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Correlation of *SMN*t and *SMN*c gene copy number with age of onset and survival in spinal muscular atrophy

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Childhood-onset autosomal recessive spinal muscular atrophy (SMA) is associated with absence of the telomeric survival motor neuron gene (*SMNt*) in most patients, and deletion of the neuronal apoptosis inhibitory protein (*NAIP*) gene in the majority of severely affected patients. Analysis of *SMNt* has been complicated by the existence of a centromeric copy, *SMNc*, which is almost identical to *SMNt* but which can be distinguished from it by restriction enzyme analysis. In this study 143 SMA patients have been genotyped for the presence or absence of the *SMNt*, *SMNc* and *NAIP* genes, and the data correlated with quantifiable clinical variables. Although a significant correlation was observed between the presence or absence of the *NAIP* gene and the severity of the clinical phenotype in SMA patients generally, there was no difference in age of onset or survival in type I patients with the *NAIP*+ or *NAIP*– genotype. Fluorimetric PCR analysis of *SMNc* gene dosage in 57 patients homozygous for the absence of the *SMNt* gene but in whom the *NAIP* gene was present showed a highly significant correlation between *SMNc* copy number and SMA subtype, and between *SMNc* copy number and both age of onset and length of survival. The data provide strong statistical support for the emerging consensus that the clinical phenotype in SMA is directed primarily by the level of functional SMN protein. The lower *SMNc* copy number in type I patients in whom the *NAIP* gene is present suggests that the *SMNt* gene is removed by deletion in the majority of such patients, rather than by gene conversion as is the case in SMA types II and III.

Keywords: spinal muscular atrophy; survival motor neuron genes; gene dosage

Introduction

Childhood-onset proximal spinal muscular atrophy (SMA) is the second most common lethal autosomal recessive disorder after cystic fibrosis, affecting approximately 1 in 10 000 births, with an approximate carrier frequency of 1 in 50. It is a neurodegenerative disease

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which affects the α motor neurons of the spinal cord, leading to wasting of the proximal muscles and death, usually due to respiratory infection. The disorder has been classified into three forms, types I (Werdnig-Hoffmann disease; severe SMA; acute infantile SMA), II (intermediate) and III (Kugelberg-Welander disease; mild SMA), which are defined by the age of onset and the clinical course of the disease.

All three forms of SMA were mapped to the long arm of chromosome 5, at 5q11.2–13.3,[1–3] indicating they could be allelic variants of the same disease. The analysis of multicopy polymorphic markers in the SMA candidate region provided the first indication that large-scale deletions could be involved in SMA.[4] This work showed both inherited and *de novo* deletions in nine unrelated SMA patients and marked heterozygosity deficiency for the loci studied in at least 18% of SMA type I patients.

Two candidate genes for all three types of SMA were identified in 1995, named the survival motor neuron (*SMN*) gene[5] and the neuronal apoptosis inhibitory protein (*NAIP*) gene.[6] Both genes were found to be duplicated within the candidate region for SMA. The *NAIP* gene was found to be homozygously deleted in 27.3% of SMA patients tested, and homozygous deletions of *NAIP* were also observed in 1.8% of SMA carriers.[6] The *SMN* gene exists in two almost identical forms, termed *SMNt* (telomeric) and *SMNc* (centromeric), which complicates the analysis of this gene. Both *SMNt* and *SMNc* encompass 20 kb of genomic DNA and have 9 exons. Sequence analysis has revealed only five single base differences between the two genes, one of which is in exon 7 and one in exon 8. PCR amplification and single-strand conformational polymorphism (SSCP) analysis allow a distinction to be made between exons 7 and 8 of the *SMNt* and *SMNc* genes. This technique was used to demonstrate that 98.6% of SMA patients studied had homozygous deletions of the *SMNt* gene.[5] No SMA carriers or normal control individuals were found to have homozygous deletions of the *SMNt* gene, although homozygous deletions of the *SMNc* gene were observed in these groups.

