Detection of the survival motor neuron (SMN) genes by FISH: further evidence for a role for SMN2 in the modulation of disease severity in SMA patients

Tiziana Vitali, Vittorio Sossi, Francesco Tiziano, Stefania Zappata, Anna Giuli, Maria Paravatou-Petsotas, Giovanni Neri and Christina Brahe

Institute of Medical Genetics, Catholic University, Largo F. Vito 1, I-00168 Rome, Italy

Received August 4, 1999; Revised and Accepted September 22, 1999

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder which presents with various clinical phenotypes ranging from severe to very mild. All forms are caused by the homozygous absence of the survival motor neuron (SMN1) gene. SMN1 and a nearly identical copy (SMN2) are located in a duplicated region at 5q13 and encode identical proteins. The genetic basis for the clinical variability of SMA remains unclear, but it has been suggested that the copy number of SMN2 could influence the disease severity. We have assessed the number of SMN2 genes in patients with different clinical phenotypes by fluorescence in situ hybridization (FISH) using as SMN probe a mixture of small specific DNA fragments. Gene copy number was established by FISH on interphase nuclei, but the presence of two SMN2 genes on the same chromosome could also be revealed by FISH on metaphase spreads. All patients had at least two SMN2 genes. We found two or three copies of SMN2 in severely affected type I patients, three copies in intermediate affected type II patients, generally four copies in mildly affected type III patients and four or eight copies in patients with very mild adult-onset SMA. No alterations of the genes were detected by Southern blot and sequence analysis, suggesting that all gene copies of SMN2 were intact. These data provide additional evidence that the SMN2 genes modulate the disease severity and suggest that knowledge of the gene copy number could be of some prognostic value.

INTRODUCTION

Proximal spinal muscular atrophies (SMAs) are a group of clinically heterogeneous motor neuron disorders characterized by the degeneration of anterior horn cells of the spinal cord which results in weakness of proximal limb and trunk muscles. On the basis of age of onset and severity of the clinical course, SMAs can be classified into three forms of childhood-onset SMA (types I–III) (1) and one adult-onset form (type IV) (2).

Childhood SMA is one of the most frequent autosomal recessive diseases, with an estimated incidence of 1 in 10 000. Type I, or Werdnig–Hoffmann disease, is the most severe form, with onset ranging from prenatal to 6 months of life; the children are never able to sit without support. Type II is an intermediate form with onset before the age of 18 months; the patients are unable to stand or walk. Type III, or Kugelberg–Welander disease, is a relatively mild, chronic form with onset after the age of 18 months. Type IV, or adult-onset SMA, is a mild, genetically heterogeneous form, with variable age of onset ranging from the third to the fifth decade.

All these conditions are in linkage with the chromosomal region 5q13 (3–5). This region contains a large duplicated, inverted segment which harbours two nearly identical copies of the survival motor neuron (SMN) gene, one telomeric (SMN1) and the other centromeric (SMN2) (6). For the vast majority of SMA patients (~95%), the SMN1 gene is not detectable by single strand conformation polymorphism (SSCP) (6) or restriction digest assay (7), regardless of the clinical severity (6). Several rare, subtle mutations in SMN1 have provided further evidence that this gene is responsible for SMA (6,8–12).

SMN1 can be distinguished from SMN2 by two base changes in exons 7 and 8 (6). It has been demonstrated recently that the exon 7 C→T transition is responsible for alternative splicing of exon 7 of the SMN2 transcripts (13,14). Hence, the two genes generate different proportions of full-length transcripts: SMN1 produces primarily full-length transcripts and a small amount (~10%) of transcripts lacking exon 5, whereas SMN2 produces primarily transcripts lacking exon 7 (40–50%) plus minor amounts of full-length transcripts and transcripts lacking exon 5 or both exon 5 and exon 7 (6,15).

