SMN1 gene duplications are associated with sporadic ALS

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ABSTRACT

Objective: To investigate the role of *SMN1* and *SMN2* copy number variation and point mutations in amyotrophic lateral sclerosis (ALS) pathogenesis in a large population.

Methods: We conducted a genetic association study including 847 patients with ALS and 984 controls. We used multiplexed ligation-dependent probe amplification (MLPA) assays to determine *SMN1* and *SMN2* copy numbers and examined effects on disease susceptibility and disease course. Furthermore, we sequenced *SMN* genes to determine if *SMN* mutations were more prevalent in patients with ALS. A meta-analysis was performed with results from previous studies.

Results: *SMN1* duplications were associated with ALS susceptibility (odds ratio [OR] 2.07, 95% confidence interval [CI] 1.34–3.20, $p = 0.001$). A meta-analysis with previous data including 3,469 individuals showed a similar effect: OR 1.85, 95% CI 1.18–2.90, $p = 0.008$). *SMN1* deletions and *SMN2* copy number status were not associated with ALS. *SMN1* or *SMN2* copy number variants had no effect on survival or the age at onset of the disease. We found no enrichment of *SMN* point mutations in patients with ALS.

Conclusions: Our data provide firm evidence for a role of common *SMN1* duplications in ALS, and raise new questions regarding the disease mechanisms involved. ***Neurology*® 2012;78:776–780**

GLOSSARY

ALS = amyotrophic lateral sclerosis; **CI** = confidence interval; **MLPA** = multiplexed ligation-dependent probe amplification; **OR** = odds ratio; **SMA** = spinal muscular atrophy.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that selectively affects motor neurons in the spinal cord and motor cortex, leading to progressive paralysis and invariably death. The majority of cases have no familial history of the disease and are said to be sporadic. The pathogenesis of sporadic ALS is thought to be an interplay of genetic and environmental risk factors contributing to increased disease susceptibility. One gene that has been claimed to modulate susceptibility and disease course in ALS is the survival motor neuron (*SMN*) gene.[1] It is present in 2 copies: *SMN1* and a centromeric copy *SMN2*, with about 10% of the biological activity of *SMN1*.[2] Homozygous deletion of *SMN1* causes spinal muscular atrophy (SMA), a congenital motor neuron disease, and higher *SMN2* copy numbers are associated with milder SMA phenotypes.[3] In a minority of cases, SMA is caused by point mutations in *SMN1*, rather than by homozygous deletion.[4]

Because of the phenotypic similarities between SMA and ALS, the role of *SMN* in ALS pathogenesis has been the subject of various studies, several of them reporting significant effects of *SMN1* and *SMN2* copy numbers on disease susceptibility or on disease duration.[1,5–8] The largest study performed so far showed that abnormal (i.e., 1 and 3) *SMN1* copy numbers were associated with ALS.[8] This investigation did, however, incorporate samples from a prior study,

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Supplemental data at www.neurology.org

Supplemental Data

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Table 1 Patient characteristics

	ALS	Controls
Copy number analyses		
No. (% female)	847 (43)	984 (48)
Mean age, y	61.9	62.8
Bulbar/spinal onset, %	33/67	—
Mutation screen		
No. (% female)	975 (40)	1,044 (47)
Mean age, y	60.4	63.4
Bulbar/spinal onset, %	32/68	—

Abbreviation: ALS = amyotrophic lateral sclerosis.

and the results were inconsistent with other reports.[5,9] Furthermore, the reported effects of *SMN* copy numbers on disease duration have been inconsistent. The role of *SMN* mutations in ALS has never been investigated.

We performed a large-scale association study to determine the effect of *SMN1* and *SMN2* copy numbers on disease susceptibility and on disease course. We genotyped a genetically homogeneous population that had not been included in previous reports. In addition, we carried out a comprehensive mutation screen to examine the role of *SMN* coding sequence mutations in ALS.

METHODS Patients with ALS and healthy volunteers participating in this study were recruited in the outpatient clinic for motor neuron diseases of the Utrecht University Medical Center, or were part of a population-based study on ALS in the Netherlands. This population has been described in detail elsewhere.[10] Patients and controls participating in previous studies on *SMN* were excluded from the copy number analyses. Patients with ALS had no family history of the disease and all fulfilled the 1994 El Escorial criteria for probable or definite ALS.[11] All participants gave written informed consent. Genomic DNA of patients with ALS and controls was isolated in the same laboratory, using a salting-out procedure. In total we included 847 patients with ALS (57% male) and 984 controls (52% male) in the copy number analyses and 975 patients with ALS (60% male) and 1,044 controls (53% male) in the mutation screen (table 1).