These results indicated that the *SMNt* gene was more likely to have a role in the pathogenesis of SMA, particularly since those SMA patients without a homozygous deletion of the *SMNt* gene were found to have a heterozygous deletion in conjunction with a point mutation in their intact copy of the *SMNt* gene. Later studies identifying various small mutations in the *SMNt* gene provided further evidence that it was likely to be the primary SMA-determining gene.[7–11] Additional work with the multicopy markers C212 and C272 showed an association of specific numbers of marker copies with the severity of the disease, and a model was proposed to account for the different phenotypes observed by a system of mild and severe alleles.[12] However, later studies[13–15] described homozygous deletions of the *SMNt* gene in a small number of unaffected individuals (siblings and parents of affected individuals), thus casting some doubt upon this gene as the only causal gene for SMA. The case for at least partial involvement of the *NAIP* gene in the SMA phenotype was supported by several studies which demonstrated that the severe SMA type I was associated with a higher frequency of homozygous deletions of *NAIP* than the milder forms[16–18] A study by Somerville *et al*[19] quoted a five-fold increased risk of type I SMA associated with absence of the *NAIP* gene.

A further complication in the molecular pathology of SMA emerged with the finding that some SMA patients who were apparently homozygously deleted for exon 7 of *SMNt*, but not for exon 8, had a chimeric gene in which exon 7 of *SMNc* was fused to exon 8 of *SMNt*, probably as a result of gene conversion.[5,7,12,20,21] Very recently, analysis of patients with SMA types II and III by pulsed-field gel electrophoresis showed physical evidence that some mutations previously classed as deletions actually resulted from gene conversions in which *SMNt* was replaced by *SMNc*.[22] These observations suggested a possible explanation for the different clinical phenotypes seen in patients who appear to have the same genotype, since the SSCP and restriction digestion assays do not distinguish between the absence of *SMNt* as a result of gene deletions or of gene conversions.[22] Thus if the loss of *SMNt* in SMA types II and III were the result of gene conversion rather than deletions, these patients would be left with a larger number of copies of *SMNc* than patients with SMA type I who had lost *SMNt* as a result of genuine gene deletions. The difference in *SMN* gene copy number would also be reflected in a different amount of functional protein, since the *SMNc* gene is known to be transcribed. This hypothesis is supported by the recent demonstration of a correlation between disease severity and SMN protein levels in the liver and spinal cord of SMA patients,[23] and by the finding of a higher ratio of *SMNc*/*SMNt* gene dosage in the parents of SMA II and III patients compared with the parents of type I patients.[16] Additionally, Coovert *et al*[24] reported a

correlation between disease severity and the amount of nuclear SMN protein, located in structures called gems, and postulated that gem formation was the key to adequate function of the SMN protein.

This study investigates the clinical and molecular characteristics of 143 SMA patients in order to establish their genotype at the *SMN*/*NAIP* loci, and to relate this information to measurable clinical parameters such as age of onset and survival. A fluorimetric PCR assay has also been developed to measure dosage of the *SMNc* gene, and demonstrates a highly significant correlation between *SMNc* copy number and clinical severity in SMA.

Materials and Methods

Patients

The patients investigated in this study were referred to the South Thames (East) Regional Genetics Centre by clinicians from the United Kingdom and continental Europe. Diagnosis rested with the individual clinicians, but review of referral details by one of us (NHT) has established that the index cases met the diagnostic criteria of the International Consortium on SMA[25,26] both in terms of clinical features and diagnostic investigations. Samples were accompanied by a standard referral form listing inclusion and exclusion criteria, and only those patients meeting these criteria were included in the study. DNA was available from 143 SMA patients, comprising 97 type I, 38 type II and eight type III individuals.

Assays

Absence of the SMNt Gene Homozygous deletion or absence of exons 7 and 8 of the *SMNt* gene was detected by restriction enzyme digestion assays based on the method published by van der Steege *et al*,[27] but with internal controls for digestion incorporated.