SMN is expressed in most if not all tissues (6). Immunofluorescence studies have shown that the protein is localized in both the cytoplasm and in the nucleus. In the nucleus, the protein appears concentrated in a few dot-like structures, designated gems (16). Western blot and immunolocalization analyses have shown a marked reduction in the level of SMN protein and number of nuclear gems, respectively, in SMA patients (17–19). There is evidence that the SMN protein is associated with other proteins, the so-called SMN-interacting proteins (SIPs) and snRNPs, and that this protein complex

*To whom correspondence should be addressed. Tel: +39 06 35500877; Fax: +39 06 3050031; Email: brahe@rm.unicatt.it
plays a role in splicingosomal snRNP biogenesis and is required for pre-mRNA splicing (20,21).

The genetic basis for the clinical variability remains unclear. Results of genotype–phenotype correlation studies have led essentially to two hypotheses. One proposes that modifying genes could modulate the severity of SMA. In favour of this hypothesis is the observation that the majority of type I SMA patients have large deletions (>70 kb) removing SMN1 and adjacent genes including the neuronal apoptosis inhibitor protein (NAIP) gene (22), the p44 subunit of transcription factor II (BTF2p44) (23,24) and a novel transcript, H4F5 (25). NAIP was shown to exert an anti-apoptotic activity in cultured cells (22) and to be deleted in 45–69% of type I individuals (22,26). However, the NAIP gene is also absent in a proportion of mildly affected patients including one patient with adult-onset SMA (5), and no phenotypic difference was observed between type I SMA patients with or without deletion of NAIP (27). As for BTF2p44 and the recently identified H4F5 genes, little is known presently about their potential involvement in SMA.

The second hypothesis is that in the milder forms of SMA, an increase in the copy number of the SMN2 gene could compensate in part for the absence of SMN1. This suggestion is supported by the observation of a correlation between SMN protein level and clinical severity (17). In fact, the number of copies supported by the observation of a correlation between SMN deletion and gene conversion events, the latter leading to the replacement of SMN1 by SMN2 (28). Velasco et al. (26), by densitometric analysis of SSCP bands, found that parents of type II and type III patients carried more copies of SMN2 than did parents of type I patients. In contrast, Schwartz et al. (29), using a solid-phase mini-sequencing method, did not find evidence that an increase in SMN2 copy number correlates with a less severe phenotype. Campbell et al. (28) showed by pulsed-field gel electrophoresis analysis that type II and type III patients have on average a greater number of SMN2 copies than type I patients. Recently, quantitative PCR-based assays have been developed by which the copy number of SMN1 and SMN2 genes is inferred from the ratios between dosages of SMN genes and internal standards. These tests have proven useful for SMA carrier detection and identification of patients with heterozygous deletions of SMN1 and have provided evidence in support of a role for SMN2 in modulating the clinical phenotype of SMA (12,27,30,31).

We have established fluorescence in situ hybridization (FISH) conditions capable of visualizing the SMN genes in metaphase chromosomes and interphase nuclei and used this method to assess the SMN2 copy number per chromosome in patients with various clinical severities. We also studied the integrity of the SMN2 genes by Southern blot and sequence analysis.

RESULTS
In our first attempts to visualize SMN genes by FISH on metaphase chromosomes and in interphase nuclei, we used either the entire PAC 215P15, which contains SMN2 and part of NAIP, or PAC 125D9, which contains SMN1. Despite the massive use of competitor DNA to block repetitive sequences, non-specific label was found throughout the nuclei with both probes. High background was also seen when we used cloned fragments from the SMN gene (13H4 of 10 kb and 1325E23 of 11.3 kb) containing SMN1 exons 7 and 8, and exon 2, respectively, or even smaller probes (5E11 of 5.6 kb and 1325E1 of 5 kb) containing SMN exons 3–6 and exon 1, respectively.

Therefore, we decided to use as probes PCR-amplified DNA fragments from the SMN gene which exclude almost completely the repetitive elements that are particularly abundant in the SMN non-coding region. The location of the probes relative to the genomic structure of the SMN gene is shown in Figure 1. For all FISH experiments, a mixture of seven fragments (fragments A–G) was used as probe (for some experiments, PCR product H was used instead of probe B) which together consist of 7.5–8.3 kb (Table 1). To determine the phase of the cell cycle in interphase nuclei, we used a cosmid located proximal to the duplicated SMA region at D5S524 as reference probe (32).

