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Variants of the heavy neurofilament subunit are associated with the development of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder primarily affecting motor neurons. The etiology of the majority of cases remains unknown. Recent findings from several laboratories suggest a role for neurofilaments in the development of motor neuron disorders. The C-terminal region of the human neurofilament heavy subunit (NEFH) contains a unique functional domain consisting of 43 repeat motifs of the amino acids Lys–Ser–Pro (KSP). This C-terminal region of NEFH forms the sidearm projections which cross-link the neurofilaments. Previously, we have demonstrated polymorphism in the C-terminal region of the human NEFH: an allelic variant of a slightly larger molecular size, containing an additional KSP phosphorylation motif. Novel mutations in this region were found in five ALS patients. We propose that changes in the KSP-repeat domain may affect the cross-linking properties of the heavy neurofilament subunit and perhaps contribute to the development of neurofilamentous swellings in motor neurons, a hallmark of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an almost invariably fatal adult-onset neurological disorder, resulting from the degeneration of the large motor neurons in brain and spinal cord. In approximately 10% of the cases, ALS is inherited as a Mendelian trait. Mutations in the copper/zinc superoxide dismutase gene located on chromosome 21 have been found in a subset of familial ALS (FALS) cases (1). Yet, for more than 95% of ALS patients, including FALS and sporadic cases, the etiology remains unknown.

While there is no evidence that neurofilaments play a primary causative role in the development of ALS, our current understanding of ALS is consistent with the hypothesis that neurofilamentous changes are an important part of a final common pathway in the degeneration of motor neurons. The abnormal accumulation of neurofilaments in the cell body and proximal axon of motor neurons is a characteristic pathological finding in ALS (2–6). Moreover, evidence for a role of neurofilaments in the pathogenesis of experimental models of motor system degeneration is increasing (7–10). Aberrant neuronal swellings that are highly reminiscent of those found in ALS have recently been reported in transgenic mice overexpressing either the neurofilament light- (NEFL; 8) or heavy-subunit (NEFH; 9). The adult-onset and progressive neurological disorder occurring in NEFH transgenics is likely the result of an impairment of neurofilament transport due to excessive levels of NEFH subunits (9).

One possible basis for neurofilaments to predispose to ALS lies in variations in the C-terminal region of the human NEFH, which contains a unique functional domain with multiple repeat motifs of the amino acids Lys–Ser–Pro (KSP). The human NEFH sequence published by Lees *et al.* (11) contains 43 KSP repeats. Recently, we have identified a polymorphic variant of this region containing (in comparison with the originally published sequence) an 18 bp insert which includes an additional KSP motif (12). The NEFH C-terminal region was examined in 356 individuals with sporadic ALS. Codon deletions in the C-terminal region were identified in five of the sporadic ALS patients; such mutations were not found in a group of 306 controls. Based on these findings, we propose that certain variant alleles of the NEFH may predispose an individual to developing ALS.

RESULTS

Mutations of *NEFH* in sporadic ALS patients

The C-terminal region of *NEFH* containing the KSP repeat motifs was amplified by the polymerase chain reaction (PCR) in DNA samples from 356 non-related individuals with sporadic ALS, and from 306 control individuals. PCR amplification of the C-terminal region of *NEFH* yielded fragments with anomalous migration after electrophoresis on non-denaturing acrylamide gels in five sporadic ALS patients (see Fig. 1 for examples). No mutations were identified in the control population of 306 individuals. The mutant *NEFH* alleles were subsequently subcloned and sequenced. Four of the patients, J.D., M.L., N.M., and J.V., each bear one *NEFH* allele with the same mutation; these patients have no ancestor in common. Their mutant *NEFH* allele contains a 3 bp deletion: bases 2368–2370, using the numbering of Lees *et al.* (11). The fifth patient, A.C., has one mutant copy of *NEFH* with a 102 bp deletion: bases