NAIP Exon 5 of the *NAIP* gene was detected using the published biplex PCR assay which includes exon 13 of the *NAIP* gene as an internal control.[6]

SMN Dosage Assay A fluorescent assay, based on a method by Yau *et al*[28] was developed to measure the ratio of *SMN* gene copy number (*SMNt* + *SMNc*) relative to a control locus, the myelin protein zero (*MPZ*) gene. The forward primers R111 and 541C960[5] were end-labelled with FAM (blue) and used with the unlabelled reverse primers 541C770 and 541C1120 to amplify *SMN* exons 7 and 8. Exons 1 and 3 of the *MPZ* gene were co-amplified with the *SMN* loci using similarly labelled primers for these exons.[29] The amplification mixture contained: R111, 541C770, 541C960 and 541C1120 at a concentration of 0.8 μM, MPZ1F, MPZ1R, MPZ3F and MPZ3R at a concentration of 0.4 μM, 500 μM dNTPs, 1.5 mM $MgCl_2$, 1.5 units *Taq* polymerase (Promega, Southampton) in a 25 μl reaction, using 'hot start'. The cycling times were as follows: 18 cycles of denaturation at 94°C for 20 s, annealing at 51.5°C for 20 s, extension at 70°C for 1 min, using the Techne Progene thermal cycler. PCR product (2.5 μl) was mixed with 3 μl of formamide loading dye and 0.5 μl of ROX-labelled size standard GS-500 and electrophoresed on an ABI 373A Genescanner (Perkin-Elmer/ABI, San Francisco, CA). The data were transferred to Genotyper software and the ratio of *SMN* to *MPZ* was calculated by dividing the sum of the peak areas for *SMN* exons 7 and 8 by the sum of the peak areas for *MPZ* exons 1 and 3.

Genotyping

The cohort of 143 patients with an unequivocal clinical diagnosis of SMA was typed with the *SMNt* exons 7 and 8 assays and with the *NAIP* exon 5 assay, in order to establish the frequencies of homozygous deletions or absence of these two genes in SMA patients. DNA from 200 SMA carriers, who were all parents of affected children, and a group of 100 unaffected controls, who were all adults referred for reasons other than neuromuscular di–sease, was also typed with these assays.

An alternative approach to evaluating the data is to classify the patients by their genotype rather than on the basis of their clinical classification as types I–III, and then to assess the clinical phenotype which is associated with a particular genotype, using measurable clinical parameters. In order to examine the relationship between *SMNt* and *NAIP* genotypes and clinical features, the patients were then divided into genotypic groups on the basis of these results, as follows:

- Group 1 (n = 64): *SMNt* exons 7 and 8 and *NAIP* exon 5 all homozygously absent (– – –);
- Group 2 (n = 64): *SMNt* exons 7 and 8 homozygously absent, at least one copy of *NAIP* exon 5 present (– – +);
- Group 3 (n = 7): *SMNt* exon 7 absent, at least one copy of exon 8 and *NAIP* exon 5 present (– + +);
- Group 4 (n = 8): At least one copy of *SMNt* exons 7 and 8 and *NAIP* exon 5 all present (+ + +).

Relevant clinical data, including age of onset, age of death and whether motor milestones (sitting and standing) were achieved, were available for these patients, although complete data for all features were not available for all patients. A statistical comparison of groups 1 and 2 was carried out, since sufficient numbers of patients had been studied.

A total of 57 SMA patients, all in genotype group 2 (– – +), was typed with the *SMN* dosage assay. Since they were known to have no *SMNt* product, this assay allowed an assessment of the relative numbers of copies of *SMNc* present. The patients comprised 26 type I, 23 type II and eight type III individuals. The assay was also used to type 27 unaffected control individuals in whom both *SMNt* and *SMNc* were present.