In dual-colour FISH analysis of metaphase chromosomes, strong red and green signals were obtained on the proximal part of chromosomes 5q which we attributed to the SMN and reference probes, respectively (Fig. 2a and b). Generally, SMN sequences were detected in red and the reference cosmid in green, but in some experiments the probes were detected

---

**Figure 1.** Schematic representation showing the position of the DNA fragments (A–H) used as probes for FISH analysis and primer pairs relative to the genomic structure of the SMN gene drawn to scale. Asterisks indicate the fragments also used for Southern blot analysis. EcoRI restriction sites and sizes of restriction fragments are also indicated.
In rare metaphases, a very faint signal was observed on one or both 5p chromosomes, presumably due to cross-hybridization with sequences on 5p homologous to the SMN gene. In mitoses of the majority of individuals, clearly four distinct signals could be seen across the width of at least one chromosome 5q, indicating that two copies of the SMN2 gene are located on the same chromosome (Fig. 2a). Exceptionally, six signals were found on chromosomes from patient M.A. (see below) (Fig. 2b). FISH on interphase nuclei showed either two single, generally distant, reference dots and, in their vicinity, one or more strong signals (see below) (Fig. 2b).

FISH on interphase nuclei showed either two single, generally distant, reference dots and, in their vicinity, one or more strong signals, representing SMN copies per chromosome of phase G1 nuclei (Fig. 2c and f), or two sets of paired reference dots and two or more adjacent signals corresponding to SMN copies on chromadits of phase G2 nuclei (Fig. 2d, e, g and h). In some rare interphase nuclei, one or two additional red signals were seen, presumably due to the 5p homologous region. These signals were, however, faint and distant from the reference signal and could thus readily be distinguished from the putative SMN gene copies.

We used this method to assess the SMN2 copy number in patients affected with different SMA types. For each patient, we analysed at least five metaphase and 30 interphase nuclei. The number of signals was determined both by direct observation at the fluorescence microscope and by elaborating digital images. Only nuclei showing an unambiguous number of signals were included into the counts, whereas those with apparently discordant copy numbers were excluded, as for example those with an odd number of SMN signals in G2 nuclei which can be explained by assuming that two copies are superimposed in the three-dimensional nucleus.

Table 2 summarizes the results of the FISH studies and NAIP analysis as well as relevant data of the patients, including current age or age at death and information on the achievement of motor milestones. Two of five SMA type I patients had two SMN2 gene copies, one on each chromosome 5. These patients were also homozygously deleted for NAIP and thus presumably carry large deletions, including both SMN1 and NAIP genes on both chromosomes 5. One SMA I patient (M.O.), who previously has been shown to carry the SMN1 Y272C mutation (19), showed three signals and is probably a compound heterozygote with one SMN2 on each chromosome 5 and a mutated SMN1 gene. As expected, this patient has retained at least one NAIP gene. Two type I SMA patients had three copies of SMN2 (one copy on one chromosome 5 and two copies on the other chromosome 5). Both had retained the NAIP gene, suggesting gene conversion events, which is supported further by the fact that one of the patients (P.I.) had a chimeric SMN gene with SMN2 exon 7 and SMN1 exon 8.

All seven SMA type II patients analysed had three copies of SMN2. Three copies were also found in one patient (A.L., see below) who was able to walk until the age of 3 years and was classified as type II/III based also on his histopathological pattern. Four type III patients had four copies of SMN2, with the exception of one patient who also had no intact NAIP gene. Two type IV patients with onset after the age of 20 years had four (I.T.) and eight (M.A.) SMN2 copies, respectively. We also included in the study one asymptomatic 15-year-old boy (M.R.G.), brother of type III patient M.R.F., who previously has been shown to have no SMN1 gene (33). This individual had four copies of SMN2 as did his haploidentical affected brother. Finally, a median copy number of four (range 3–6) SMN genes (SMN1 and SMN2) was detected in four controls, which is identical to that found by Taylor et al. (27) on a larger sample. For one control with three genes, genotype analysis has shown the absence of SMN2 genes.

