared to individuals with only one to three *SMN2* copies.

Our study has refined genotype–phenotype correlations in mild SMA. Enhanced knowledge on how the age of disease onset and that of disease progression are genetically determined is essential to design potential

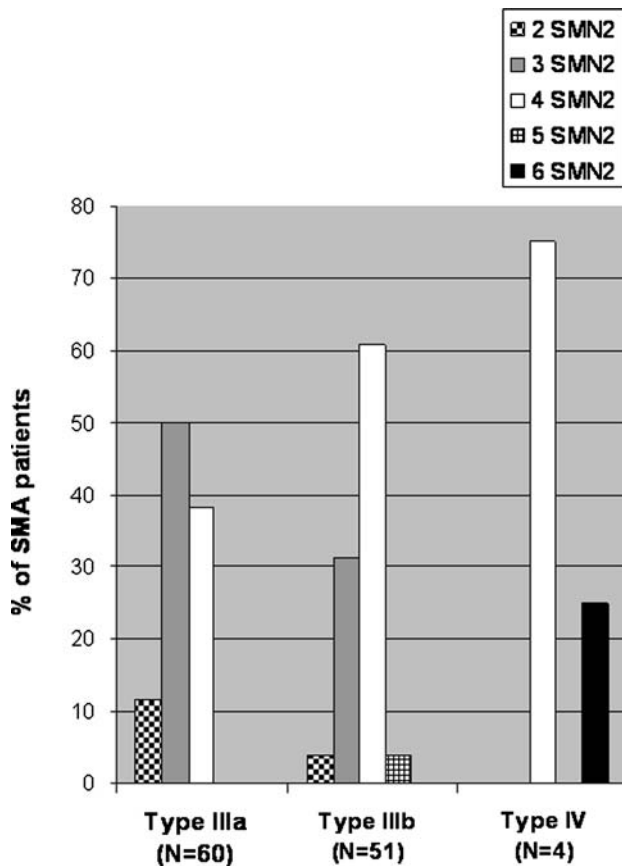


Fig. 1 Graphical representation of the correlation between *SMN2* copy number and phenotype in 115 spinal muscular atrophy (SMA) patients

therapeutical trials. An important question in this context will be when to start treatment with the drug on trial. We still have to learn much more about the

presymptomatic phase of SMA in mild and adult SMA. Nevertheless, if the currently ongoing clinical trials show clear benefits and improvements in the SMA patients, a neonatal screening for *SMN1* deletions could be the next crucial step. In neonates carrying homozygous absence of *SMN1*, the *SMN2* copy number would be an important predictor of the most likely phenotypic outcome. Although others and we have shown that this correlation is not absolute, there is a very strong correlation of one to two *SMN2* copies with a severe SMA phenotype, and four or more *SMN2* copies with a mild SMA phenotype. Especially for VPA, it is well known that treatment of children < 2 years holds a comparatively higher risk of side effects. Therefore, in neonates with four or more *SMN2* copies, one should carefully balance between the start of a drug therapy and the higher risk of side effects due to drug administration in the first 2 years of life.

We conclude with an example illustrating the impact that genotypic information can have on clinical management decisions in families with SMA. The child of a father with mild SMA type IIIb (phenotype: discrete symptoms from 19 years of age, only slight problems with climbing stairs at 38 years of age; genotype: homozygous loss of *SMN1*, five *SMN2* copies) and a heterozygous mother was prenatally diagnosed to have a homozygous deletion of *SMN1* (Fig. 2). The couple decided to continue the pregnancy. Immediately after birth, the physician started an individual experimental therapeutic approach of the healthy child with valproic acid according to Section 41 German Drug Act (AMG). At the age of 3 weeks, we measured five *SMN2* copies in an EDTA blood sample from this child. With this result strongly in favour of a very mild/adult SMA, it was decided together with the parents to suspend the VPA therapy. As expected, at the age of 14 months the child is

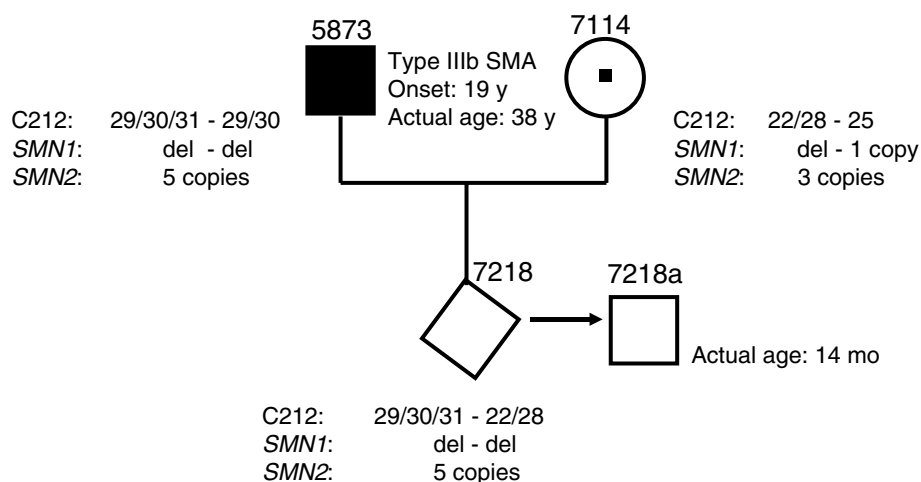


Fig. 2 Prenatal diagnosis in an SMA family. Direct genetic testing in the foetus revealed homozygous absence of *SMN1*. The parents decided to continue the pregnancy and to start valproic acid (VPA) therapy immediately after birth. Quantitative analysis of *SMN2* copies at 3 weeks revealed five *SMN2* copies in the newborn.

Haplotype analysis with the multicopy polymorphic microsatellite marker C212 (localised 12 kb upstream of *SMN*) also revealed five different alleles in the foetus. Based on the genotype-phenotype correlation in patients with five *SMN2* copies, the experimental therapy is on hold at the present time

still healthy. The physician may consider restarting the therapy in 1–2 years based on newer data to be expected from clinical trials.

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