Statistical Analyses

For genotypic groups 1 and 2 (64 patients in each group), the mean ages of onset and death and the proportion of patients who achieved sitting and standing were calculated, and statistical analyses were performed. A histogram was produced showing the ages of onset for individuals in groups 1 and 2, and the difference in onset age between the two groups was assessed using the non-parametric Wilcoxon rank sum test. The differences in the proportion of individuals in these groups who achieved sitting and standing were assessed using a *t*-test.

Many patients were still alive at the most recent follow-up, and in order to make use of this censored information, survival time rather than age of death was examined. Survival was modelled using a Kaplan-Meier survival curve, which takes into account observations from the individuals known still to be alive at a certain age.

The *SMN*:*MPZ* ratios were calculated for each patient in genotypic group 2, and these were subdivided depending on the clinical classification of the disease severity. Analysis of variance was used to determine whether the *SMN*:*MPZ* ratios had a significant effect on the clinical subgroup. The effect of *SMN*:*MPZ* ratios on age at diagnosis and on survival was also investigated.

Results

The results of the analysis of *SMNt* and *NAIP* genotypes for 143 SMA patients, 200 SMA carriers and 100 unaffected controls were similar to previous studies, with homozygous absence of the *SMNt* gene in 94.4% of SMA patients tested, both exons 7 and 8 absent in 89.5%, and exon 7 alone absent in a further 4.9% of patients. The (– + +) genotype was only seen in patients with SMA type II. At least one copy of the *SMNt* gene was present in all SMA carriers and controls tested. No SMA patients had homozygous absence of the *SMNc* gene, but it was absent in 11% of controls. Exon 5 of the *NAIP* gene was absent in 44.8% of SMA patients and 4% of SMA carriers. The (– – – –) genotype was seen in 63.9% of SMA type I patients, but in only 5.3% of type II and no type III patients studied. The data confirm the high frequency of apparent deletions of the *SMNt* gene in all classes of SMA, and the association of *NAIP* gene deletions with the more severe phenotype, in agreement with previous studies.[5,16–19]

Figure 1 shows a histogram representing the distribution of ages of onset in groups 1 and 2 (– – – and – – + genotypes respectively). Where both *SMNt* and *NAIP* are absent, onset occurs earlier, and all patients with this genotype are clearly affected before the age of 10 months (mean = 2.16 months). By contrast, in patients where *NAIP* is present, onset occurs significantly later ($P < 0.0001$) with a mean of 5.8 months, and 18% of patients are diagnosed between 1 and 2 years. The proportion of patients in groups 1 and 2 who achieved the clinical milestones of sitting and standing was 2.9% versus 49.2% and 0% versus 16.1%, respectively ($P < 0.0005$ in each case). The mean age of death in group 2 was higher than in group 1 – 30.36 months compared with 8.93 months. However, this figure is distorted by one outlier in group 2 – a type II patient who survived until the age of 39 years. The survival curve for these two groups (Figure 2) clearly shows that patients with the – – +) genotype have a prolonged survival time compared with those with the (– – –) genotype. The median survival time, beyond which 50% of patients are still alive, was 7 months in the (– – –) genotype group, compared with 72 months in the (– – +) genotype group. Ten patients in group 2 were still alive after 10 years.