Patients M.A. and A.L. belong to the same family and were born to consanguineous parents. M.A. has very mild adult-onset SMA and four SMN2 genes per chromosome 5 and previously has been shown to be homozygous by descent (34). His nephew,
type II/III patient A.L., was found to have three SMN2 genes. If we assume that he has inherited one gene from his father, he must have lost two copies of SMN2 present in the haplotype of his mother and uncle. Loss of at least one gene was confirmed by Ag1-CA genotyping (Fig. 3).

To investigate the possibility that some SMN2 genes may be functionally different due to sequence variations or rearrangements, Southern blot analysis of DNAs from 14 patients was performed using five DNA fragments as probes which detect all EcoRI fragments containing the entire SMN gene (Fig. 1, Table 2). No variation in size of the EcoRI fragments was found (data not shown), suggesting that none of the SMN2 genes had gross deletions or rearrangements. Sequence analysis was performed for all exons and intron–exon boundaries as well as part of the promoter (from Ag1-CA to the first codon) for the two SMA type I patients with three copies of SMN2. Furthermore, since the level of full-length transcripts of SMN2 depends primarily on the extent of exon 7 skipping and since most of the rare mutations reported for the SMN1 genes are confined to exons 6 and 7, we have sequenced these exons and...
the adjacent intronic regions of most patients (and for some cases also exon 8). No single sequence variation was detected.

**DISCUSSION**

We describe here a FISH analysis which allows visualization of the SMN genes on metaphase chromosomes and interphase nuclei. In a previous effort to detect SMN genes by FISH, Rajcan-Separovic et al. (35) reported on a variable number of hybridization signals in interphase nuclei from three SMA type I patients with 1–2, 2 and 5–6 signals, respectively. One explanation for this variability may be the use of a cosmid as SMN probe. When we used large DNA fragments from the SMN gene region as probes, we obtained strong background signal, presumably caused by the abundant repetitive sequences. In fact, the 28 kb DNA fragment harbouring the SMN gene contains 36 repeat elements, of which 33 are Alu sequences and the others are members of the THEI, L1 and MER4 repeat families. By choosing short DNA fragments consisting mainly of SMN exons and adjacent regions which exclude almost completely the repeat elements, we were able to obtain specific SMN signals on metaphase chromosomes and interphase nuclei. A faint label on 5p was observed in some preparations; this region has been shown previously to contain homologous sequences from the SMA region (36).

We used FISH on interphase nuclei to assess the exact number of SMN genes in 19 SMA patients, one asymptomatic individual with no SMN1 gene and four controls. Interestingly, the minimum number of genes could be deduced directly from the observation of metaphase spreads. In fact, chromosomes 5q frequently showed four SMN signals (two double dots per chromatid) on one or both chromosomes from individuals who were shown to have three or more SMN genes by interphase FISH. Similar to the data reported by Lawrence et al. (37), these signals generally were seen across the width of contracted chromosomes, whereas on longer chromosomes from less condensed metaphase spreads the signals could not be resolved. Lawrence et al. (37) have shown previously that sequences 500 kb–1 Mb apart frequently are resolved as two signals, and those separated by 1.1 Mb appear distinct in ~50%