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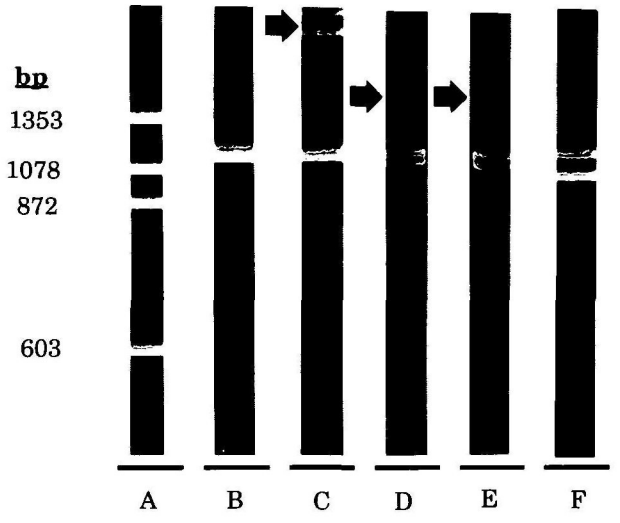

Figure 1. PCR amplification from genomic DNA of the KSP repeat motif region of human *NEFH*. Lane **B**: Control individual, homozygous for the 44 KSP repeat allele of *NEFH*. Lane **C**: Control individual, heterozygote, with one allele containing 43 KSP repeats, and one allele containing 44 KSP repeats. Lane **D**: Patient J.D. Lane **E**: Patient N.M. Lane **F**: Patient A.C. The arrow indicates heteroduplex formation seen after electrophoresis on 8% polyacrylamide, from any heterozygous individual. Lane **A** contains *Hae*III digested ϕX174 DNA, with fragment sizes shown at the left.

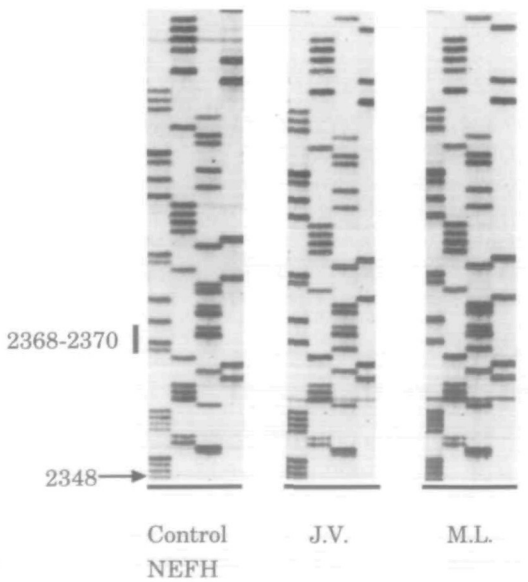

Figure 2. Sequencing gels showing the control *NEFH* allele (bases 2348–2407), and the mutant *NEFH* allele from ALS patients J.V. and M.L. Bases 2368 through 2370 (numbering of Lees *et al.*, ref. 11) are deleted in these individuals. In each sequencing panel, the lanes are ordered **A–C–G–T**.

1582–1683. Figures 2 and 3 show the sequence of these mutant alleles.

The 3 bp deletion in the NEFH gene creates a new *Taq*I site. The mutation in four patients was further confirmed by Southern hybridization of genomic DNA digested with *Taq*I enzyme using a probe spanning 1.2 kb of the *NEFH* KSP repeat region (data

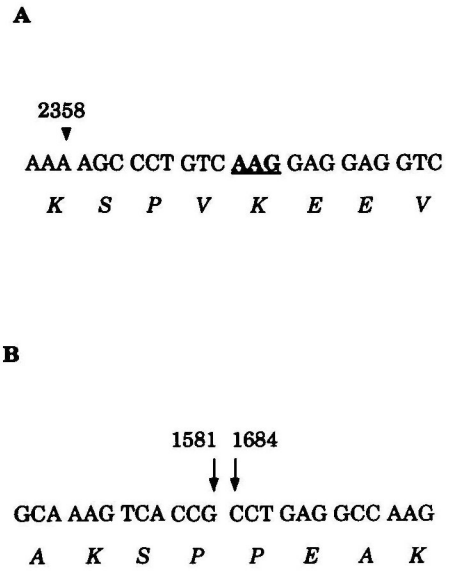

Figure 3. (**A**) The sequence of human *NEFH*, 2356–2379 bp. The underlined trinucleotide, AAG, encoding Lys (K), is deleted in four ALS patients: J.D., M.L., N.M., and J.V. (**B**) The sequence of the mutant *NEFH* allele from ALS patient A.C., beginning with nucleotide 1570.