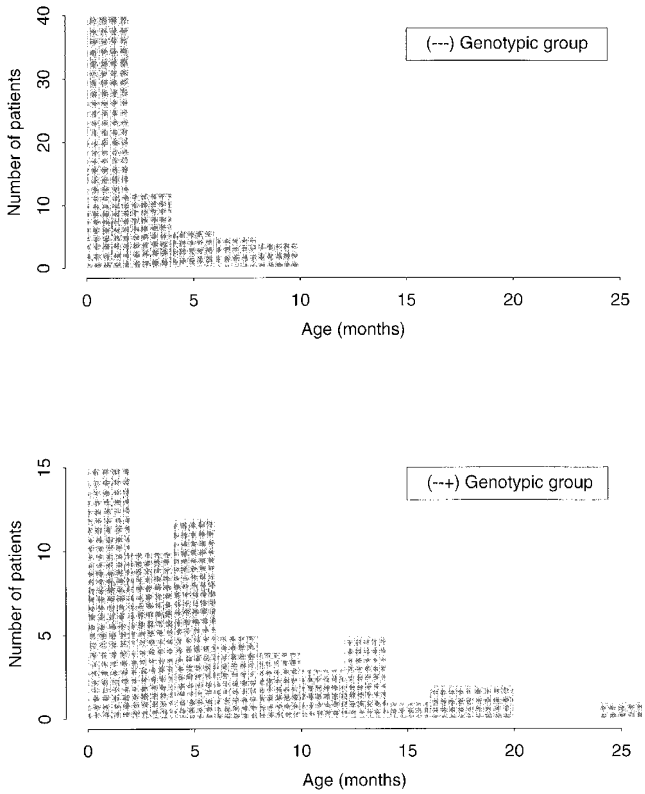

Figure 1 *Histograms showing earlier age of onset with absence of NAIP*

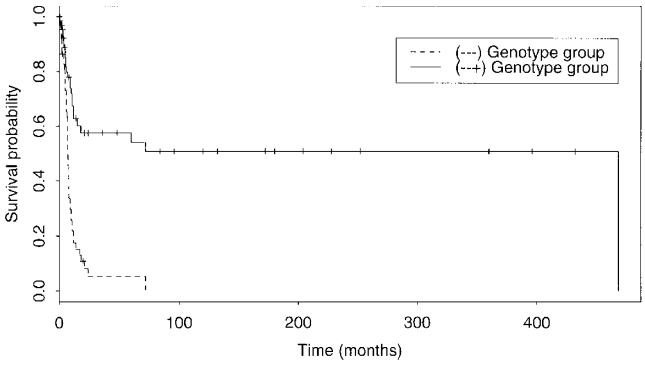

Figure 2 *Survival curves showing significantly reduced survival with absence of NAIP. The short vertical lines each represent one individual*

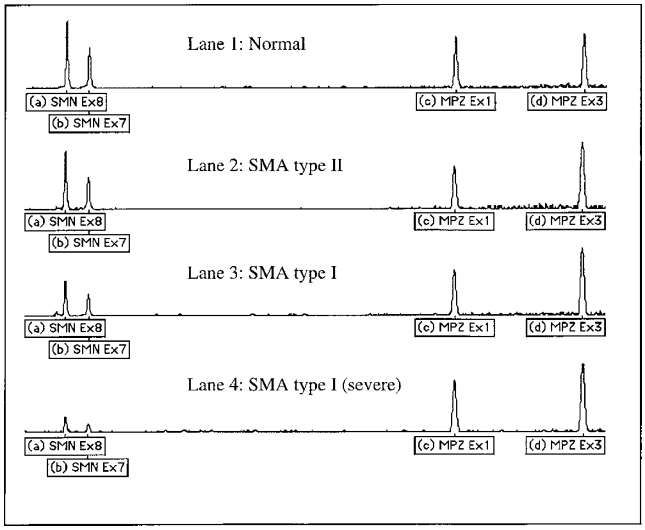

Figure 3 *Fluorescent dosage assay of SMNc, showing reduced peak areas for SMN exons 7 and 8 associated with a more severe phenotype*

Although the correlation between the presence or absence of *NAIP* and the severity of phenotype is highly significant, the (−−+) genotype group contains a substantial number of SMA type I patients. The phenotypes in type I patients in whom *NAIP* was present or absent were therefore compared. The (−−−) group had a mean age at onset of 2.1 months (n = 61), and a mean age at death of 8.1 months (n = 44), whereas in the (−−+) group the mean age at onset was 2.0 months (n = 32) and mean age at death was 8.0 months (n = 20). Thus the *NAIP* deletions had no effect on the phenotype of type I patients.