<table>
<thead>
<tr>
<th>Patient</th>
<th>SMA</th>
<th>SMN2 genes</th>
<th>NAIP</th>
<th>Southern analysis</th>
<th>Sequenced exons</th>
<th>Current age/death/motor milestones</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.N.</td>
<td>I</td>
<td>2</td>
<td>–</td>
<td>+</td>
<td>6–7–8</td>
<td>6.5 months at death</td>
</tr>
<tr>
<td>M.N.</td>
<td>I</td>
<td>2</td>
<td>–</td>
<td></td>
<td></td>
<td>4.1 months at death</td>
</tr>
<tr>
<td>M.O.</td>
<td>I</td>
<td>2</td>
<td>+</td>
<td></td>
<td>4.5 years (under ventilation)</td>
<td></td>
</tr>
<tr>
<td>C.T.</td>
<td>I</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>All</td>
<td>15 months at death</td>
</tr>
<tr>
<td>P.I.</td>
<td>I</td>
<td>3</td>
<td>+</td>
<td></td>
<td>All</td>
<td>Unknown</td>
</tr>
<tr>
<td>M.R.</td>
<td>II</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>6–7–8</td>
<td>8 years, sits, never walked</td>
</tr>
<tr>
<td>C.L.</td>
<td>II</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>7 years, sits, never walked</td>
<td></td>
</tr>
<tr>
<td>L.N.</td>
<td>II</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>4 years, sits, never walked</td>
<td></td>
</tr>
<tr>
<td>W.O.</td>
<td>II</td>
<td>3</td>
<td>n.d.</td>
<td></td>
<td>6 years, sits, never walked</td>
<td></td>
</tr>
<tr>
<td>P.P.</td>
<td>II</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>6–7–8</td>
<td>24 years, sits, never walked</td>
</tr>
<tr>
<td>L.V.</td>
<td>II</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>2.5 years, sits, never walked</td>
<td></td>
</tr>
<tr>
<td>F.R.</td>
<td>II</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>6 years, sits, never walked</td>
<td></td>
</tr>
<tr>
<td>A.L.</td>
<td>II/III</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>6–7–8</td>
<td>33 years, walking until 3 years</td>
</tr>
<tr>
<td>M.G.</td>
<td>III</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>6–7</td>
<td>&gt;40 years, walked until 22 years</td>
</tr>
<tr>
<td>T.S.</td>
<td>III</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>6–7–8</td>
<td>19.5 years, walked until 5 years</td>
</tr>
<tr>
<td>A.I.</td>
<td>III</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>6–7</td>
<td>12 years, walking</td>
</tr>
<tr>
<td>M.R.F</td>
<td>III</td>
<td>4</td>
<td>+</td>
<td></td>
<td>6–7</td>
<td>12.5 years, walking</td>
</tr>
<tr>
<td>M.R.G.</td>
<td>III</td>
<td>4</td>
<td>+</td>
<td></td>
<td>6–7</td>
<td>15 years, asymptomatic</td>
</tr>
<tr>
<td>I.T.</td>
<td>IV</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>6–7–8</td>
<td>40 years, walking well</td>
</tr>
<tr>
<td>M.A.</td>
<td>IV</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>72 years, walking with aid since age 58</td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 2</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 4</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All patients have no SMN1 genes except for M.O. who has a mutated SMN1 gene.

*All copies are SMN1.

\[Table 2. FISH and molecular studies in SMA patients with different clinical phenotypes\]
copy number in siblings from SMA families with discordant phenotypes. Wirth et al. (12), in a study of compound heterozygous patients with one mutated SMN1 gene and absence of SMN1 on the other chromosome, found that in some families segregating the same mutation the severity was determined by the SMN2 copy number whereas others had phenotypic discrepancies despite the same number of SMN2 copies.

Absence of correlation in such particular families may be explained by functional differences between SMN2 genes. Coovert et al. (18) showed that type II patients with the same SMN2 copy number as type I patients produce more protein, and they hypothesized that sequence differences 5′ to exon 7 could account for these discrepancies. We did not find rearrangements and sequence differences around exon 7, suggesting that, if functionally different SMN2 genes exist, differences in splicing efficiency of exon 7 are unlikely to be involved. However, the presence of point mutations may not be detectable due to a higher ratio of normal versus mutated DNA and thus cannot be ruled out completely.