not shown). Similarly, it was possible to distinguish the 102 bp deletion in one *NEFH* allele of patient A.C. by Southern hybridization of genomic DNA digested with *Hin*fI (Fig. 4). Most of the KSP repeat-encoding sequence of *NEFH* is contained in a 997 bp *Hin*fI fragment. Figure 4 shows the Southern blot demonstrating normal and mutant *NEFH* alleles in A.C.

Family studies of sporadic ALS patients

DNA was obtained from first-degree relatives of the five ALS patients with *NEFH* deletions. The mother of A.C. died of breast cancer at 54; no information is available for the father or sole sibling, who are also deceased. DNA was available from three children. Both parents of J.D. are deceased: her mother at 63 from a cerebral tumor and her father at 82 from a heart attack. DNA was available from a number of relatives: one sibling, four children, two grandchildren, and four nephews. DNA was available from three siblings of M.L. Unfortunately, both parents are deceased, one from a stroke at 78 years, and one from a heart attack at 61. Both parents of N.M are also deceased: her mother from bile duct cancer and her father from a heart attack. DNA was available from two siblings and one child. Among the relatives of these four patients, two children (ages 45 and 50) and two siblings (ages 62 and 65) carry a mutant *NEFH* allele.

Neither the parents nor the three siblings of J.V. carried the mutant NEFH allele, indicating that this must be a new mutation in the patient J.V. Paternity was verified by testing the family members with 15 informative CA-repeat polymorphic markers located throughout the genome (data not shown).

DISCUSSION

Mechanism of mutation

We report two variant NEFH alleles which may predispose to ALS. One peculiarity of the variant NEFH alleles described here in ALS patients is that they originate from codon deletions rather

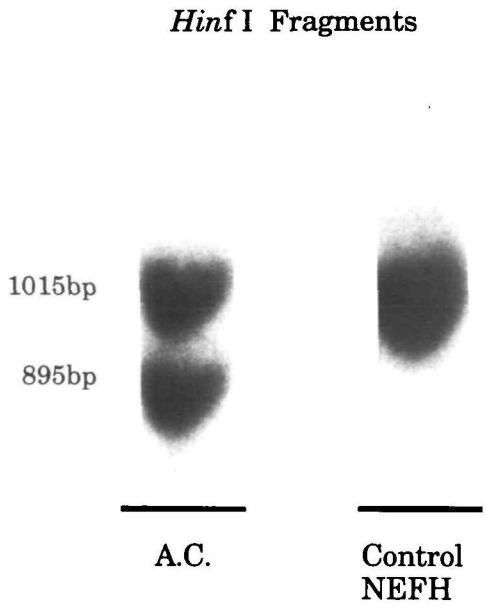

Figure 4. Southern blots with *Hinf*I-digested genomic DNA from ALS patient A.C. (left panel) and from a control individual (right panel). Southern blots were hybridized with a 1.2 kb fragment including the entire KSP repeat motif region of human *NEFH*. The control individual is a heterozygote for the 43 and 44 KSP repeat alleles of *NEFH*. Patient A.C. displays one allelic fragment of normal size (44 KSP repeats), and a second fragment which contains the 102 bp deletion.

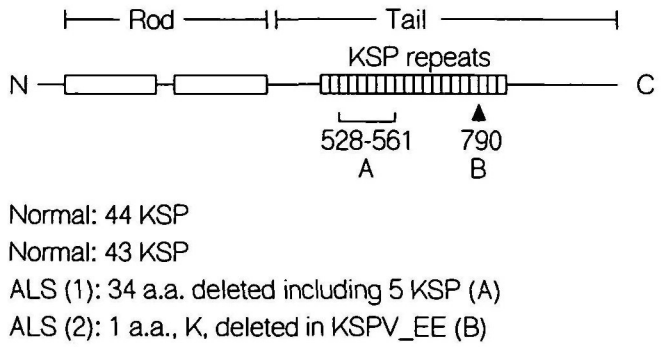

Figure 5. This schematic depicts the locations of the changes in the neurofilament heavy subunit protein resulting from the two mutations which have been identified in ALS patients.