The hypothesis that copy number of the *SMNc* gene influences the clinical phenotype was therefore investigated. A dosage assay was devised to measure the copy number of this gene in patients who had homozygous absence of the *SMNt* gene, but in whom the *NAIP* gene was present (group 2). These included 26 patients classified as SMA type I, 23 as type II and eight as type III. The type of data produced by this assay is shown in Figure 3, which clearly illustrates a reduced *SMNc* dosage in SMA type I. Lane 1 shows a control individual, with lanes 2–4 showing patients in increasing order of severity – SMA type II, type I and severe type I. It can be seen that the peak areas for *SMN* exons 7 and 8 are reduced in more severely affected patients.

The boxplot in Figure 4 shows mean *SMN*:*MPZ* ratios for each group of patients with the (−−−+) genotype, and for the control group (+++) of 27 unaffected individuals, with 95% and 99% confidence intervals. The median of the ratios reduces progressively from type III to type I, and the ratios are substantially lower for SMA type I. The median and range for type III is similar to the controls, although the control values include one or more copies of *SMNt* which cannot be distinguished from *SMNc* in this assay.

The lowest ratio observed (0.11) was in a type I patient who was unusually severely affected, with reduced foetal movements apparent from 30 weeks of gestation, extreme hypotonia at birth, and death on day 4. Pairwise testing of dosage ratios between the four groups showed significant differences between all groups except between type III patients and controls (P < 0.002 in each case). Fitting a linear model with the subgroup as a factor was highly significant and accounted for 40% of the variance in the data. Analysis of variance showed a highly significant effect of the subgroup on the values obtained, with P < 0.0001. The *SMNc* gene dosage is plotted as a function of age of onset and survival in Figure 5 and Figure 6. These figures indicate that high *SMN*:*MPZ* ratios are significantly associated with both a later age of onset of SMA and a longer survival time. The age of onset increases approximately linearly with gene dosage (r^2 = 0.48, P < 0.0001). For survival, there was a sharp cut-off at a gene dosage ratio of 0.4, and a ratio lower than this is associated with significantly reduced survival, having an effect similar to that of patients in the (−−−) genotypic group.

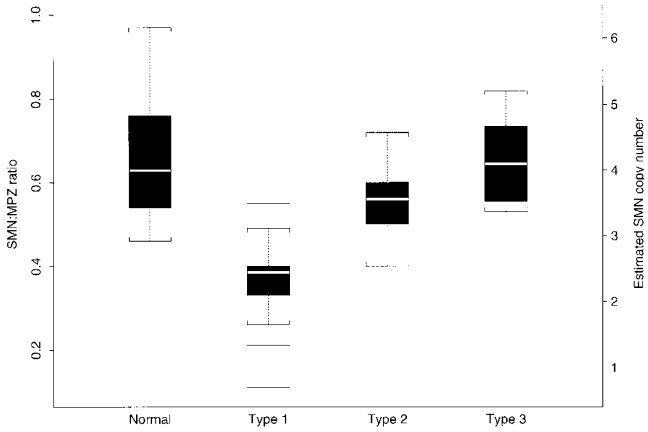

Figure 4 *Boxplot of SMNc ratios vs SMA types I, II and III (all of these patients lack SMNt). The median ratio is represented by a white horizontal line. The 95% confidence intervals are represented by the solid box, with the 99% confidence intervals being represented by the vertical dotted lines. Outliers are represented by the horizontal lines above and below the boxes. (Note that in the controls the dosage ratio reflects the copy number of both the SMNc and SMNt genes)*