Multiplexed ligation-dependent probe amplification (MLPA) assays were run using standard protocols (www.mlpa.com). We used the SALSA P060 MLPA kit (MRC Holland, the Netherlands), containing 2 probes specifically targeted to *SMN1*, 2 probes targeted to *SMN2*, and control probes targeted to other chromosomal loci for normalization and assay quality control. A total of 50–100 ng of genomic DNA was used in each MLPA assay. Data normalization and analysis were performed with GeneMarker software (SoftGenetics, State College, PA) using standard parameters.

To determine the reproducibility of our MLPA assay, we ran 90 samples twice, in separate reactions, and calculated the copy numbers for both replicates of each sample as described below. For the *SMN1* probes, the percentage of agreement was 99% (1 of 90 samples had different copy numbers between the 2 replicates), and 98% for *SMN2* (2 out of 90 samples showed different copy numbers between replicates).

For mutation screening, we used PCR and sequencing protocols described elsewhere.[12] In short, we designed 2 nested primer pairs for each amplicon, amplified exonic sequences and intron-exon boundaries, and sequenced the amplicons using dideoxy sequencing. Sequencing was done on ABI 3,730 capillary sequencers with Big Dye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA). Sequence data were imported in PolyPhred software,[13] and sequences were visually inspected for heterozygous sites. All putative mutations were confirmed with an independent PCR and sequencing reaction. Functional impact of identified mutations was predicted using PolyPhen software (http://genetics.bwh.harvard.edu/pph/). Primer sequences are available upon request. These primers are not specific to *SMN1* or *SMN2*, but amplify sequences from both genes. Identified mutations cannot, therefore, be mapped specifically to 1 of the 2 genes. We chose this method because approaches to specifically sequence either *SMN1* or *SMN2* would be extremely laborious, and would only be justified in the case of a suspected association. Two SMA patients with known *SMN1* mutations (in the presence of normal *SMN2* copy numbers) were used as positive controls. The software called both mutations, thus demonstrating that our method reliably detects mutant alleles at least in a 1:3 ratio.

All statistical procedures were carried out in *R* 2.10.1 statistical environment (http://www.r-project.org). Because the quantitative measurement of copy number data is prone to systematic bias leading to false-positive associations,[14] we used 2 different methods to test *SMN1* and *SMN2* copy number state for association with ALS susceptibility. First, we determined *SMN1* and *SMN2* copy number states for each individual using Gaussian mixture modeling with the CNVtools software package in *R*.[14] The mean signal of the 2 probes for each gene was used as the input signal. Gaussian distributions were fitted on the signal intensity distributions and individuals were assigned to copy number states based on the highest a posteriori probability. For *SMN1* a 3-component model was used (corresponding to 1, 2 and 3 copies) and for *SMN2* a 5-component model was used (corresponding to 0, 1, 2, 3, and 4 copies). These copy number states were then used in a multivariate logistic regression model including *SMN1* and *SMN2* copy number state and with age at onset and gender as covariates. Secondly, we used a likelihood ratio association test employing CNVtools, using a linear trend model. This method was specifically designed to handle intensity data from quantitative measurements, and allows for differential bias due to possible differences in data quality between cases and controls, causing spurious associations.[14] Cox regression was used to test for effect of *SMN1* and *SMN2* copy number on survival, using age at onset, gender, and site of onset as covariates. For the effect on age at onset, we used Cox regression with gender and site of onset as covariates. For the combined analysis of the different studies, we used the random-effects meta-analysis (DerSimonian-Laird) in the rmeta package in *R*. We used the Woolf test to test for significant heterogeneity between different studies. In order to test for difference in frequency of *SMN* mutations between patients and controls, the Fisher exact test (2-sided) was applied.