than point mutations, and they occur in a region of NEFH consisting of a repeat sequence motif. It is well established that repeat sequences are prone to such mutations. For instance, deletions or additions of trinucleotide repeats have been described in Kennedy's disease (13), the fragile X syndrome (14), myotonic dystrophy (15), Huntington's disease (16), and spinocerebellar ataxia type 1 (17). The mechanism proposed for this type of mutation involves the loss or gain of repeats by a process involving slipped mispairing during DNA replication (18,19). Such a mechanism is possibly responsible for the codon deletions detected in the multiple sequence repeats of the *NEFH* gene, albeit the rate of mutations in *NEFH* is extremely low compared with those found in minisatellite repeats (18) and trinucleotide repeats (14,15).

NEFH phosphorylation domain and predisposition to ALS

NEFH mutations were found in five unrelated ALS patients diagnosed as sporadic cases (summarized in Fig. 5). With the exception of relatives of known ALS patients, no healthy individual was found to have mutations in the carboxy-terminal region of *NEFH*. The ALS family members carrying the *NEFH* mutations are close to or below the average age at onset for sporadic ALS of 61 years. As no mutations were identified in the 306 controls tested, we can rule out the possibility that the mutations represent a common polymorphism. *NEFH* has been mapped to human chromosome 22q12.1 (20); to date, no FALS kindred has been definitively linked to this locus.

This report is the second example of a class of intermediate filaments involved in human disease. Mutations which introduce inappropriate amino acid substitutions have been described in human keratin genes, leading to skin disorders (21–24). In these disorders, mutations occur at the ends of the highly evolutionarily conserved rod domain, and affect filament elongation. Similarly, it has been recently demonstrated in transgenic mice that accumulation of a modest level of NEFL bearing a point mutation in the rod domain leads to an early-onset degeneration of spinal motor neurons, with relative sparing of sensory neurons (25). How could mutations in *NEFH* underlie the pathogenesis of ALS? A hallmark of ALS is the presence of neurofilamentous swellings in the cell body and proximal axon of motor neurons (2–6). Transgenic mice overexpressing the human *NEFH* progressively develop neurological defects and abnormal neurofilamentous swellings in motor neurons similar to those found in ALS (9). The carboxy-terminal domain of NEFH forms the side-arms which appear, at the level of electron microscopy, to cross-link neurofilaments (26,27). The results in *NEFH* transgenics led to the proposal that an impairment of neurofilament transport by extra NEFH cross-linkers was responsible for the gradual accumulations of neurofilaments in the perikarya and proximal axons. In another neurological disorder, giant axonal neuropathy, NEFH side-arms appear to be flattened leading to more close spacing of the assembled filaments and giant axonal swellings (28). Moreover, recent evidence suggests that there may be a physical arrangement of phosphorylated NEFM and NEFH which is unique to motor neurons (29). Consequently, irrespective of the nature of the neurofilament derangement, the neurofilament network of motor neurons may be inherently more vulnerable to disruption than that of other large neurons.

It is plausible that the above mutations in the human NEFH phosphorylation domain cause slight changes in the cross-linking properties of this protein resulting in a late-onset neurodegenerative disorder due to a tendency to gradually develop aberrant neurofilamentous accumulations. It is interesting that four of five of the ALS patients reported here bear the same 3 bp deletion. This deleted lysine residue is part of the five amino acid consensus sequence which serves as a substrate for the recently identified cdc2-like kinase (30) which has been isolated from rat spinal cord. Perhaps, the disruption of this substrate recognition site, or the lack of 102 bp encoding 5 KSP phosphorylation sites may predispose to neuronopathy through the formation of shorter and stronger neurofilament cross-linkages by NEFH proteins. In this model, one would expect that the onset and development of ALS, even in individuals bearing *NEFH* mutations, depends on additional environmental and/or genetic

factors unique to each individual. For instance, patient J.V. with a 3 bp *NEFH* deletion has a documented record of long-term exposure to toxic compounds (hydrazine and morpholine) which may have accelerated the onset of the disease.