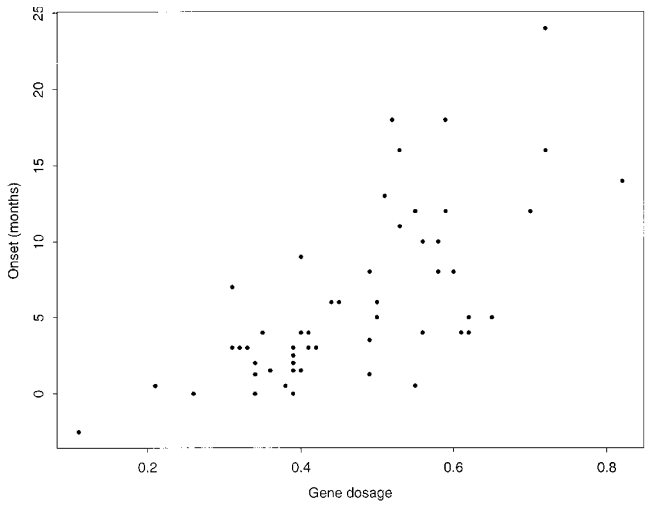

Figure 5 *Onset by gene dosage, showing a positive correlation between gene dosage ratio and age at onset of disease*

A wide range of *SMN*:*MPZ* ratios was seen in each group, and it is not possible to assess exact copy numbers from these ratios. However, if one assumes that the median dosage ratio observed in the normal control group (0.63) is most likely to represent four copies of *SMNc* + *SMNt* genes, an extrapolation could be made from this to estimate *SMN* copy number in SMA patients (see right-hand axis in Figure 4). This would give the following results:

- Normal: median copy number 4, range 2.9–6.2 copies (*SMNt* and *SMNc*);

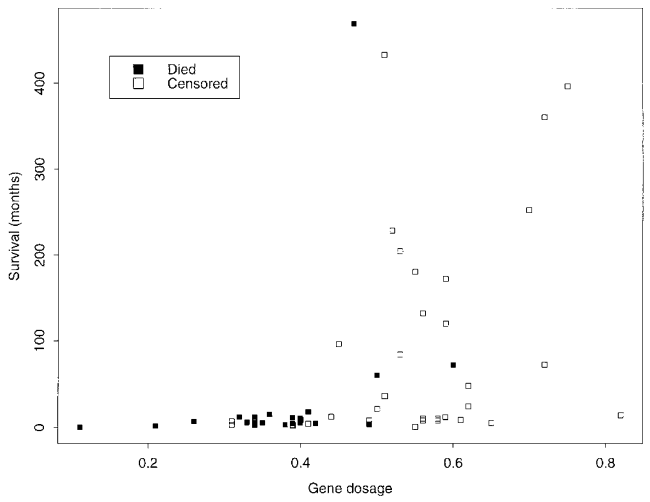

Figure 6 *Survival by gene dosage, showing a positive correlation between gene dosage ratio and survival time. A gene dosage ratio less than 0.4 is strongly associated with poor survival time*

- Type III: median copy number 4.1, range 3.4–5.2 copies (*SMNc* only);
- Type II: median copy number 3.6, range 2.5–4.6 copies (*SMNc* only);
- Type I: median copy number 2.4, range 0.7–3.5 copies (*SMNc* only).

The reproducibility of the assay was examined by dosage analysis of one SMA type I patient on six different occasions. This produced an estimated copy number of 2–2.7 copies. Thus variation in experimental conditions could account for the non-integral values of copy number observed in some patients.

Discussion

It is now well established that the *SMNt* gene is absent in a high proportion of SMA patients, and that deletions of the *NAIP* gene are found in the majority of type I patients, but only rarely in types II and III.[5,16–19,30] This correlation has been extended by assessing the clinical phenotype of the four genotypic groups defined by the *SMNt* and *NAIP* PCR assays in terms of the measurable clinical parameters, and subjecting the data to formal statistical analysis. It is apparent that the (−−−) genotype is associated with an earlier age of onset of the disease and survival relative to the (−−+) genotype, and that these differences are statistically highly significant. However, the fact that there was no difference in age of onset or survival in type I patients with the (−−−) and (−−+) genotype indicates that it is not the absence of the *NAIP* gene that determines the clinical phenotype.