Although the number of patients studied is small, our data, together with those from previous studies, may allow some prognostic considerations. A single copy of the SMN2 gene is rare and is likely to be associated with a very poor prognosis. None of our patients had fewer than two genes. Taylor et al. (27) found a dosage ratio of 1:1, presumably reflecting a single SMN2 gene, in a patient with an unusually severe phenotype who survived for only 4 days. Also, the presence of two SMN2 genes was found to be associated with poor survival. The two patients with two SMN2 genes survived for 4 and 6.5 months, respectively, whereas type I patient C.T. with three SMN2 copies survived for 15 months in relatively good condition (no information was available for the other type I patient with three copies). Patients with three SMN2 genes either never gain the ability to walk or generally become wheelchair bound in childhood. Four or more genes generally are associated with a mild to a very mild phenotype.

In conclusion, we show that SMN genes can be detected by FISH on both metaphase chromosomes and interphase nuclei. Furthermore, our data provide additional evidence that the clinical phenotype of SMA is influenced by the copy number of SMN2 genes.

MATERIALS AND METHODS

Patients

All patients were diagnosed with SMA using the criteria defined by the International SMA Consortium (1). Clinical details of patient M.R.F. and his asymptomatic sib M.R.G. have been reported elsewhere (33). Patient M.A. corresponds to individual 4 of a group of patients with adult-onset SMA described earlier (5).

Molecular analysis

Molecular analysis was performed on DNA extracted from lymphocytes of peripheral blood. Exons 7 and 8 of the SMN gene were analysed by SSCP (6) and restriction digest assay (7). Exons 5 and 13 of NAIP were studied by multiplex PCR using the primer pairs 1863 + 1864 and 1258 + 1343, respectively (22). Sequence analysis of the SMN gene was performed using
a dye terminator cycle sequencing kit (Perkin Elmer, Warrington, UK) and an automatic sequencer (Applied Biosystem 373). Segregation analysis of the polymorphic multi-copy marker Ag1-CA was performed as described earlier (9).

Probes used for FISH analysis

Preliminary experiments were performed by using as probes PAC 215P15 and PAC 125D9 (kindly provided by Dr C. DiDonato, Ottawa General Hospital Research Institute, Canada), and plasmids 132H4, 132SE23, 5E11 and 132SE1 (kindly provided by Dr J. Melki, IGBMC, INSERM U184, Strasbourg, France). The probe used for FISH analysis of all patients and controls consisted of a mixture of DNA fragments (A–H) from the SMN gene region (Fig. 1), which were obtained by PCR amplification using the primers given in Table 1. Template DNAs for PCR amplification were PAC 215p15 (A, B, C and H), plasmids 132SE11 (D) and 5E11 (E and F), and a 5.2 kb fragment subcloned from 212P15 (G). PCR was performed using 50 µM primers, 200 µM dNTPs, 1.25 U of Taq polymerase (Boehringer Mannheim, Mannheim, Germany), 2 mM MgCl₂ and the annealing temperature of 56°C, except for fragment H for which the annealing temperature was 60°C. For some experiments, maxi-preparations of PCR products cloned in Bluescript vector were used as templates for PCR amplification with vector primers KS and SK at an annealing temperature of 48°C. Cosmid cos2 at locus D5S524 (32) was used as reference probe.

FISH

Metaphase chromosome and interphase nuclei preparations from SMA patients and controls were made from peripheral blood lymphocytes, lymphoblastoid cell lines or fibroblasts according to standard protocols.

For two-colour FISH analysis, ~300 ng of each amplification product was mixed and labelled using standard conditions. Hybridization to metaphase chromosomes or interphase nuclei were performed using standard conditions. Hybridization to repetitive sequences was suppressed by incubation of the labelled probe with 250 µg of sonicated human placenta in 4x SSC for 45 min at 65°C.

ACKNOWLEDGEMENTS

We thank the patients, clinicians and the Italian Union for Muscular Dystrophies (UIILDM) for their kind cooperation. We are grateful to M. Rocchi and N. Gandolfi for their advice and help in FISH analysis, to J. Melki for providing plasmids 132SE11, 5E11, 134H and 132SE23 and to C. DiDonato for providing PACs 215P15 and 125D9. The financial support of Telethon Italy (grant 828) is gratefully acknowledged.

REFERENCES

the spinal muscular atrophy phenotype by censMN copy number. Am. J. Hum. Genet., 63, 1712–1723.