RESULTS We included 847 patients with ALS and 984 controls in the copy number analyses (table 1). First we tested both *SMN1* and *SMN2* copy number states for association with ALS susceptibility using

Table 2 *SMN1* and *SMN2* copy number association analysis

Copy no.	ALS (n = 847), n (%)	Controls (n = 984), n (%)	OR (95% CI)	p
SMN1				
1	16 (1.9)	23 (2.3)	0.83 (0.42–1.65)	0.60
2	771 (91.0)	926 (94.1)	—	—
3	60 (7.1)	35 (3.6)	2.07 (1.34–3.20)	1.1×10^{-3}
SMN2				
0	62 (7.3)	78 (7.9)	0.84 (0.58–1.22)	0.36
1	329 (38.8)	372 (37.9)	1.00 (0.81–1.22)	0.98
2	416 (49.1)	486 (49.5)	—	—
3	39 (4.6)	46 (4.7)	0.99 (0.63–1.58)	0.98
4	1 (0.1)	2 (0.2)	0.62 (0.05–7.22)	0.70

Abbreviations: ALS = amyotrophic lateral sclerosis; CI = confidence interval; OR = odds ratio; *p* = logistic regression *p* value.

logistic regression. *SMN1* duplications (i.e., 3 copies) were significantly associated with ALS (odds ratio [OR] = 2.07, 95% confidence interval [CI] = 1.34–3.20, $p = 0.001$) (table 2). There was no effect of *SMN1* deletions (i.e., 1 *SMN1* copy) on disease susceptibility (OR = 0.83, 95% CI = 0.42–1.65, $p = 0.60$), and we found no effect of *SMN2* copy number states on disease susceptibility. The removal of *SMN2* copy number states from the model that tested *SMN1* and vice versa did not change the results (not shown).

We then used a likelihood ratio test for association to investigate the significance of *SMN* copy numbers on ALS susceptibility. Using this approach, we obtained similar results for both genes: $p = 0.001$ for *SMN1*, $p = 0.99$ for *SMN2*, thus corroborating the results obtained from the logistic regression model.

When combining data from the current study with previously published data, *SMN1* duplications were significantly associated with ALS (OR = 1.85, 95% CI = 1.18–2.90, $p = 0.008$) (figure). There was no significant heterogeneity between different studies ($p = 0.17$, Woolf test). The effect of *SMN1* and *SMN2* deletions was not significant (*SMN1:* OR = 2.23, 95% CI = 0.93–5.32, $p = 0.07$; *SMN2:* OR = 1.41, 95% CI = 0.87–2.27, $p = 0.16$).

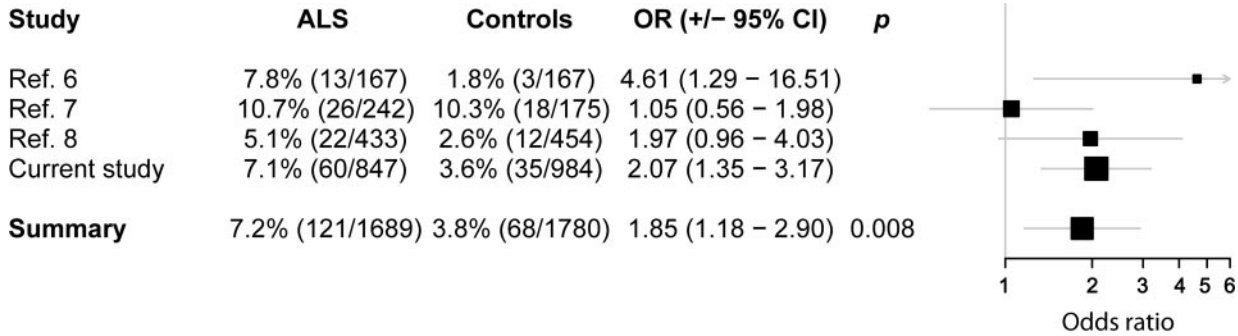
Then the effect of SMN copy number on phenotypic characteristics was tested. Complete clinical data were available for 814 patients with ALS. We found no effect of either deletions or duplications of *SMN1* or *SMN2* on disease duration or age at onset (*p* values >0.1, not shown).

Sequence data were available for 975 patients with ALS and 1,044 controls. In our mutation screen we identified 15 heterozygous sequence variants, excluding known single nucleotide polymorphisms (dbSNP build 129) (table e-1 on the *Neurology*® Web site at www.neurology.org). There was no enrichment of *SMN* mutations in patients with ALS: $p = 0.30$, 2-tailed Fisher exact test. Two variants, G26D and P198L, will result in amino acid changes. A G26D mutation was identified in 1 patient with ALS; this variant is predicted to be "possibly damaging" by PolyPhen. A P198L mutation was found in a healthy control, and is predicted to be "probably damaging." Both individuals had "normal" (i.e., 2) copy number of both *SMN* genes.