The hypothesis that the extensive variation in the clinical phenotype of patients with apparently identical genotypes as defined by the *SMN* and *NAIP* PCR assays results from differences in copy number of the *SMNc* gene was then investigated. Fifty-seven patients from genotype group 2 (homozygous absence of the *SMNt* gene with *NAIP* present) were typed with a fluorescent dosage assay which measures the relative number of copies of the *SMNc* gene. Highly significant differences in *SMNc* copy number were observed among all three SMA types (Figure 4), and only type III patients were not significantly different from controls. The correlation of *SMNc* copy number and phenotype was also highly significant when analysed with respect to age of onset and survival (Figure 5 and Figure 6). Dosage values of less than 0.4, estimated to be

equivalent to 1–2 copies of *SMNc*, were associated with particularly early onset and poor survival. The most severe phenotype in our series was associated with a dosage ratio of 0.11, which is likely to reflect a single copy of the *SMNc* gene. Since no individuals with absence of both *SMNt* and *SMNc* genes have been reported, it is likely that disruption of both genes would constitute a lethal mutation. The fact that type III patients with the (−−+) genotype had *SMN* dosage values similar to those of controls can be explained by assuming that while the total number of *SMN* genes of any type is similar in these two groups, normal individuals (who have at least one copy of the fully functional *SMNt*) have fewer copies of *SMNc* than these type III patients. If the model of copy number proposed above is valid, normal individuals could have as many as 6 copies of *SMNt* and *SMNc* genes, in agreement with the study of Velasco *et al*.[16] Although the correlation between the *SMNc* dosage and measurable clinical parameters of clinical severity was not absolute, the results do indicate that in the absence of *SMNt*, the phenotype is strongly related to *SMNc* copy number.

This analysis is consistent with the recent study of Lefebvre *et al*, who used antibody staining to show both that the *SMNc* gene is expressed in SMA patients, and that SMN protein levels are correlated with the severity of the disorder.[23] It also supports the earlier study of Velasco *et al*,[16] who demonstrated an increased *SMNc*/*SMNt* ratio in the parents of type II and type III patients relative to the parents of type I patients. Additionally, McAndrew *et al*[31] have recently used a quantitative PCR assay to show that an asymptomatic SMA carrier with no copies of the *SMNt* gene had four copies of the *SMNc* gene, in keeping with the findings of this study. However, three haploidentical siblings from SMA families with discordant phenotypes showed no difference in *SMNt* or *SMNc* copy numbers. The correlation between phenotype and protein level is also not absolute. Coovert *et al*[24] found that in five type I patients the mean SMN protein level was 17% of normal (range 9–27%), whereas in eight type II patients it was 28% (range 15–41%). However, there was a very strong correlation between the nuclear staining pattern as reflected in the number of gems from 100 nuclei, which was 5 in type I and 20 in type II patients. Thus, all *SMNc* genes are not likely to be functionally equivalent.[24]

This study also addresses the question of the molecular basis of the absence of the *SMNt* gene in SMA type I patients in whom the *NAIP* gene is present. Burghes[32] points out that whereas the (−−−) genotype in SMA type I is likely to reflect a deletion and the (−−+) genotype in types II and III a conversion, the (−−+) genotype in SMA type I could be either a deletion or a conversion. The data in Figure 4 show that most type I patients with the (−−+) genotype have approximately two copies of *SMNc*, whereas the type II and III patients have 3–5 copies. This suggests that the *SMNt* gene is removed by a deletion in the majority of (−−+) type I patients.

In conclusion, this study demonstrates that although there is a highly significant correlation between the presence or absence of the *NAIP* gene and the severity of the clinical phenotype in SMA, its presence does not influence age of onset or survival in a subgroup of severely affected patients. This is consistent with the fact that small mutations within the *SMNt* gene do not disrupt *NAIP*, but may still give rise to a severe phenotype.[7–9] The study also confirms the strong correlation between *SMNc* copy number and phenotype in patients lacking an *SMNt* gene, and demonstrates the value of assessing clinical phenotype in terms of quantifiable variables which can be subjected to formal statistical analysis. This is particularly relevant in the context of the difficulties that may be encountered in classification of SMA patients into defined subgroups.[33,34]

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