The production and analysis of transgenic mice bearing the variant human *NEFH* alleles on an *NF-H*-knockout mouse background will represent the next step in the effort to understand the *in vivo* effects of the mutations on neurofilament organization and neuronal function.

MATERIALS AND METHODS

Patient material

Blood samples were obtained from sporadic ALS patients, seen at the Centre de Diagnostic, Centre SLA, Hotel-Dieu de Paris, or the ALS Clinic, Department of Neurology, Henry Ford Hospital, Detroit MI, between December of 1991 and February of 1993. None of the 356 individuals are inter-related. The diagnosis of definite ALS was made using the El Escorial classification (31). Control samples were taken from 306 individuals with no known neurological disorder, and no family history of neurological problems.

Patient A.C. (female). A.C. displayed the first symptoms of ALS at age 72, with dysarthria, mild cramps and weight loss, and a mix of hypo- and hyper-reflexia. Over the course of 1 year, she has shown mild progression which includes the development of pseudobulbar signs, bilateral Babinski, diffuse atrophy and fasciculations.

Patient J.D. (female). J.D. presented at the age of 65 with weakness of the left hand, followed shortly thereafter by weakness of the left leg. Her clinical condition worsened over the course of 2 years. Symptoms included difficulty walking, diffuse fasciculations, brisk tendon reflexes, a bilateral Babinski, and atrophy of the tongue. J.D. died at the age of 68 years, 7 months.

Patient M.L. (male). M.L. was admitted to the ALS Clinic at the age of 52 with the complaint of leg weakness and cramps. At this time, mild weight loss, diffuse fasciculations, and some spasticity were present. Over the course of 1 year, severe and rapid progression was observed to include spasticity of all four limbs, brisk tendon reflexes, bilateral Babinski, and severe dysarthria. M.L. died at the age of 53 years, 5 months.

Patient N.M. (female). N.M. was first seen at the ALS clinic at the age of 47 when she received the definitive diagnosis of ALS. The disease progressed over the course of 2 years to complete quadriplegia and prominent pseudobulbar signs.

Patient J.V. (male). J.V. presented at the age of 46 with lower limb involvement—cramps, fasciculations, and weakness. There was no upper limb involvement, and no sensory signs. The disease has progressed over the course of a year and a half to include both upper and lower limb atrophy, a bilateral Hoffmann sign, and a mixture of hypo- and hyper-reflexia. It is of interest that J.V. spent 5 years as a member of a maintainence staff during which time he was exposed up to five times a day to a highly toxic cleaning solution containing 24% hydrazine and 8% morpholine (tetrahydro-1,4-oxacine, oxide diethyleneimide).

PCR amplification and electrophoresis

PCR amplification of the KSP repeat motif region of human *NEFH* (bases 1336 through 2508; numbering of Lees *et al.* (11)) was carried out for all DNA samples as previously described (12). PCR products were visualized by ethidium bromide staining after electrophoresis in an 8% (acrylamide:bis; 39:1) neutral acrylamide gel. Sample preparation, loading, and electrophoresis were all carried out at room temperature.

Cloning and sequencing

PCR-amplified *NEFH* fragments from the patients J.V., M.L., and A.C., were subcloned and sequenced as previously described. Clones from J.V. and M.L. were sequenced using oligo B (12) as primer. Clones from A.C. were sequenced using the T3 primer: 5'-A-A-T-A-A-C-C-C-T-C-A-C-T-A-A-A-T-3'.

Southern blot and hybridization

Genomic DNA prepared from patient A.C., and a control individual, was digested with *Hin*fI (Gibco BRL) according to manufacturer's recommendations. Digested samples were electrophoresed on 1.5% agarose gels at 20 V overnight, and a Southern transfer was prepared. Probe was prepared by PCR amplification of bp 1336–2508 of *NEFH* from a control individual, and labelled by the random priming method. Filters were hybridized at high stringency. These methods have been described previously (32–35).

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ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; KSP, lysine–serine–proline; NEFH, neurofilament heavy subunit; NEFL, neurofilament light subunit; NEFM, neurofilament medium subunit; PCR, polymerase chain reaction.

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