DISCUSSION We found a significant effect of *SMN1* duplications on ALS susceptibility, which can be considered a major risk factor for sporadic ALS. The effect size of *SMN1* duplications, obtained from the combined analysis of almost 3,500 individuals, is one of the highest, compared to other established risk factors for ALS. The large sample size and the robust techniques used for data acquisition and analysis provide confidence in the solidity of the data.

Our results are in line with previous reports that showed that abnormal *SMN1* copy numbers are as-

Figure Combined analysis of *SMN1* duplications



The table shows per study the frequency and absolute numbers and risk estimate of *SMN1* duplications. ALS = amyotrophic lateral sclerosis; CI = confidence interval; OR = odds ratio; *p* = logistic.

sociated with an increased susceptibility to ALS. In fact, all 3 studies measuring *SMN1* duplications in the context of ALS reported higher frequency in patients with ALS, compared to controls (see figure); in 2 of the 3 studies this difference was statistically significant.[6–8] This is reflected by a significant result in the combined analysis, without evidence for heterogeneity between the different studies. With these data, we conclude there is now firm evidence for an association between *SMN1* duplications and ALS susceptibility.

In the current study we find no evidence for an effect of *SMN1* deletions on disease susceptibility. Additionally, the results of the combined analysis weaken previous reported associations of *SMN1* deletions and ALS. The same holds true for the effect of *SMN2* copy number variants, which was not confirmed in our study. One possible explanation for the nonreplication of previous reported results is the fact that studies using quantitative PCR-derived data, such as in copy number studies, are inherently sensitive to different sources of bias.[14] For example, DNA isolation and handling can introduce differential bias between cases and controls, leading to spurious associations.[15] For this reason we used MLPA, a technique that allows simultaneous quantification of multiple probes with one primer pair.[16] This reduces the chances of spurious results due to different PCR reaction properties of target and normalization primers, as might be obtained with standard quantitative PCR assays. Furthermore, we used a recently developed statistical framework, providing a robust means of association testing of copy number data. The fact that we obtained similar results using different association tests adds to the validity of our data.

In addition to the copy number analyses, we undertook a mutation screen to examine the role of *SMN* point mutations in ALS pathogenesis, prompted by the fact that a minority of SMA cases are caused by subtle point mutations rather than by gross deletions of *SMN1*. The role of *SMN* mutations in ALS has not been studied before. We find no evidence for a role of point mutations in ALS pathogenesis. Although our approach does not discriminate between mutations in *SMN1* and *SMN2*, and therefore a differential clustering of *SMN1* mutations in patients with ALS and *SMN2* mutations in controls cannot be excluded, the lack of enrichment of mutations in patients with ALS does make it very unlikely that these mutations contribute significantly to ALS pathogenesis.

The mechanism of *SMN1* duplications on disease susceptibility remains elusive. Given the initial hypothesis that low SMN protein levels increase risk for ALS, in analogy to SMA, this is counterintuitive. An explanation would be that *SMN1* duplications actually produce lower amounts of SMN protein. A total of 5%–15% of copy number variants are negatively correlated with gene expression[17] but it is not known if such a relation exists for *SMN*. If a lower amount of SMN protein mediates ALS risk, one would also expect an overrepresentation of *SMN1* deletions in ALS. An alternative and intriguing explanation would be that *SMN* duplications produce higher SMN protein levels that are toxic to motor neurons, but to our knowledge there is as yet no experimental evidence to support this theory. Another explanation is that *SMN1* duplications confer a risk factor independent of SMN protein, e.g., because of duplication of other local genomic regions, which we cannot exclude. Our MLPA assay did not include probes that are targeted at SMN-flanking genes. Therefore, this question remains to be answered and could be subject to further research in the future.

Our data provide firm evidence that *SMN1* copy number variants are involved in ALS pathogenesis. Further research is needed to explain the increased risk of *SMN1* duplications, rather than deletions. Given the large effect size, as compared to other established risk factors for sporadic ALS, *SMN* duplications are an important risk factor for ALS and further functional studies would be highly justified.

AUTHOR CONTRIBUTIONS

Dr. Blauw: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data, statistical analysis, study supervision. Dr. Barnes: analysis or interpretation of data, statistical analysis. Dr. Van Vught: analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data. Dr. Van Rheenen: analysis or interpretation of data, acquisition of data. M. Verheul: study concept or design, acquisition of data. Dr. Cuppen: study concept or design, analysis or interpretation of data, acquisition of data. Dr. Veldink: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data, statistical analysis, study supervision, obtaining funding. Dr. Van Den Berg: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, acquisition of data, study supervision, obtaining funding.

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